PROJECT 1 : ANATOMICAL LOCALISATION OF ANTIGEN-SPECIFIC CD8+ T CELLS IN TISSUES

BY

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This project is submitted in partial fulfilment of the requirements for the award of the MRes' plus.

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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):

EBV-specific CD8+ T cells have previously shown to be present in high frequencies within the tonsil compared to the peripheral blood of healthy EBV carriers. The anatomical location of these EBV-specific CD8+ T cells in the tonsil is currently unknown and could be the means of identifying lytically infected cells. Class I tetramer staining can now be adapted for in-situ staining of EBV-specific CD8+ T cells.

We have utilized Quantum dot (Qdot) nanocrystal technology to form EBV-specific Qdot multimers. Each multimer has a potential to form strong MHC-TCR interactions and are extremely photo stable. Here, we have optimized and validated the use of EBV-specific Qdot multimers in-situ staining to visualize EBV-specific CD8+ T cells in human tonsil sections from healthy EBV carriers.

Saturation of streptavidin conjugated Qdot655 (StpQdot655) was achieved at a ratio of 3.9µg of MHC class I monomer to 1pmol of StpQdot655 forming EBV-specific Qdot multimers. In all experiments EBV-specific Qdot multimers were shown to be extremely stable and demonstrated specific binding in flow cytometry and microscopy analysis. Qdot multimers also showed comparable staining intensity to conventional tetramers in flow cytometry analysis of multiple T cell populations. Optimized confocal microscope settings, using Qdot655 and Alexa488 reagents, allowed for the visualization of EBV-specific CD8+ T cell clones using corresponding EBV-specific Qdot multimers and anti-CD8 Alexa 488 antibodies.

The use of EBV-specific Qdot multimers in an optimized staining protocol has now been validated for the visualization of EBV-specific CD8+ T cell via confocal microscopy. Once an EBV+ HLA matched tonsil specimen can be obtained the localization of EBV-specific CD8+ T cells in the tonsil can for the first time be determined.

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I would firstly like to especially thank my supervisor Andrew Hislop whose help, advice and guidance has been highly valued all through my project.

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1 INTRODUCTION

1.1 Epstein-Barr virus (EBV) biology.

EBV, a double stranded DNA virus, is a member of the herpesviridae family which chronically infects >90% of the world's population. Infection with EBV occurs via saliva transmission usually in childhood which results in an asymptomatic infection. If primary infection is delayed into adolescence/adulthood infectious mononucleosis (IM) can result in 25% of infections(Crawford, Macsween et al. 2006). IM is an acute self limiting disease which presents with flu like symptoms characterised by a large expansion of CD8+ T cells specific for EBV, which has been shown to persist for months(Niederman, McCollum et al. 1968). IM patients have allowed for the study of primary infection and revealed what we know today about the early events of EBV infection.

EBV enters the oropharynx and infects a permissive cell type of unknown identity but is most likely an epithelial cell of the oropharynx or a resting B cell. EBV is known to establish infection in the B cells population of the oropharyngeal lymphoid tissue. Upon entry into the B cell (Li, Spriggs et al. 1997) the viral genome traffics to the nucleus and circularises to form an episome and a restricted set of genes are expressed and transforms resting B cells to an immortalised latently infected lymphoblastoid cell. Following the B cell transformation process, viral gene expression is down regulated and the infected cells exit the lymphoid tissues as a long lived memory B cell (Figure 1.1). The virus can then persist within its host for life as a latent infection with the infected B cells recirculating between the oropharyngeal lymphoid tissues and the blood.

During transit through the oropharanyx latently infected EBV cells can spontaneously undergo reactivation into lytic cycle producing new virions. The identification of lytically infected cells has not been established fully, however, epithelial cells were thought to account for the detection of virus in throat washings of healthy infected individuals(Hutt-Fletcher 2005). Consistent with this, B cells *in vitro* were able to efficiently transfer surface bound EBV to epithelial cells of the

oropharynx(Shannon-Lowe, Adland et al. 2009). Regardless, virus is shed into the oropharynx enabling transmission from host to host as new infection of B cells.

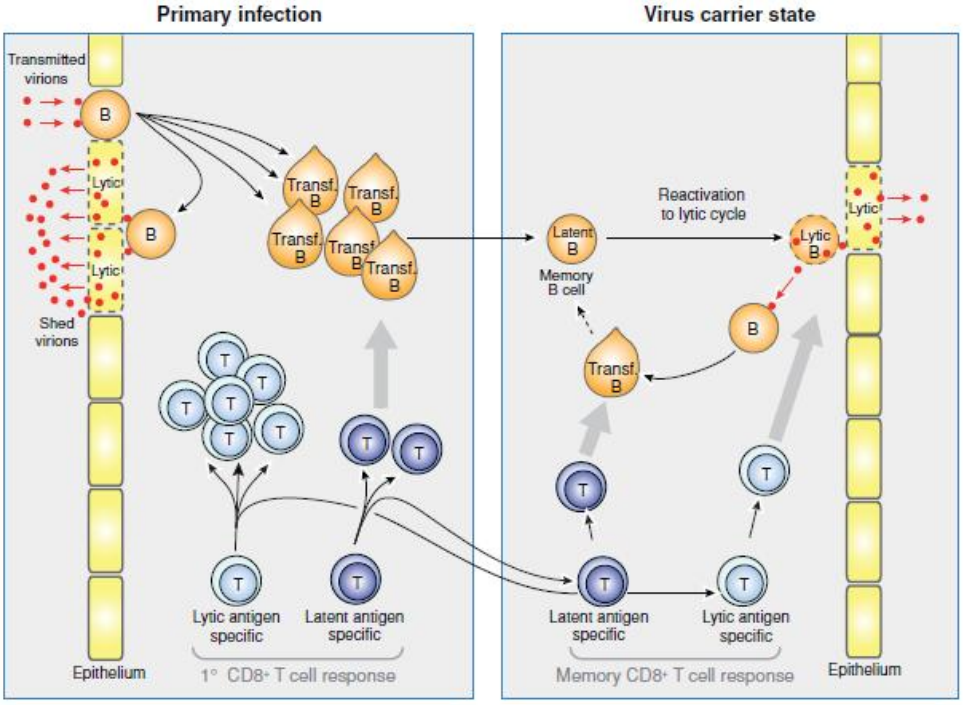


Figure 1.1 - schematic representation of EBV primary infection through to carrier and reactivation of the virus. (Hislop, Taylor et al. 2007)

1.2 EBV and Cancer.

EBV was the first virus associated with human cancer and was discovered in Burkitt's lymphoma, in which all cells contain EBV genome(Epstein, Achong et al. 1964). EBV has since been implicated in a number of B and epithelial cell malignancies. Approximately 40% of Hodgkin's lymphoma in the developed world contains EBV (Deacon, Pallesen et al. 1993) as well as some T and natural killer (NK) cell lymphomas. Nasopharyngeal carcinoma (NPC) is an epithelial malignancy in which virtually all cases are EBV positive with a high incidence in China and South East Asia. Genetic or environmental factors such as immunosuppression or diet in an EBV positive individual can allow for the development of EBV associated malignancies (Young and Rickinson

2004). Cellular immunity is important in controlling EBV and when suppressed outgrowth of EBV transformed B cells can occur manifesting as post transplant lymphoproliferative disease (PTLD)(Haque, Wilkie et al. 2002) .

1.3 T cell Immunity.

Innate and adaptive immunity provide effective defence against pathogens and work together to eliminate an infectious agent. However it is the cells of the adaptive immune system that are able to form immune memory to pathogens and T lymphocytes are thought to be particularly important in the control of EBV infection as evidenced by studying immunosuppressed patients. CD8 T cells (known as effecter cells) provide the cytotoxic response, contain perforin and can secrete inflammatory cytokines upon activation *in vitro* in order to kill target cells (Callan, Fazou et al. 2000). T cells can only see pathogens when peptides derived from the pathogen have been processed and presented to them via major histocompatibility complex (MHC) termed human leukocyte antigen (HLA) in humans. CD8+ T cells see peptide presented by MHC class I and each peptide sequence is specific to the pathogen it originates from and is restricted to the HLA type of the host and thus creates a highly specific immune response.

1.4 EBV targets of the T cell response.

Targets of the T cell response can be derived from proteins expressed from lytic and latent proteins. Overall, lytic replication occurs in three phases starting with the production of immediate early proteins (IE), BZLF1 and BRLF1, which activate a cascade of gene expression resulting in production of early proteins (E) and late proteins (L) and induce strong CD8+ responses(Hislop, Taylor et al. 2007). Latent infection manifests in B cell memory pools resulting in the expression of 8 latent viral proteins with CD8+ T cells targeting the EBNA 3 family in preference over other latent proteins. The identification of different CD8+ T cell targets has helped develop successful immunotherapy, PTLD for example can be cured via the adoptive transfer of HLA matched

PBMCs enriched in the EBNA 3A,3B and 3C specific CD8+ T cells (Heslop, Ng et al. 1996; Khanna, Bell et al. 1999) The identification of new EBV epitopes is important in the development of future immunotherapy of other EBV associated diseases.

1.5 Anatomy of the tonsil.

The tonsil is a secondary lymphoid organ and is thought to be of particular use in the defence against pathogens invading the oropharynx. The tonsil is not fully encapsulated and the epithelium that is exposed to the throat contains a tough stratified squamous epithelium which is underlined by a layer of connective tissue protecting it from its external environment. The outer surface folds in on itself and forms tonsillar crypts which act as a passage to capture antigen from the pharyngeal lumen. In contrast, the tonsillar crypts contain sponge like epithelia and show a lack of a thick connective tissue band known as the lymphoepithelium. Direct antigen stimulation occurs here between antigens from the oropharynx and lymphocytes of the tonsil. Follicles are present through out the tonsil and are arranged parallel to the crypts. The outer layer of the follicle is the follicular mantle which contains small lymphocytes beneath which lies the germinal centres (GC) formed for the purpose of B cell maturation. GC's contain dark zones, containing blast cells, and light zones, which are packed with antigen presenting cells (APC). Between and below the follicular mantle is the T zone containing high endothelial venules enabling T lymphocytes to migrate from the blood to the lymphatic system (Figure 1.2). The structure and cell population of the tonsil enables it to be an efficient immune competent organ.

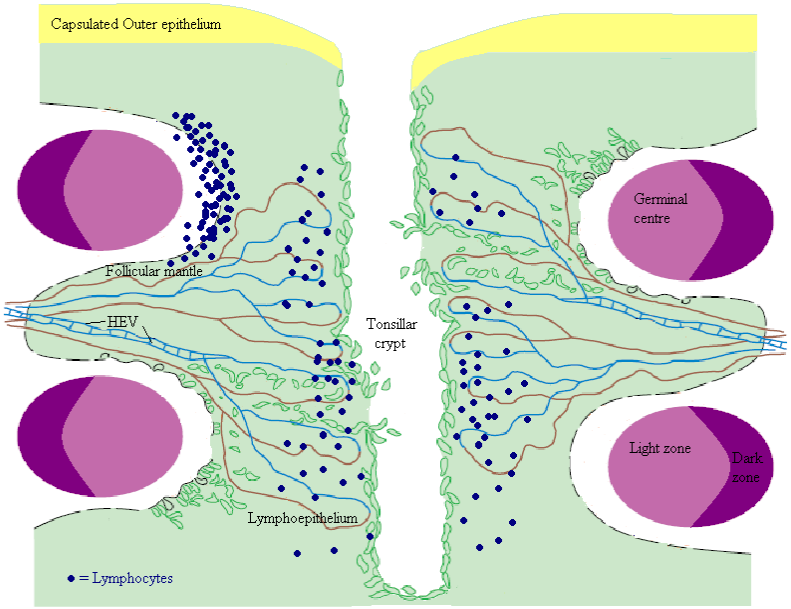


Figure 1.2 Schematic representation of tonsil structure. The exposed outer epithelium is an encapsulated, stratified squamous epithelium set upon a layer of connective tissue. The tonsillar crypts are not encapsulated and lack the connective tissue layer. Instead a sponge like structure can be seen containing a mixture of epithelial cells, lymphocytes and blood vessels forming the lymphoepithelium. The germinal centres are aligned parallel to the tonsillar crypts containing light and dark zones. (Perry and Whyte 1998)

1.6 Tetramer Technology

Since its introduction in 1996 (Altman, Moss et al. 1996) the use of soluble MHC class I tetramers has allowed for the in depth analysis of CD8+ T cells in terms of specificity, frequency and phenotype in a wide range of diseases. Activation of T cells specific for an antigen occurs through the interaction between MHC class I and TCR, however this is a weak interaction. Conventional MHC class I tetramers consist of 4 biotinylated MHC class I molecules lacking a transmembrane domain bound to a fluorochrome conjugated to streptavidin (Figure 1.3a). This complex act as a soluble probe for specific TCR's with the 4 MHC molecules on a tetramer resenting the same peptide. This allows for binding to multiple TCR and therefore slows the dissociation rate of the TCR-MHC interaction(Altman, Moss et al. 1996). The fluorescence can be monitored by flow cytometry and have been used to discover frequencies of CD8+ T cells in hosts.CD8+ tetramer stained cells have been sorted and used in cytotoxicity and proliferation assays, their apoptotic

potential can be determined and by co-staining with other antibodies allows for the discovery of T cell homing phenotypes (Appay and Rowland-Jones 2002).

The use of tetramers in investigating EBV biology has revealed more about EBV-specific T cell numbers as well as their phenotype. Past studies have shown EBV infected healthy carriers contain significantly higher frequencies of EBV-specific CD8+ T cells, up to 20%, in the tonsil as compared to the peripheral blood of the same individual(Hislop, Kuo et al. 2005). Co-staining of tetramer positive CD8+ T cells have shown the EBV-specific CD8+ T cells in the tonsil have increased expression of CD103, an integrin associated with retention in mucosal epithelium(Woodberry, Suscovich et al. 2005). This suggests the tonsil epithelium as a potential site of viral infection and looking at the localisation of EBV-specific CD8+ T cells may uncover more about EBV biology.

1.7 In situ Tetramer staining.

Tetramer technology can now be adapted to look at the localization of these specific T cells in tissue. Skinner *et al* successfully developed the use of tetramers in tissue and were able to visualise, in mouse spleens, peptide specific CD8+ T cells, whose numbers were comparable to flow cytometry data(Skinner, Daniels et al. 2000). The optimisation of this technique has enabled for the identification of virus-specific CD8+ T cells and also be combined with techniques to identify cells infected with the target virus and discover the spatial relationships between viral replication and the CD8+ responses *in situ*(Bauquet, Jin et al. 2009; Li, Skinner et al. 2009). This protocol has since been used to look other chronic virus infections, from Simian Immunodeficiency Virus (Reynolds, Rakasz et al. 2005) to Herpes Simplex Virus - 2(Zhu, Koelle et al. 2007). Indirect *in situ* staining relies on amplification of tetramer signal (Skinner, Daniels et al. 2000) however direct tetramer staining methods using different fluorochromes have been used(Haanen, van Oijen et al. 2000). The

direct method allows for the visualisation of low frequency antigen specific CD8 cells without the problem of background fluorescence.

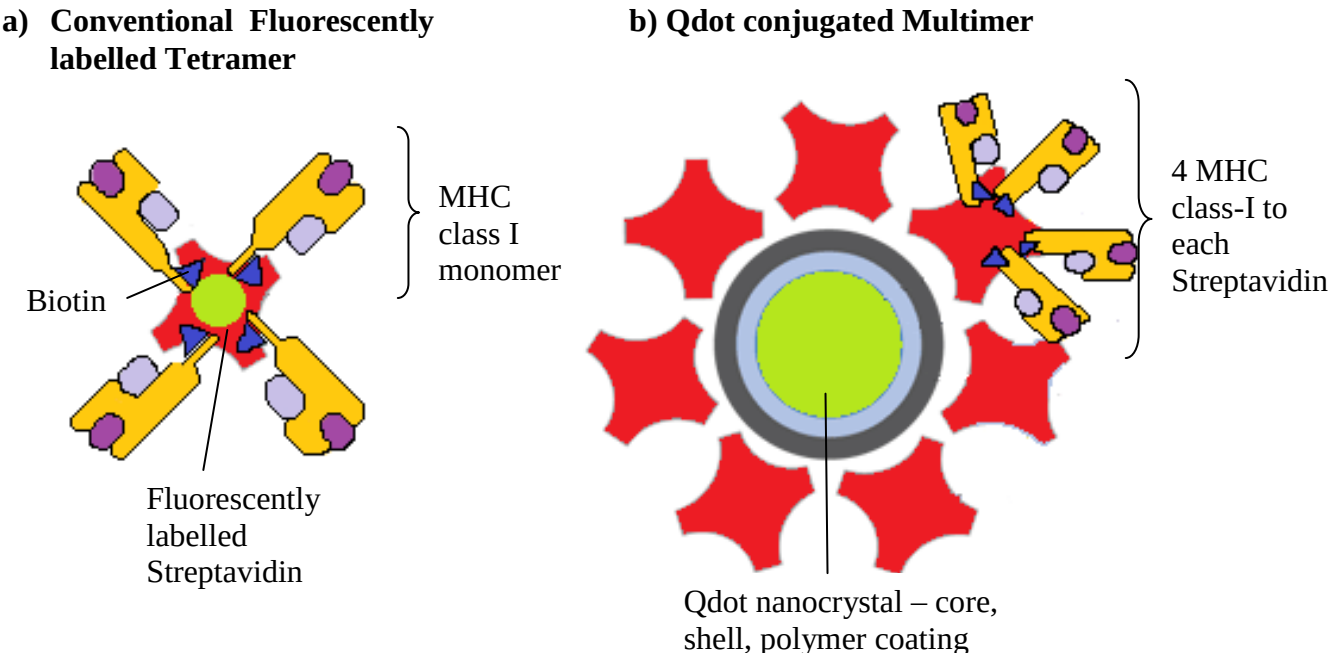


Figure 1.3 Structure of Conventional Tetramer and Qdot Multimer. (a) A conventional, fluorescently labelled tetramer, which contains one strepatvidin molecule conjugated to a fluorochrome. Up to 4 biotinylated MHC class I monomers can be bound to a streptavidin molecule. (b) Qdot conjugated multimer – Qdot nanocrystal core is protected by an outer shell and polymer coating which has 5-10 streptavidin molecules attached. Each steptavidin molecule can have up 4 biontinylated MHC class I molecules resulting in each Qdot molecule with a potential 20-40 MHC class I molecules attached. The increased MHC molecules on the Qdot multimer results in stronger binding.

1.8 Qdot Multimer technology.

Quantum dots (Qdots) are fluorophore nanocrystals which are extremely resilient to photo bleaching having a broad absorption spectra, with optimum absorption at UV wavelengths, but with narrow emission peaks (Figure 1.4) preventing bleed through into other fluorophore spectra and therefore make excellent tools in co staining experiments(Deerinck 2008). Qdots can be conjugated to streptavidin and upon addition of biotinylated MHC class I molecules can create Qdot multimers (Figure 1.3b). The multiple MHC class I monomers (a potential of 20-40 per Qdot) should therefore bind with more TCR potentially strengthening the interaction between TCR-MHC even more than

conventional tetramers (Altman, Moss et al. 1996). These Qdot Multimers have proved to be successful in the detection of low frequency CD8+ T cells specific for HSV-2 in skin biopsies with all the advantages of direct *in situ* staining and the photo stability of these nanocrystals(Zhu, Koelle et al. 2007).

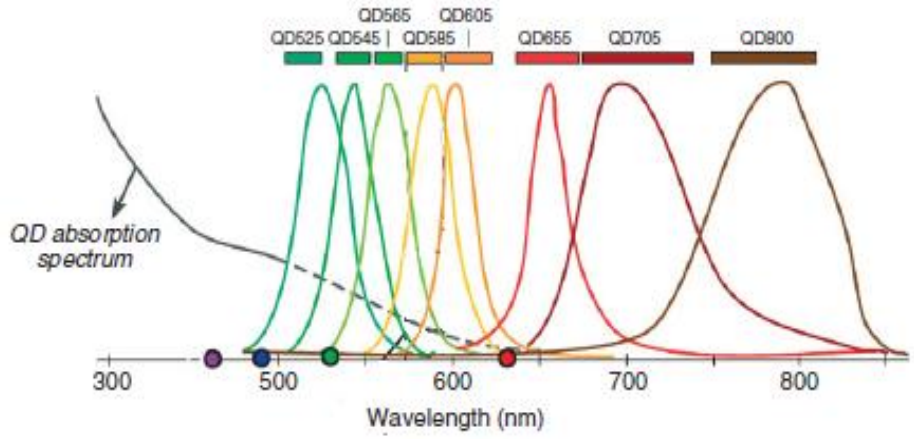


Figure 1.4 Emission spectra of Qdot dyes(Chattopadhyay, Perfetto et al. 2010)

1.9 Aims

This project aims to utilise Qdot multimer technology in *in situ* staining of human tonsil sections from healthy carriers. The high proportion of EBV-specific CD8+ T cells in the tonsil compared to the peripheral blood insinuates an importance of the tonsil as an EBV harbouring organ and a potential EBV replication site. The localization of these EBV-specific CD8+ T cells within the tonsil may reveal more about the immune regulation of EBV but also reveal the locations of cells replicating EBV in which the T cells target.

2 MATERIALS AND METHODS.

2.1 Human tissue specimens and PBMCs.

Eight tonsil specimens (each containing two whole tonsils) were collected from either the Birmingham Children's Hospital NHS trust or University hospital of Birmingham NHS trust with all ethical considerations approved. Consent was taken from each patient and stored in the patient notes. Neither the HLA type nor the EBV status was known at the time of collection. One of the tonsils was cut into 0.5 x 0.5cm pieces and embedded in 4% Agarose and sectioned via vibratome to 200µm thick sections and used immediately for Immunofluorescence staining. The other tonsils were homogenised manually with a scalpel and the unfractionated mononucleocytes (UMs) separated by Lymphoprep (AXIS – SHIELD). The harvested cells were then either used for flow cytometry or stored in liquid nitrogen. Whole blood (60ml) was taken from healthy lab donors and PBMCs were separated with Lymphoprep (AXIS-SHIELD) and used in either flow cytometry or stored in liquid nitrogen.

2.2 T cell clones

T cell clones, previously established with a defined specificity, were removed from liquid nitrogen and activated with buffy coat cells and incubated at 37ºC for 10-14 days. After activation the clones were then fed twice weekly with T cell cloning media (10% foetal calf serum, 1% human serum, 30% MLA, 1% penicillin/streptomycin, 50µl/ml IL2 and 58% RPMI) and kept at 37ºC. The clones used were B8 FLR 213, B8 FLR IM59.1, A2 GLC IM225.8 and A2 YVL IM226.

2.3 Synthesis of Qdot multimers and PE tetramers.

MHC class I – EBV peptide biotinylated monomers were made previous to these experiments. A2 restricted EBV peptide GLC and B8 restricted EBV peptide RAK and FLR biotinylated monomers were used to saturate streptavidin conjugated Qdot 655 (StpQdot655) (Invitrogen) via hourly

additions of saturating amount of monomer to a predetermined volume of StpQdot655, forming GLC-Q, RAK-Q and FLR-Q. For the conventional streptavidin conjugated PE (Invitrogen) tetramers hourly additions of streptavidin conjugated PE was added to a predetermined volume of monomer, forming GLC-PE, RAK-PE and FLR-Q.

2.4 Staining of T cell clones for microscopic analysis.

0.2 x10^6 T cell clones were stained with either mouse anti human CD8-Qdot 655 (Invitrogen 1:100), mouse anti IgG2a -Qdot 655 (Invitrogen 1:100), human CD8 purified antibody (Invitrogen 1:100) or FITC mouse anti human CD8 (BD biosciences 1:50), purified mouse anti IgG2a k (BD Biosciences 1:100) or FITC mouse anti IgG2a k (BD Biosciences 1:50) for 30mins on ice or Qdot multimer (1:25 GLC-Q, FLR-Q) for 15mins at 37ºC. Qdot multimer was added first in co-staining experiments and cells washed before the addition of co-staining antibody. Donkey anti human Alexa 488 (Invitrogen 1:100) was used as a secondary antibody in experiments using human CD8 purified antibody as a primary antibody. 10µl of stained cells was then air dried onto a microscope slide and fixed in 50% acetone/ 50% methanol for 20mins at -20ºC. The slides were then washed in PBS and mounted with DABCO for viewing by confocal or epifluorescence microscopy.

2.5 Immunofluorescence in situ staining of Tonsil

Fresh 200µm thick tonsil sections were stained over night with either mouse anti human CD8-Qdot 655 (Invitrogen 1:200), mouse anti IgG2a -Qdot 655 (Invitrogen 1:200), human CD8 purified antibody (Invitrogen 1:200) or FITC mouse anti human CD8 (BD biosciences 1:20), purified mouse anti IgG2a k (BD Biosciences 1:200) or FITC mouse anti human CD20 (BD biosciences 1:20) or Qdot multimer (1:50 RAK-Q, GLC-Q, FLR-Q) at 4ºC on a rocking platform. For co-staining of tissue human CD8 purified antibody was added alongside Qdot multimer in the initial overnight incubation. Tissues were then washed in MACS buffer and fixed in 4% paraformaldehyde for

30mins at room temperature. The sections were then washed and incubated with donkey anti mouse Alexa Fluor 488 (Invitrogen 1:200) (if required) for 1 hour at 4ºC on a rocking platform. Sections were then washed and mounted onto a microscope slide and mounted in DABCO for viewing with confocal or epifluorescence microscopy.

2.6 Confocal and Epifluorescence microscopy

Samples stained Qdot 655 reagents were viewed by epifluorescence microscopy (Nikon E600) using the Texas red filter set and FITC was viewed by the green filter set. Confocal Microscopy was carried out using the Zeiss Axiovert 200m. Fluorescent dyes were excited by the following lasers: Qdot655 excited with Enterprise364nm and Alexa488 with Argon 488. A single track configuration was used with an NFT545 beam splitter with 505 Long Past (LP) filter for Alexa 488 and a 650 LP for Qdot 655.

2.7 Flow cytometry

T cell clones ($0.1x10^6$), tonsil UM's ($1 x10^6$) and PBMCs ($0.5 x10^6$) were used in each flow cytometry experiment where relevant. The flow cytometers used were the XL-MCL for PE-tetramers and the LSR II for Qdot multimers. Cells were stained with PE tetramer (1:50 FLR-PE, GLC-PE or RAK-PE) or Qdot multimer (1:50 GLC-Q, FLR-Q) for 15mins at 37ºC. Cells stained with Qdot multimer were then co stained with PE mouse anti human CD8 (1:40 BD Biosciences) cells stained with PE tetramer were co stained with Human CD8 Tri Colour conjugate (1/40 Invitrogen) for 30mins on ice. Cells are then washed and resuspended in PBS (2% fcs) and analysed by flow cytometry.

3 RESULTS.

3.1 Determination of the amount of MHC class I monomer required to be added 1µl of 1µM Qdot 655 to achieve optimal staining of a T cell clone with this reagent

Few studies have been conducted using Qdot reagents as fluorochromes bound to MHC class I multimers for staining T cells. It is unclear what amount of class I molecule should be added to the Qdot streptavidin that allows stable binding to T cell receptors without causing non-specific binding. As such, an empirical experiment was conducted in which different amounts of an HLA A2 monomer was titrated onto a constant amount of Streptavidin conjugated Qdot 655 (StpQdot655) and these reagents were then used to stain specific T cell clones and analysed by flow cytometry. Here monomers containing the GLC peptide from the EBV BMLF1 protein were used and tested against a cognate T cell clone IM225.8. StpQdot655 (1pmol) was mixed with either1.3ug, 2.6ug, 3.9ug, 5.2ug or 6.5ug of GLC monomer. Flow cytometry analysis of the clones stained with the GLC Qdot multimer (GLC-Q) and anti-CD8 antibodies suggested that mixing 3.9ug of HLA class I protein with 1pmol of StpQdot655, gave the clearest staining in that the population of cells stained the brightest with the least amount of background staining (Figure 3.1). In future experiments Qdot multimers were made at a ratio of 3.9ug monomer to 1pmol of StpQdot655.

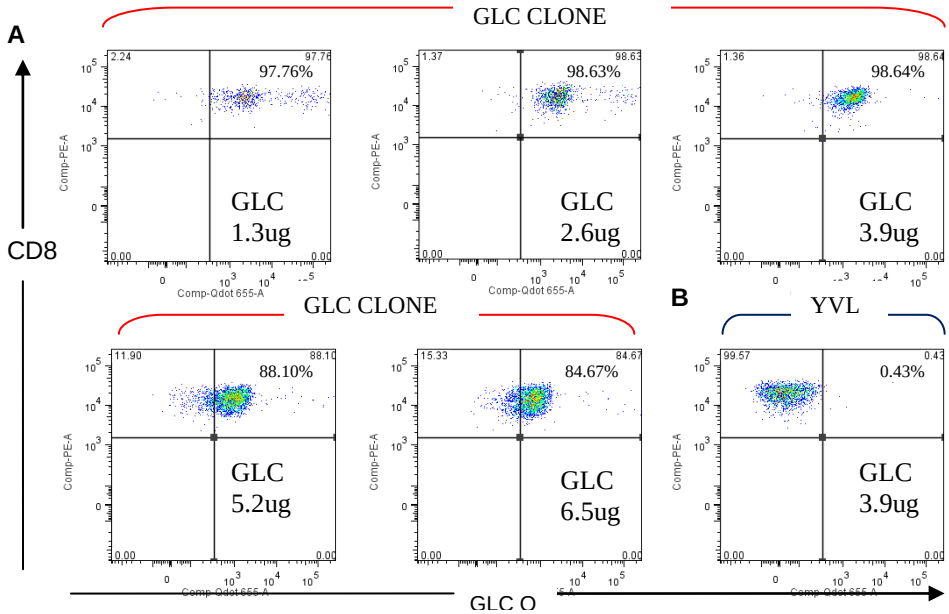


Figure 3.2 Saturation of Streptavidin conjugated Qdot with A2 GLC monomer.
Frequency of GLC-specific CD8+ T cells in (A) a GLC-specific clone or (B) an irrelevant HLA A2 restricted YVL-specific clone. Clones were stained with GLC-Q and further stained with anti-CD8 mAbs. The results shown have been gated on CD8 + cells. Values shown refer to the percentage of CD8+ cells stained with Qdot multimer.

3.2 Validation and titration of GLC Qdot vs. PBMCs from HLA A2EBV sero positive and sero negative donors and a GLC-specific T cell clone.

In order to further validate the GLC-Q reagent made under the optimal conditions determined above, this reagent was tested against the same GLC T cell clone for consistency. A new batch of GLC-Q was made at a concentration of 3.9ug monomer to 1pmol of StpQdot655 and was also tested against the GLC T cell clone as well as an HLA A2 EBV seropositive donor PBMCs to test the ability to bind to resting GLC-specific T cells and an HLA A2 EBV seronegative donor PBMC's to determine background staining. The GLC-Q was titrated at 1/25, 1/50 and 1/100 dilutions on the above cell types in parallel experiments. The new batch of GLC-Q showed comparable results to the original batch of GLC-Q against IM225.8 GLC clones (Figure 3.2a) showing consistency between the batches. Interestingly, at higher concentrations of GLC-Q an increase in double negative GLC clone populations was seen (Figure 3.2a). The lower concentrations of the GLC-Q showed less fluorescence intensity, giving an apparent reduction in

the CD8+ clonal population specific for GLC to 91.46%. This was shown in the EBV seropositive donor and again a titration effect of decreasing intensity and percentage positivity was observed with decreasing concentration of the GLC-Q multimer (Figure 3.2b). At lower dilutions it was difficult to separate discrete populations of GLC positive cells from the multimer negative cells in the seropositive donor due to the low intensity staining. PBMCs from the seronegative donor showed no GLC-Q staining at all concentrations tested thus showing the absence of EBV derived GLC-specific T cells in the peripheral circulation (figure 3.2c). The absence of GLC-Q staining cells in the seronegative donor was consistent with the specificity data of GLC-Q staining against an irrelevant YVL clone (Figure 3.1b) confirming the GLC-Q was extremely specific and does not non-specifically bind to other cells. Interestingly, the high concentration of GLC-Q did not substantially increase the double negative population of PBMCs when tested against the seropositive and seronegative donors, suggesting this phenomenon was specific to the highly activated GLC clone. Due to the effect of higher concentrations of GLC-Q increasing the double negative population and the less intense fluorescence seen at lower concentration all flow cytometry Qdot multimer experiments was performed using a 1/50 dilution.

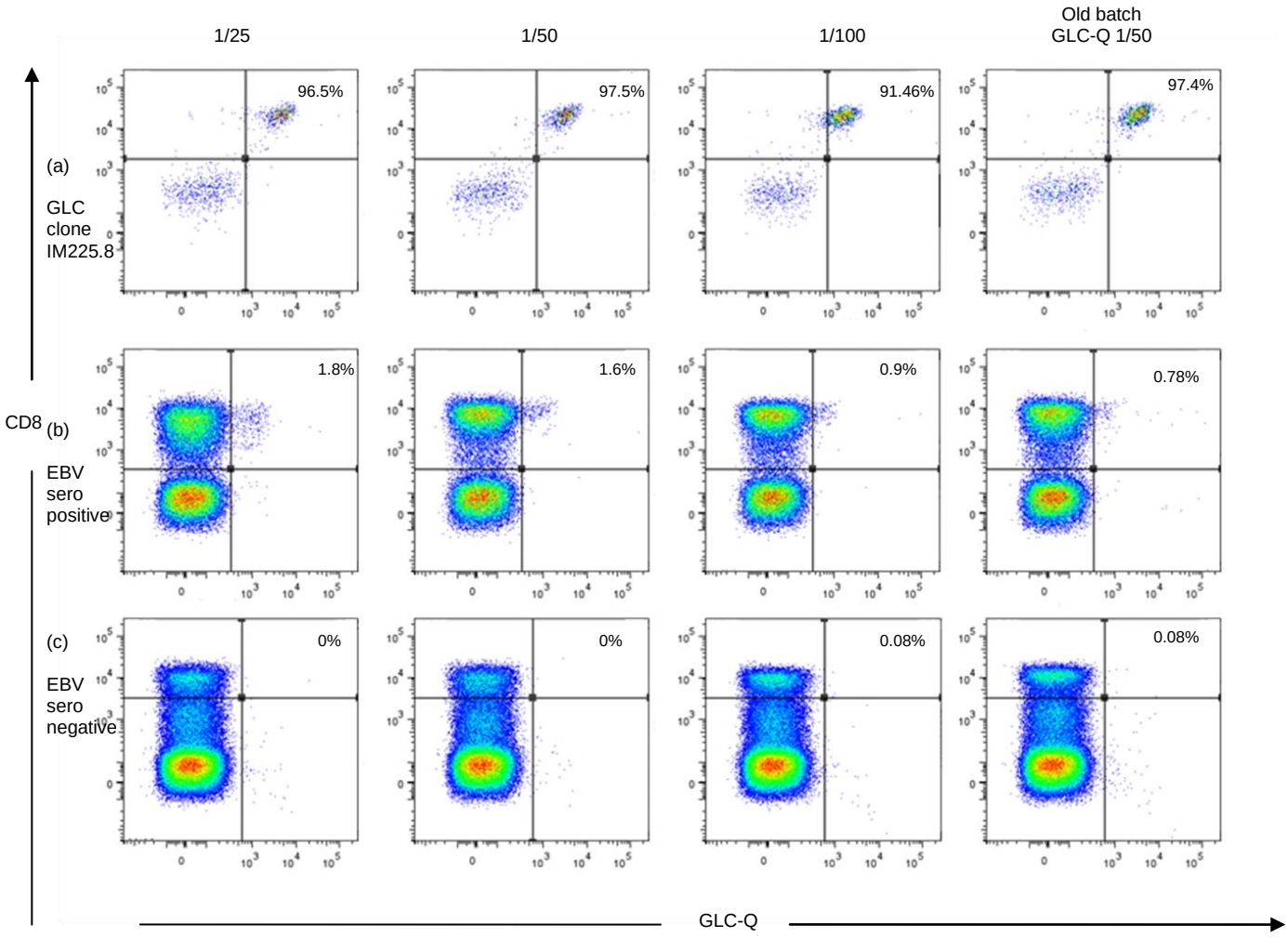


Figure 3.2. Frequency of EBV-specific CD8+ T cells in (a) GLC clone IM225.8, (b) PBMC's of a Sero positive donor and (c) PBMCs of a Sero negative donor.
GLC clones were stained with either 1/25, 1/50 or 1/100 dilutions of GLC loaded Qdot multimers (GLC-Q) and further stained with anti-CD8 mAbs. GLC clones and PBMCs were gated on the lymphocyte population. Values shown refer to the percentage of CD8+ cells stained with Qdot multimer.

3.3 Validation of Qdot multimer versus conventional PE tetramer staining of tonsillar Unfractionated Mononuclear (UM) populations.

In the next series of experiments, the capability of Q-dot multimers to detect the same frequency of EBV peptide-specific CD8+ T cells was compared to conventional Class I MHC tetramers in tonsillar UM populations by flow cytometry. To conduct this work additional Qdot multimers were made using the conditions determined above. Here HLA B8 monomers were made containing the HLA B8 presented peptides either RAK or FLR, from BZLF1 or EBNA3A, giving additional reagents to A2 GLC-Q for use in subsequent experiments. These new reagents were validated as described above (data not shown).

Tonsil 26 (T26) UM's was known to have relatively high numbers of CD8+ T cells specific for FLR (Hislop, Kuo et al. 2005) and were stained with FLR-Q multimer or a conventional FLR tetramer labelled with phycoerythrin (PE) and co stained with CD8 antibody. As a control, a culture of cells known to have FLR-specific cells present (IM63.1) was also stained with these reagents. Figure 3.3 shows that the FLR-PE and FLR-Q tested on FLR cells showed comparable results of 2.83% positive with FLR-PE and 2.96% positive with FLR-Q. Analysis of the T26 UMs showed a frequency of 5.3% of CD8+ T cells stained positive with FLR-Q and FLR-PE (Figure 3.3). The fluorescence intensity shown when staining T26 UMs was remarkably less bright compared to the FLR culture. The comparable results allows for the confidence of the use of Qdot multimers to stain for CD8+-T cells specific for EBV peptides in tonsils and is also comparable to conventional tetramer.

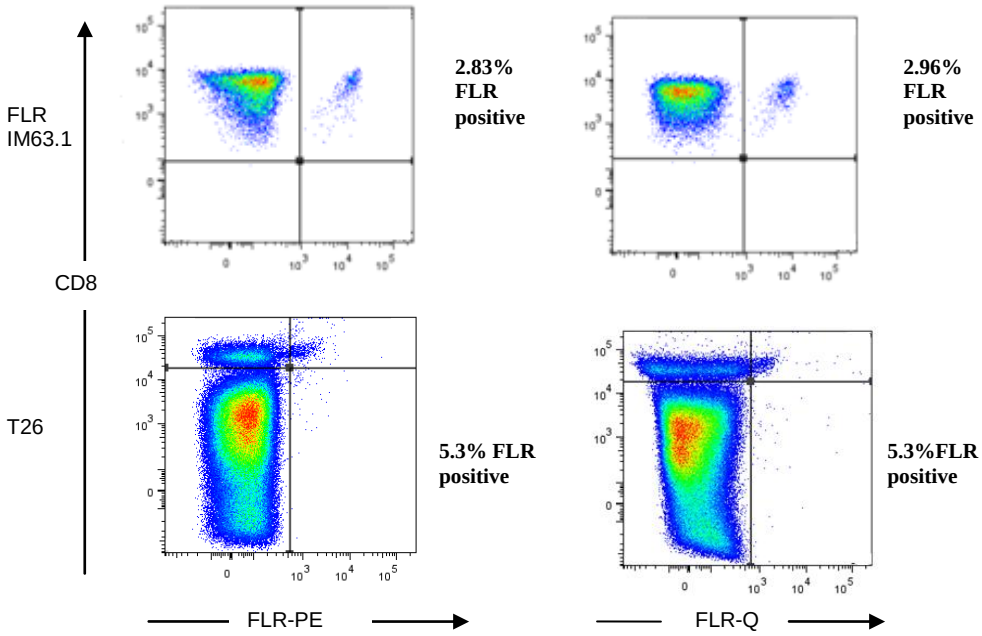


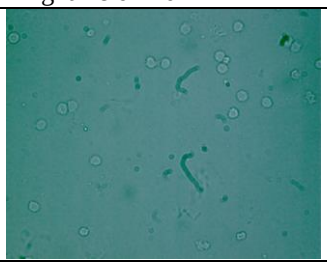
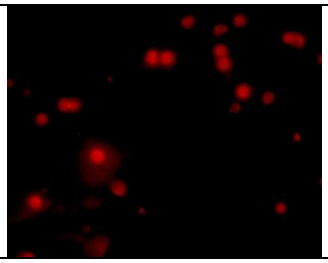
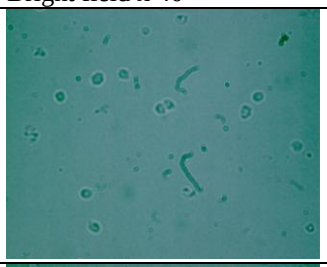
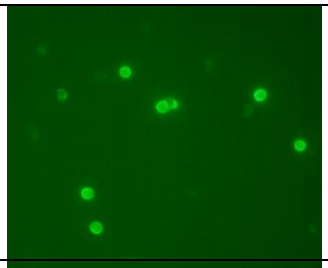
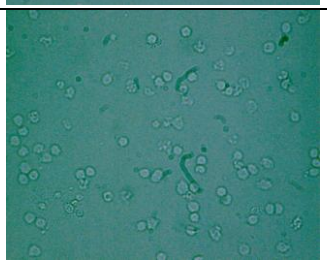
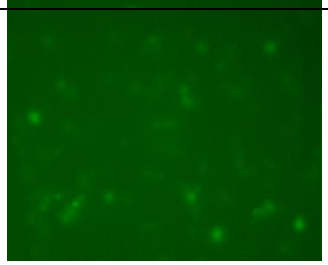
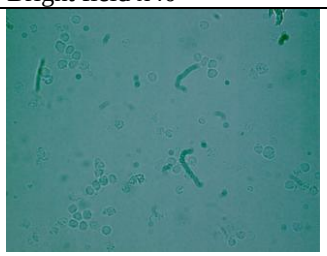
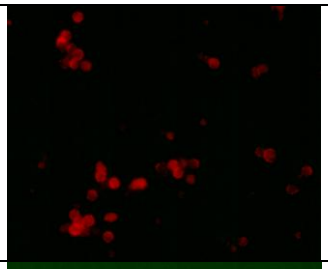
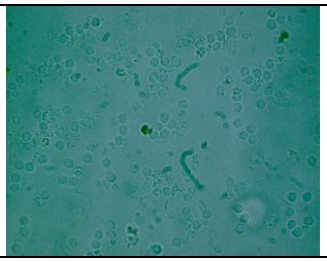
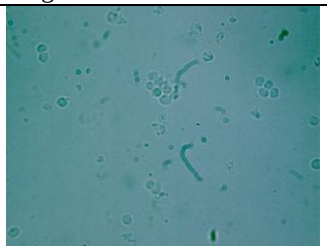
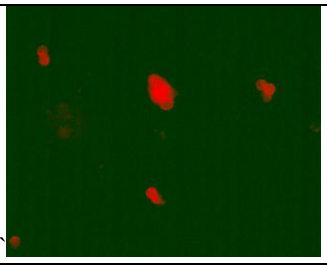
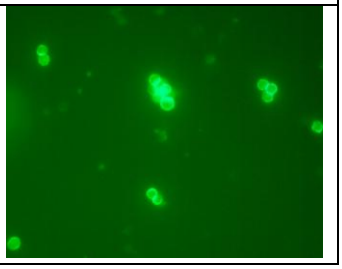
Figure 3.3 Validation of Qdot and PE tetramers vs. Tonsil Sample T26. FLR-PE and FLR-Q multimer stained T26 Unfractionated Mononuclear cells (UM) and FLR clones IM63.1. T26 UM and IM631 cells were stained with either FLR-Q multimer or FLR-PE tetramer and further stained for with CD8 conjugated antibody. The lymphocyte population was gated and the percentages shown refer to the percentage CD8 T cells which show FLR peptide specificity.

3.4 Epifluorescence analysis of GLC-specific clone staining using the Qdot multimer.

It was important to determine whether these Qdot multimers could be visualised by microscopic, and later, confocal analysis. GLC-specific clones were firstly stained with either directly conjugated anti-CD8-Qdot655 (CD8-Q) or CD8-FITC alone, GLC-Q alone or the combination of GLC-Q and CD8-FITC. The stained cells were then dried onto microscope slides, fixed using acetone/methanol and mounted with DABCO before viewing under the epifluorescent microscope. Firstly the clones stained with CD8-Q were viewed in order to determine if the microscope filters could detect the Qdot655 emission. Using the Texas red emission filter (600-650nm), overlapping with the emission of Qdot655 (600-700nm – figure 1.3), GLC clones stained for CD8-Q showed clear staining (Figure 3.4a). CD8-FITC staining alone of GLC clones was viewed under the FITC filter of the microscope (Figure 3.4b). The use of an isotype control (IgG2a) showed little non specific binding and cells stained with CD8-FITC can easily be distinguished from background staining (Figure 3.4b). Next, GLC clones stained with GLC-Q alone were viewed using the same settings as for CD8-Q. Using

these cells and GLC-Q combination, GLC-Q staining could be easily visualised (Figure 3.4c). To test for non specific staining, the GLC-Q was also used on an irrelevant YVL-specific clone. No fluorescence could be seen (Figure 3.4c), indicating that any fluorescence seen by the GLC-Q on the GLC clone is specific. As the ultimate goal for this project is to use the Qdot655 multimers alongside a T cell marker (CD8-FITC) for *in situ* staining, clones were stained with both GLC-Q and CD8-FITC and analysed by visualising using the FITC and Texas red filter sets. Figure 3.4d shows that cells that fluoresce for GLC-Q under the Texas red filter also fluoresce for CD8-FITC under the FITC filter. This result allows confidence in the use of GLC-Q in microscopic analysis and appropriately stained EBV-specific CD8 positive cells could be viewed using the combination of EBV specific peptide Qdot655 multimer and CD8-FITC co-stain.

Figure 3.4 – Epifluorescence microscopy of GLC clones. Stained with fluorescently labelled CD8 antibody alone (a & b), GLC-Q alone against GLC clones and YVL clones (c) or combined GLC-Q and anti CD8-FITC antibody staining on GLC clones (d).

	Filter sets		
	Bright field x40	Texas Red filter x40	
(a) GLC clone CD8-Qdot (1/100)			
	Bright field x 40	FITC filter x40	
(b) GLC clone CD8-FITC (1/50)			
GLC clone IgG2a FITC (1/50)			
	Bright field x40	Texas Red filter x40	
(c) GLC-Q alone (1/25) Vs GLC clone			
GLC-Q alone (1/25) Vs YVL clone			
	Bright Field x 60	Texas Red Filter x 60	FITC filter x 60
(d) GLC-Q (1/25) & CD8-FITC (1/50) Vs GLC clone			

3.5 Tissue staining with Qdot655 multimer, anti-CD8 and anti-CD20 and analysis by epifluorescence microscopy.

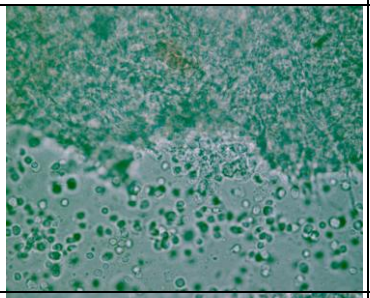
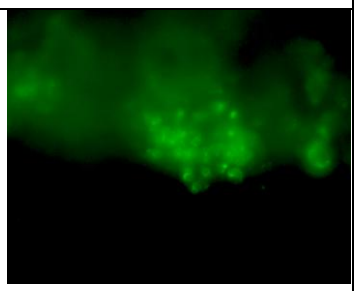
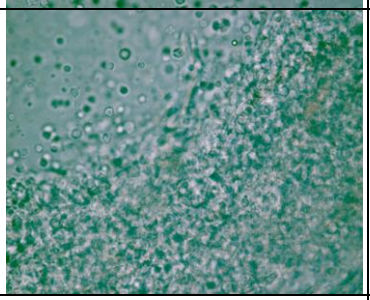
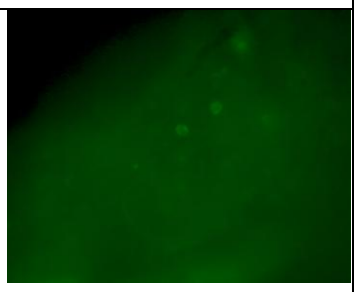
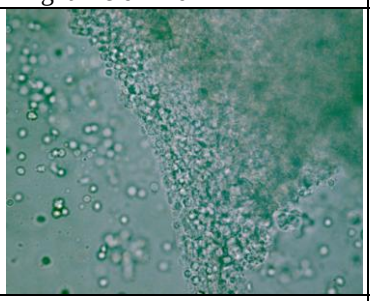
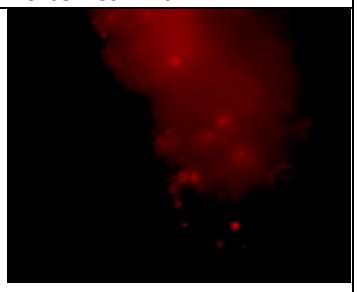
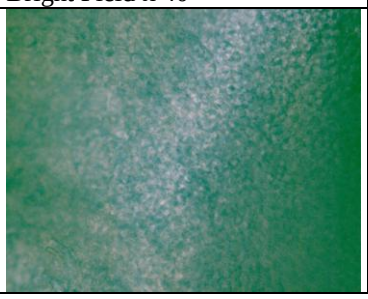
As the fluorochromes FITC and Qdot655 had been validated for use on the epifluorescence microscope, Qdot655 multimers were tested by conducting *in situ* tissue staining of a fresh tonsillectomy specimen (T43). T43 was sliced using a scalpel into thin sections and individual pieces stained overnight with, CD8-FITC, CD8-Q, GLC-Q alone or CD20-FITC. CD20 is an activation marker found on B cells, a prominent cell type within the tonsil due to presence of germinal centres. This staining was undertaken to give some anatomical context as to where cell types other than CD8+s could be found. The slices were then fixed and mounted on a microscope slide with DABCO and viewed under the epifluorescence microscope.

The first observation was that the tissue sections were too thick to view individual cells or tonsil structure under the epifluorescence microscope and it was only at the edges of the tissue sections which were thinner that cells could be seen either on bright field or UV light settings. CD20 staining of sections indicated a relatively high abundance of CD20 cells within the section stained as expected for a tonsil tissue (Figure 3.5a). However the CD8+T cells were more difficult to find within the tissue slice and were not frequent in areas where single cells could be viewed. However, those cells that fluoresced with CD8-FITC and CD8-Q were obvious compared with background staining seen throughout the tissue (Figure 3.5a). CD8-FITC became less bright overtime whilst the CD8-Q staining remained bright and appeared to be a more stable fluorophore. T43 was also stained with GLC-Q alone, however no fluorescence was seen when multiple fields of view were examined (Figure 3.5a). To confirm the absence of GLC-specific CD8+ T cells within the tonsil, a single cell homogenate of T43 made at the time of sectioning was subjected to conventional GLC-PE tetramer staining, analysed via flow cytometry gating on the lymphocyte population (Figure 3.5b). T43 showed a CD8+ frequency of 3.91% of the total lymphocyte population of which none were GLC-PE tetramer positive; this supports the microscope findings in Figure 3.5a. This demonstrates that using these reagents on such tissue sections gave low background staining. However given the low

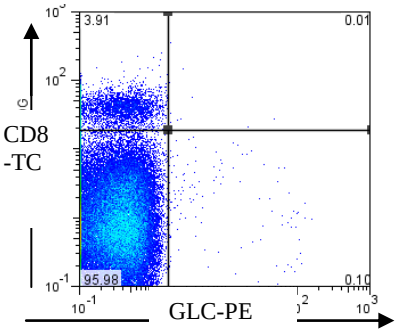
percentage of CD8+ T cells within T43 and also the fact that most of the tissue was too thick to view individual cells clearly meant that an alternative method to viewing thick tissue would be more successful and so attentions turned towards using confocal microscopy.

Figure 3.5 – In-situ tissue staining of Tonsil (T43) and Flow cytometry GLC-PE tetramer staining of T43 UM's

(a) **In Situ tissue staining of Tonsil T43, with CD20 FITC (green), CD8-FITC (green), CD8-Q (red) and GLC-Q (red)**

	Bright Field x 40	FITC x 40
CD20 FITC		
CD8 FITC		
	Bright Field x 40	Texas Red x 40
CD8 Q		
	Bright Field x 40	Texas Red x 40
GLC Q		

(b) **GLC-PE tetramer stain of T43 UM's**

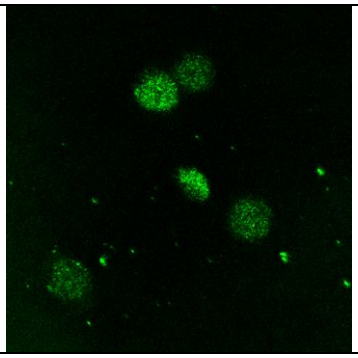
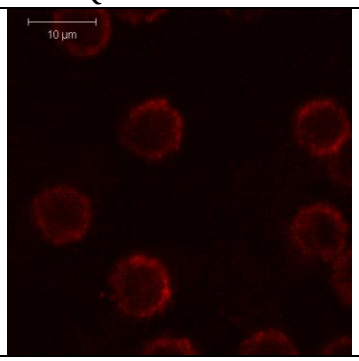
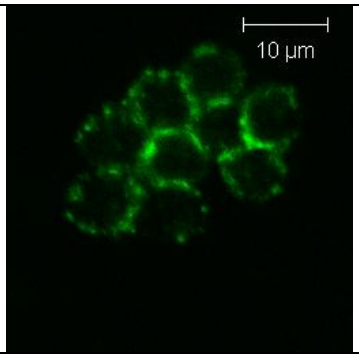
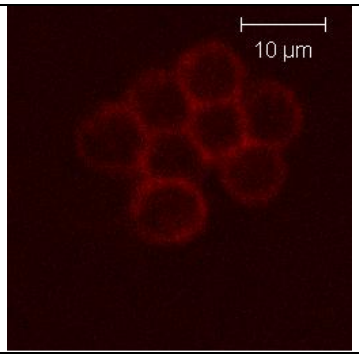
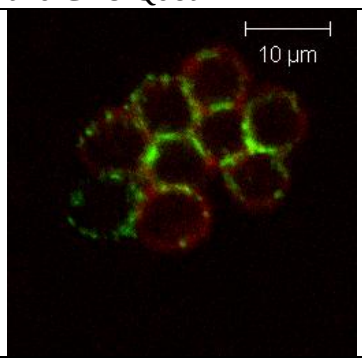


3.6 Optimisation of Confocal Microscopy using T cell clones stained with Qdot655 and Alexa488 labelled antibodies.

In order to ensure the correct laser and filter sets were in place on the confocal microscope GLC-specific clones were firstly stained with either CD8 conjugated to FITC (CD8-FITC) or CD8 conjugated to Qdot655 (CD8-Q). GLC IM225.8 T cell clones were stained individually with these reagents, fixed onto microscope slides and mounted with DABCO. FITC is excited at a wavelength of 488nm and emits at a wavelength of 525nm, the 488 laser was therefore used to excite and a 505LP green filter was used to capture emission. The CD8-FITC antibody was generally not stable enough for longer exposures as although staining was seen it quickly became photo bleached by the 488 laser and images deteriorated before they could be captured (Figure 3.6a).

In a second series of optimisation experiments an alternative fluorophore was sought that would not photo bleach under the 488 laser and so Alexa fluor 488 (Alexa488), which is a more stable equivalent to FITC, was used. The Alexa488 conjugated secondary antibody used was in conjunction with an anti MHC class I primary antibody against the GLC-specific clones. This fluorchrome proved to be more photo stable and gave clear staining of GLC clones (Figure 3.6b). The optimum excitation of Qdot655 nanocrystals is 364 nm and emits at 655 nm and so the CD8-Q could be viewed using the UV laser with a 650 LP red filter which let light with a wavelength of >650nm pass through and fluoresces red upon excitation and gave clear staining of GLC clones(Figure 3. 6a & b). GLC clones were also co-stained for class I and CD8 using the same reagents and the use of the 488 laser and the UV laser together with the correct filters (red and green) allowed for cells expressing Class I and CD8 to be viewed (Figure 3.6b). These results confirmed the settings on the confocal microscope were now in place in order to view Qdot655 and Alexa488 fluorochromes as a co-stain marker (Figure 3.6b).

Figure 3.6 – Confocal images of GLC clones stained with different excitation fluorochromes. (a) GLC clones IM225.8 stained with CD8 FITC or CD8-Q. CD8-FITC deteriorated under the 488 laser and staining was not clear. CD8-Q staining remained strong and extracellular staining could be seen in red. (b) GLC clones IM225.8 with class I Alexa 488 (green) co stained with CD8-Q (red) and mounted with DABCO.

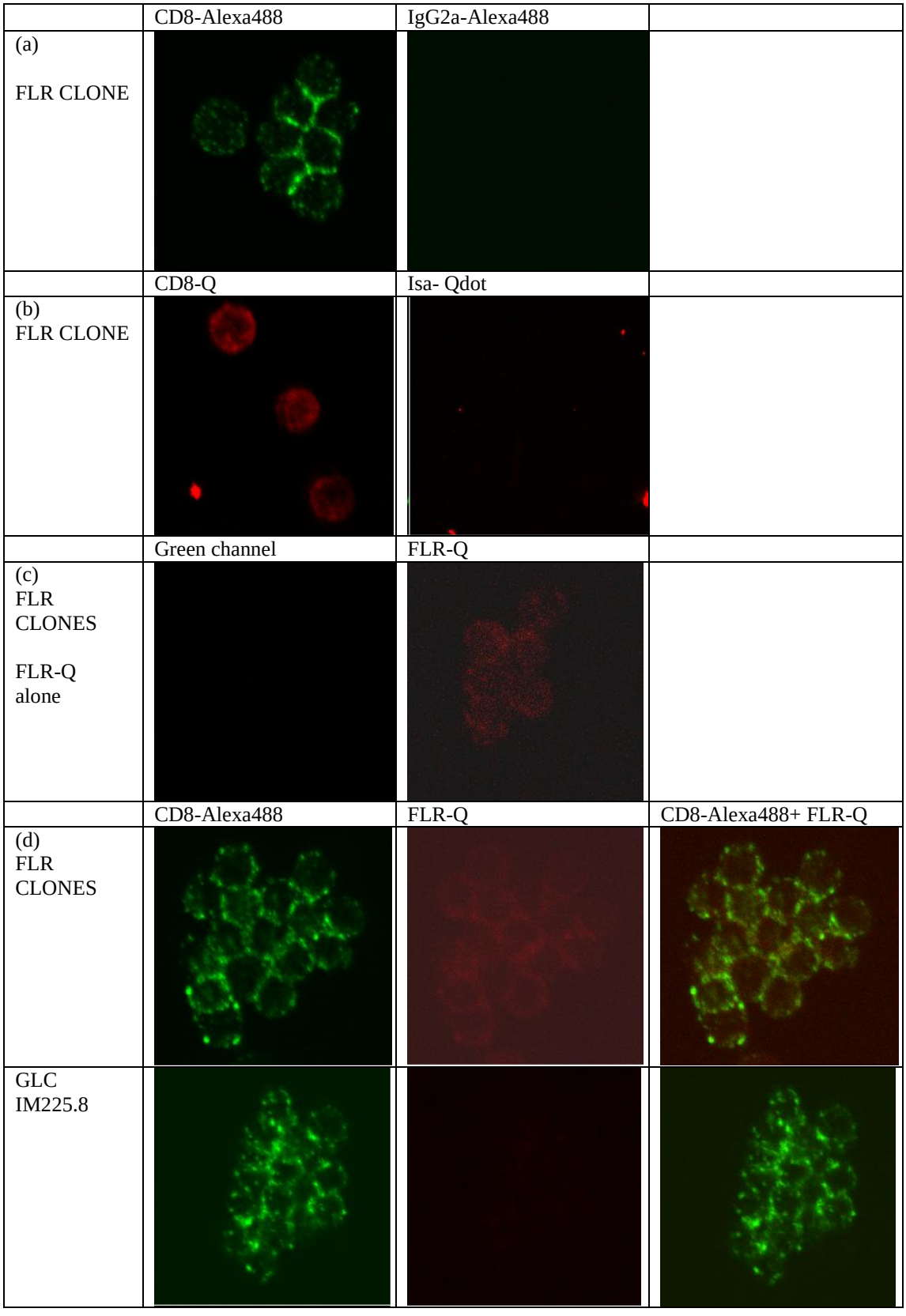
	CD8 - FITC	CD8-Qdot	
(a) GLC IM225.8			
	Class I Alexa488	CD8-Qdot	Combined Class I alexa488 and CD8-Qdot
(b) GLC IM225.8 DABCO			

3.7 Determining if the Qdot multimers can be viewed using the optimised confocal configuration.

It was vital to determine the Qdot655 multimers could also be viewed along side CD8-Alexa488. In this series of experiments FLR-specific clones were firstly stained with CD8-Q or an unlabelled CD8-specific antibody followed by the anti-mouse-Alexa488 antibody and fixed onto slides in order to validate the confocal configuration. Figure 3.7a shows clear staining of the CD8-Alexa488 and staining with isotype matched control antibodies in parallel showed no background staining. Figure 3.7b also showed clear staining with the CD8-Q of the FLR clones and showed absence of staining when using the isotype matched control. An aliquot of FLR-specific clones were also stained with FLR-Q alone and under the same configuration showed fluorescence of the FLR-Q in the red channel (Figure 3 7c) although it was not a bright as the CD8-Q staining (Figure 3.7b).

Finally the clones were co-stained with FLR-Q and CD8-Alexa488, were fixed and viewed under the confocal microscope. Figure 3.7d shows FLR-specific clones stained positive for CD8-Alexa488 (green) and those same cells also stained positive for FLR-Q (red) and could be viewed under this confocal configuration. Furthermore when an irrelevant T cell clone (GLC IM225.8) was stained with CD8-Alexa488 and FLR-Q, the clones only stained postive for CD8 (green), and showed no staining of FLR-Q (red) thus confirming the specificity of the multimer. This gave confidence in the ability to view CD8+ T cells specific for EBV peptides the Qdot multimers present in tissue sections under these microscope settings.

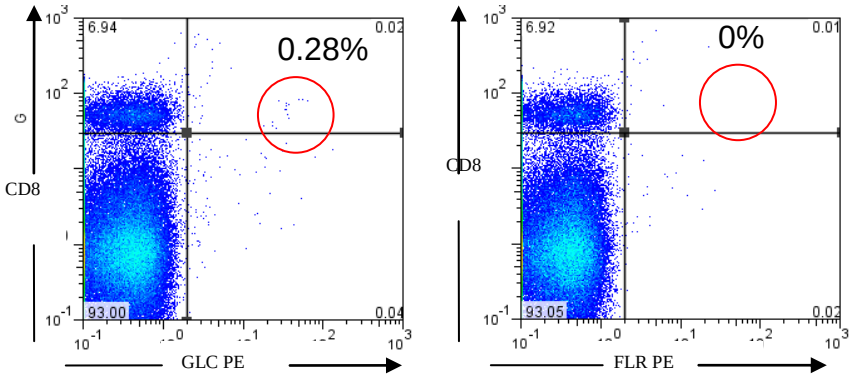
Figure 3.7 – Confocal images of FLR-Q, CD8-Alexa488 and CD8-Q staining using FLR clones. FLR clones were stained with CD8-Alexa488 alone (a) CD8-Q alone (b) FLR-Q alone (c) and with the combination of CD8-Alexa488 and FLR-Q or irrelevant GLC-Q (d).



3.8 In situ staining of tonsil sections with Qdot multimers and confocal imaging.

In the final series of experiments a fresh tonsil was collected for sectioning and staining with Qdot multimers. Firstly to determine if any GLC- or FLR-specific CD8+ T cells were present in the T48 tonsil flow cytometry analysis was performed on a homogenised sample of the tonsil an stained with GLC-PE or FLR-PE tetramers followed by anti-CD8. This analysis showed a cluster of cells appeared when GLC-PE tetramer was used and accounted for 0.28% of the CD8+ population, however in the presence of FLR-PE no cluster could be seen (Figure 3.8).

Figure 3.8 – Flow cytometry analysis of T48 UMs. Staining of T48 UM's with GLC-PE and FLR – PE tetramer. Percentage represents the number of CD8+ cells stained positive for tetramer.



As before, the remainder of the tonsil was sliced into 200µm sections using a vibratome and these were stained overnight with either unlabelled anti-CD8 followed by anti-mouse 488, or CD8-Q alone, or with Qdot multimer alone (FLR-Q or GLC-Q), or with Qdot multimer and unlabelled anti CD8 antibody followed by anti-mouse Alexa488 co-stain. The sections were then fixed, mounted with DABCO and viewed by confocal microscopy. Firstly, CD8-Alexa488 staining (green) could be distinguished in spite of more background fluorescence of the tissue in the presence of CD8-Alexa488 (Figure 3.9a). Interestingly, the CD8-Alexa488 positive cells appeared to be found in clusters towards the outer edge (Figure 3.9a) of the tissue and less frequently in the centre of the tissue, however without any other anatomical landmark staining the importance of this was

unknown (Figure 3.9b). The absence of staining in the presence of isotype-Alexa488 (Figure 3.9c) indicates specific binding of the CD8-Alexa488 antibody (Figure 3.9a).

T48 was also stained with CD8-Q in order to ensure Qdot655 multimers would be able to be visualised in tissue under the current confocal configuration. CD8-Q stained cells were clearly visualised in the T48 sections (Figure 3.9d) however the same distribution of CD8-Q stained cells was seen as before with the CD8-Alexa488, showing to be more frequent towards the edge of the tissue compared with the centre along the same focal plane (Figure 3.9e). Isotype matched IgG2a Qdot655 antibody showed no staining within the T48 sections showing the specific binding of CD8-Q (Figure 3.9f).

Next, the sections that were stained with A2 GLC-Q or B8 FLR–Q were viewed to look for any CD8+ T cells specific for these two EBV derived peptides. Confocal microscopy revealed although CD8-Alexa488 stained T cells could be seen, none of these CD8-Alexa488 positive cells showed co staining with GLC-Q or FLR-Q in the multiple fields viewed (Figure 3.10c & d). In other sections from the same tonsil FLR-Q and GLC-Q were used alone to eliminate any interference that may be caused by the CD8-Alexa488 and again no GLC-Q or FLR-Q positive cells could be seen verifying the lack of CD8+ T cells staining for multimer (Figure 3.10 a &b).The low frequency of GLC specific CD8+ T cells in the flow data (Figure 3.8) where approximately 7% of UMs were CD8 positive could account for the lack of GLC-Q positive cells in the tissue section that was stained (Figure 3.9b).

Figure 3.9 – Confocal images, validating Alexa488 and Qdot655 reagents for use in in-situ staining of the tonsil (T48). T48 sections were individually stained with anti CD8 antibodies or isotype control conjugated with Alexa488 (a,b + c) or with Qdot655 reagents (d,e +f).

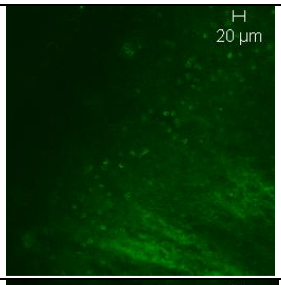
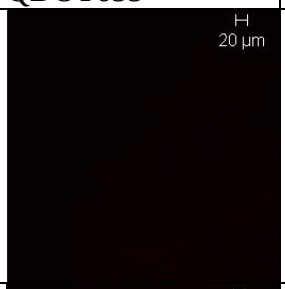
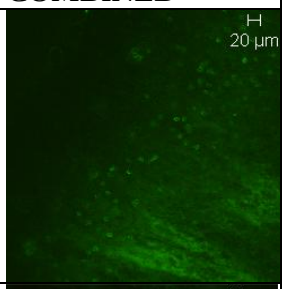
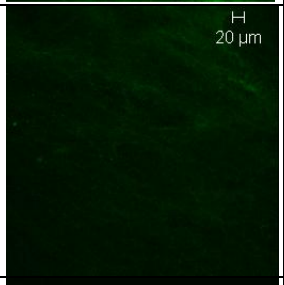
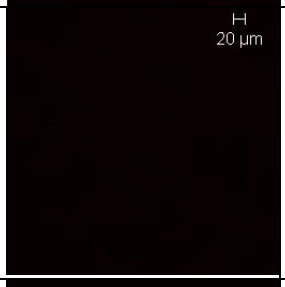
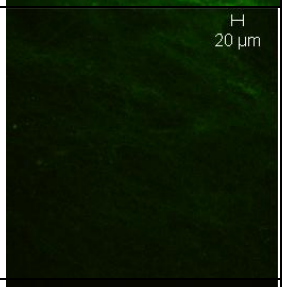
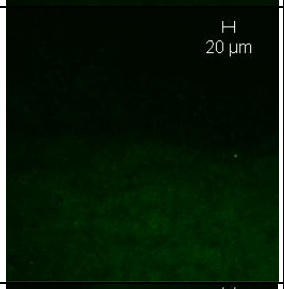
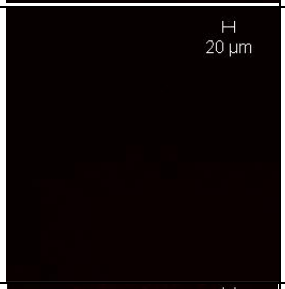
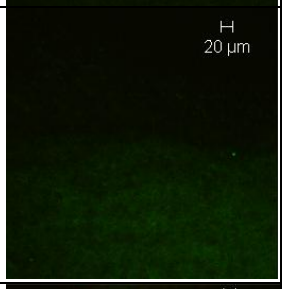
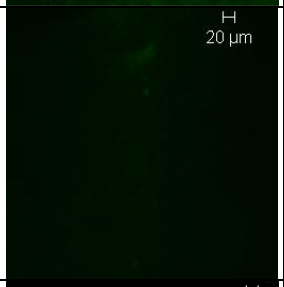
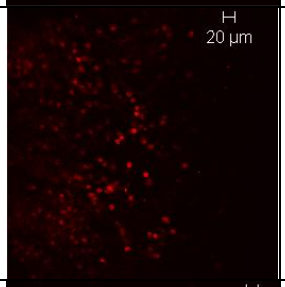
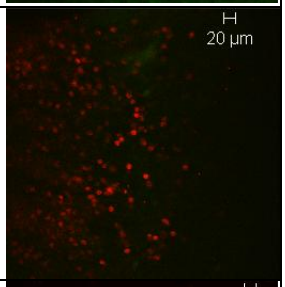
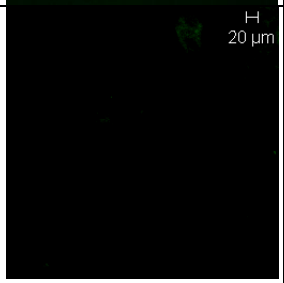
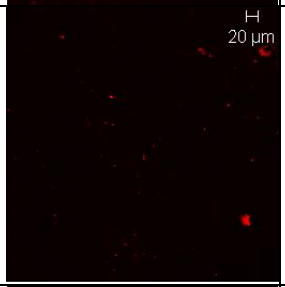
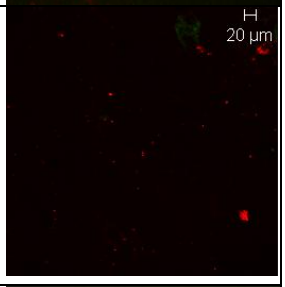
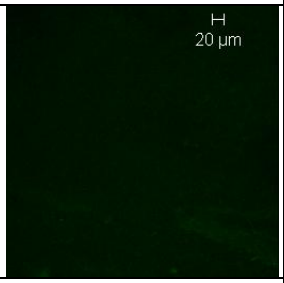
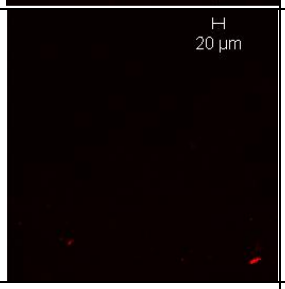
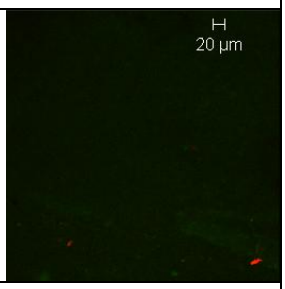
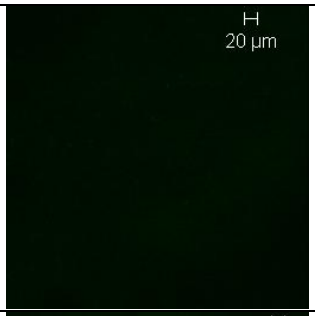
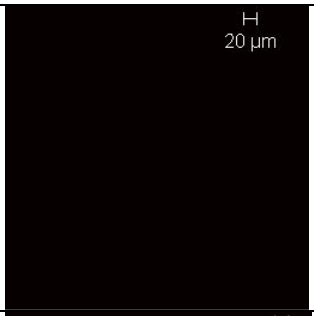
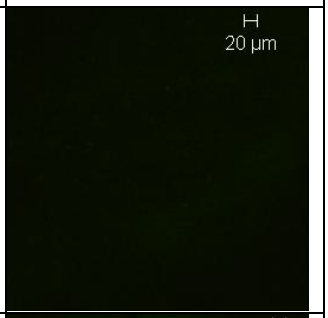
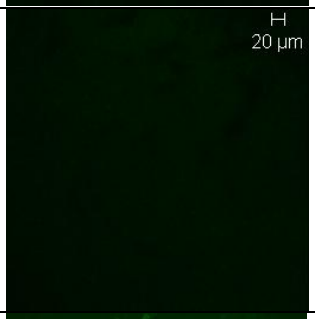
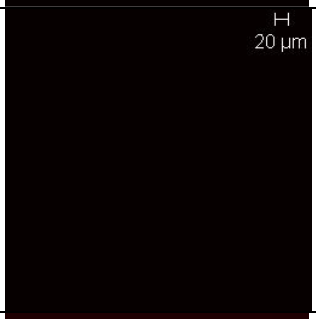
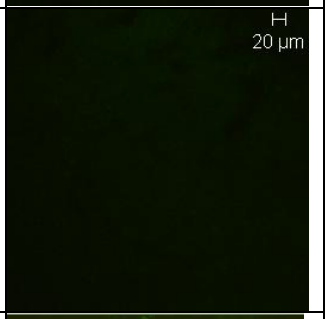
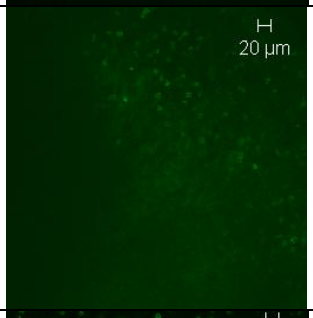
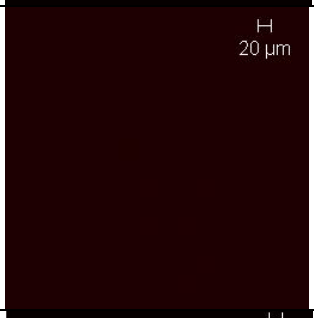
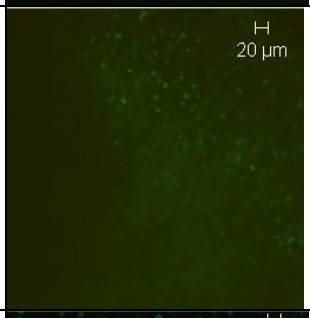
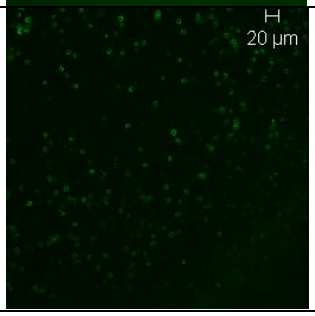
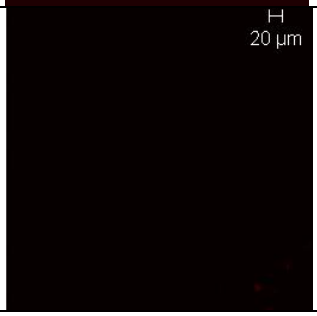
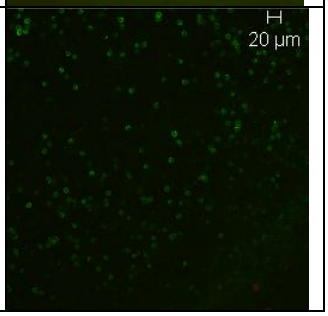
	488	QDOT655	COMBINED
(a) CD8 488 Outer edge			
(b) CD8 Alexa488 Centre of tissue			
(c) IgG2a Alexa488			
(d) CD8 Q Outer edge			
(e) CD8 Q Centre of tissue			
(f) IgG2a QDOT655			

Figure 3.10 – confocal images of combined antibody and tetramer *in situ* staining of T48. T48 was stained with FLR-Q alone (a) GLC-Q alone (b) and combination staining with CD8-Alexa488 and either FLR-Q (c) or GLC-Q (d).

	Green filter	Red filter	COMBINED
(a) FLR-Q alone			
(b) GLC-Q alone			
(c) CD8- Alexa488 and FLR-Q			
(d) CD8 Alexa488 and GLC-Q			

4 DISCUSSION

Hislop *et.al* 2005 and Woodberry *et.al* 2005 previously discovered that the human tonsil harbours high amounts of EBV-specific CD8+ T cells compared to the peripheral blood of healthy EBV infected carriers. This has led to the aims of this project to discover the localisation of these EBV-specific CD8+ T cells within the tonsil. Tetramer technology has been adapted for use in, *in situ* tissue staining and has allowed the visualisation of virus-specific CD8+ T cells in tissue (Skinner, Daniels et al. 2000) and has also enabled the discovery of T cell localisation and interactions of virus-specific CD8+ T cells with surrounding tissue cells(Zhu, Koelle et al. 2007; Hong, Reynolds et al. 2009). Tetramers are shown to work best in fresh tissue and using them in direct staining reduces background fluorescence (Haanen, van Oijen et al. 2000). Therefore, direct tetramer staining of fresh tonsil tissue was to be implemented in this project but, before this can be achieved much validation and problem solving was performed.

The tonsil proved to be a structurally tough organ due to a stratified squamous nonkeratinized epithelium which is underlined by a band of thick connective tissue (Perry and Whyte 1998). In contrast the lymphoepithelium, which lines the tonsillar crypts and contains lymphocytes(Perry and Whyte 1998), can be described as quite spongy. The tough outer epithelium and spongy lymphoepithelium made slicing of fresh tonsil into thin enough slices for viewing structure as well as single T cells quite challenging. The tissue needed to be sliced into small sections (0.5cm/0.5cm), embedded in 4% agarose and kept chilled, maintaining the freshness of the tissue but also making the tonsil tissue more firm and therefore easier to cut. Even so it took many attempts with a vibratome to obtain satisfactory sections, often producing a limited number of sections mostly due to the elasticity of the tissue, which is extremely problematic for further use. In future studies an option may be to keep the tissue stored in PBS at 4˚C overnight which may make the tonsil tissue more rigid. This method has been done in past studies showing no difference in tetramer staining

compared to tissue that is stained shortly after sectioning (Skinner, Daniels et al. 2000) and prevents the interference of chemicals and maintaining the freshness of the tissue as much as possible.

The generation of more tonsil sections would have made this project more feasible, but to account for the difficulties in slicing of the tonsil may mean it would have had to be processed in a different way. Snap freezing tissue proves unsuccessful for tetramer staining as this kills the cells (Skinner and Haase 2002). However staining small tissue sections with tetramer first before snap freezing and sectioning has been shown to provide sections with tetramer staining comparable to that of fresh sections (Vyth-Dreese, Kim et al. 2006) however cryosections of tissue are generally much thinner than fresh sections and cause problems in looking for low frequency T cells(Skinner, Daniels et al. 2000).

Out of the 8 tonsils that were collected only 1, T48, showed a response to GLC tetramer by flow cytometry however this response was quite low at 0.28% of the total CD8+ which is only 7% of the total UM population in T48. (Figure 3.8). This indicates that out of 5000 UM's in the tonsil only 1 will be a CD8+ T cell specific for the EBV GLC-epitope, if these cells were homogenously distributed through the tissue. The fact that only a limited number of sections were obtained from one piece of tonsil dissected in which only two slices were stained with GLC-Q multimer, made the odds of discovering the low frequency of GLC-specific CD8+ T cells remote. It was therefore not so unexpected that the 0.28% GLC-specific CD8+ T cells could not be visualised by microscopy (Figure 3.10). However, it cannot be excluded that the staining protocol was not successful. In future experiments visualising other specificities, which maybe more in enriched in the patient, could be studied.

The lack of medical history of the tonsil specimens collected throughout this study meant that the EBV status of the patient and whether the donor had responses to the epitopes used was unknown. Another complication was that the HLA type of the patient was also unknown at the time of staining. As such the multimer staining was done blindly using HLA A2 GLC, HLA B8 RAK and

HLA B8 FLR multimers. Furthermore, even if a patient was the correct HLA type and is EBV infected they may not necessarily mount a response to a known HLA restricted EBV peptide (Bihl, Frahm et al. 2006). As described above the small snapshot of tonsil viewed by microscopy can not provide the full picture and so the remainder of each tonsil collected was homogenised and the UM's analysed by flow cytometry with the same peptide panel of tetramers/multimer. Flow data and *in situ* staining have been shown to be comparable (Skinner and Haase 2002) and one success of the Qdot655 multimers is a lack of non-specific binding as background levels were low in flow cytometry and microscopy experiments. It also revealed that the majority of tonsils collected in this project did not contain T cell specific to the tetramers tested and is consistent with the lack of multimer positive cell staining in the tissue (all data not shown).

Qdot nanocrystals were used due to them characteristically being a more photo stable, brighter alternative to traditional fluorescent dyes (Chattopadhyay, Price et al. 2006) and can be conjugated to streptavidin and saturated with MHC class I to produce Qdot multimers for use in direct *in situ* staining. Zhu, et al 2007 have used Qdot multimers to identify the localisation of low frequency HSV-2 specific CD8+ T cells in skin lesions and prompted the use of Qdot multimers in this study to look at the localisation of EBV-specific CD8+ T cells in tonsil tissue. Validation experiments using flow cytometry show that although the Qdot655 multimers showed comparable specificity they did not appear to be much brighter in fluorescence intensity compared to conventional tetramers (Figure 3.3). When used on T cell clones to validate the use of the Qdot655 multimers in confocal microscopy, they did not appear very bright at all especially when compared to the CD8-Q counterpart perhaps reflecting the affinities with which these reagents bind to their ligands.

The results in Figure 3.9 showed an apparent difference in CD8+ staining, more frequent at the edge of the tissue compared with the low frequencies in the centre of the tissue, along the same focal plane. The antibody not infiltrating the tissue effectively maybe a cause of this anomaly and only staining cells towards the edge of the tissue might be due to thinning of the tissue at the edges.

If this is the case, the same effects maybe seen with use of the Qdot655 multimers in tissue however this is all speculative. Validating the use of Qdot655 multimers in microscopy has been confirmed with the use of matched CD8+ T cell clones to Qdot655 multimers which have been an indispensible tool throughout the project.

A limitation in using the Qdot655 multimer in *in situ* tissue staining is that its use in tissue could not be validated. One reason for this is the lack of a tonsil sections thin enough for analysis, but another reason is the lack of tonsil samples collected containing tetramer positive CD8+ T cells making it difficult to determine if the Qdot655 multimers produced would work in this staining protocol. Other groups have used TCR transgenic mice to ensure T cells expressing the matched TCR's are present for the tetramers to bind(Skinner, Daniels et al. 2000), however this was not conceivable for this project. Future experiments maybe to incubate tonsil sections with T cell clones, relying on migration into the tonsil sections and then stain with the matched Qdot655 multimers.

The use of EBV-specific T cell targeting tetramers in in-situ tissue staining can provide much more information about their localisation within tonsil tissue by co staining for other cells in the environment. It is still unclear if the EBV-specific CD8+ T cells are located near to the germinal centres of B cells or located near to Epithelium of the tonsillar crypts or tonsil surface. Once the tetramers have been optimised for use in tonsil tissue these questions will be answered.

5 LIST OF REFERANCES:

Altman, J. D., P. A. Moss, et al. (1996). "Phenotypic analysis of antigen-specific T lymphocytes." Science **274**(5284): 94-6.

Appay, V. and S. L. Rowland-Jones (2002). "The assessment of antigen-specific CD8+ T cells through the combination of MHC class I tetramer and intracellular staining." J Immunol Methods **268**(1): 9-19.

Bauquet, A. T., H. Jin, et al. (2009). "The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells." Nat Immunol **10**(2): 167-175.

Bihl, F., N. Frahm, et al. (2006). "Impact of HLA-B alleles, epitope binding affinity, functional avidity, and viral coinfection on the immunodominance of virus-specific CTL responses." J Immunol **176**(7): 4094-101.

Callan, M. F., C. Fazou, et al. (2000). "CD8(+) T-cell selection, function, and death in the primary immune response in vivo." J Clin Invest **106**(10): 1251-61.

Chattopadhyay, P. K., S. P. Perfetto, et al. (2010). "The use of quantum dot nanocrystals in multicolor flow cytometry." Wiley Interdiscip Rev Nanomed Nanobiotechnol **2**(4): 334-48.

Chattopadhyay, P. K., D. A. Price, et al. (2006). "Quantum dot semiconductor nanocrystals for immunophenotyping by polychromatic flow cytometry." Nat Med **12**(8): 972-7.

Crawford, D. H., K. F. Macsween, et al. (2006). "A cohort study among university students: identification of risk factors for Epstein-Barr virus seroconversion and infectious mononucleosis." Clin Infect Dis **43**(3): 276-82.

Deacon, E. M., G. Pallesen, et al. (1993). "Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells." J Exp Med **177**(2): 339-49.

Deerinck, T. J. (2008). "The application of fluorescent quantum dots to confocal, multiphoton, and electron microscopic imaging." Toxicol Pathol **36**(1): 112-6.

Epstein, M. A., B. G. Achong, et al. (1964). "VIRUS PARTICLES IN CULTURED LYMPHOBLASTS FROM BURKITT'S LYMPHOMA." The Lancet **283**(7335): 702-703.

Haanen, J. B., M. G. van Oijen, et al. (2000). "In situ detection of virus- and tumor-specific T-cell immunity." Nat Med **6**(9): 1056-60.

Haque, T., G. M. Wilkie, et al. (2002). "Treatment of Epstein-Barr-virus-positive post-transplantation lymphoproliferative disease with partly HLA-matched allogeneic cytotoxic T cells." Lancet **360**(9331): 436-42.

Heslop, H. E., C. Y. C. Ng, et al. (1996). "Long-term restoration of immunity against Epstein-Barr virus infection by adoptive transfer of gene-modified virus-specific T lymphocytes." Nat Med **2**(5): 551-555.

Hislop, A. D., M. Kuo, et al. (2005). "Tonsillar homing of Epstein-Barr virus-specific CD8+ T cells and the virus-host balance." J Clin Invest **115**(9): 2546-55.

Hislop, A. D., G. S. Taylor, et al. (2007). "Cellular responses to viral infection in humans: lessons from Epstein-Barr virus." Annu Rev Immunol **25**: 587-617.

Hong, J. J., M. R. Reynolds, et al. (2009). "Localized populations of CD8 MHC class I tetramer SIV-specific T cells in lymphoid follicles and genital epithelium." PLoS One **4**(1): e4131.

Hutt-Fletcher, L. (2005). EBV entry and epithelial infection. Epstein-Barr Virus. E. S. Robertson, Caister Academic Press**:** 359-378.

Khanna, R., S. Bell, et al. (1999). "Activation and adoptive transfer of Epsteinâ€“Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease." Proceedings of the National Academy of Sciences **96**(18): 10391-10396.

Li, Q., P. J. Skinner, et al. (2009). "Visualizing antigen-specific and infected cells in situ predicts outcomes in early viral infection." Science **323**(5922): 1726-9.

Li, Q., M. Spriggs, et al. (1997). "Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes." J. Virol. **71**(6): 4657-4662.

Niederman, J. C., R. W. McCollum, et al. (1968). "Infectious mononucleosis. Clinical manifestations in relation to EB virus antibodies." JAMA **203**(3): 205-9.

Perry, M. and A. Whyte (1998). "Immunology of the tonsils." Immunol Today **19**(9): 414-21.

Reynolds, M. R., E. Rakasz, et al. (2005). "CD8+ T-lymphocyte response to major immunodominant epitopes after vaginal exposure to simian immunodeficiency virus: too late and too little." J Virol **79**(14): 9228-35.

Shannon-Lowe, C., E. Adland, et al. (2009). "Features distinguishing Epstein-Barr virus infections of epithelial cells and B cells: viral genome expression, genome maintenance, and genome amplification." J Virol **83**(15): 7749-60.

Skinner, P. J., M. A. Daniels, et al. (2000). "Cutting edge: In situ tetramer staining of antigen-specific T cells in tissues." J Immunol **165**(2): 613-7.

Skinner, P. J. and A. T. Haase (2002). "In situ tetramer staining." J Immunol Methods **268**(1): 29-34.

Vyth-Dreese, F. A., Y. H. Kim, et al. (2006). "In situ visualization of antigen-specific T cells in cryopreserved human tissues." J Immunol Methods **310**(1-2): 78-85.

Woodberry, T., T. J. Suscovich, et al. (2005). "Î±EÎ²7 (CD103) Expression Identifies a Highly Active, Tonsil-Resident Effector-Memory CTL Population." The Journal of Immunology **175**(7): 4355-4362.

Young, L. S. and A. B. Rickinson (2004). "Epstein-Barr virus: 40 years on." Nat Rev Cancer **4**(10): 757-68.

Zhu, J., D. M. Koelle, et al. (2007). "Virus-specific CD8+ T cells accumulate near sensory nerve endings in genital skin during subclinical HSV-2 reactivation." J Exp Med **204**(3): 595-603.