Cytotoxic T lymphocyte immunodominance to Epstein-Barr virus infection

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Abstract

EBV lytic replication involves the sequential expression of a large array of antigens that potentially provides a complex antigenic challenge. Despite this number, the primary CD8+ T cell response in infectious mononucleosis (IM) patients appears focused against epitopes found in immediate-early and some early expressed antigens with responses to late antigens typically subdominant. However previous approaches have only focused on a limited number of lytic antigens. To resolve these issues, this study examines the CD8+ T cell repertoire to all EBV lytic antigens in different phases of infection.

Polyclonal CD8+ T cell lines were mitogenically expanded from IM patients or expanded in an antigenically unbiased way from post-IM patients and healthy carriers using autologous dendritic cells loaded with lysates of lytically infected cells. Target cells expressing individual lytic antigens along with donor HLA class I molecules were used to challenge polyclonal lines with responses measured by the release of IFNγ.

These studies show that the pattern of target antigen choice varies with the phase of infection and is consistent with the idea that CD8+ T cell responses in primary infection are driven by lytically infected B cells. However over time the repertoire of responses may be influenced through antigen cross-presentation.

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ABC	ATP-binding cassette
ACV	Acyclovir
AP-1	Activator protein-1
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
β2m	Beta-2-microglobin
BCR	B cell receptor
BFA	Brefeldin A
CARD	Caspase recruitment domain
CBF-1	Cp binding factor-1
CDR	Complementarity-determining regions
CLIP	Class II peptide
CLRs	C-type lectin receptors
CSF-1	Colony-stimulating factor-1
CTL	Cytotoxic lymphocyte
DAMPs	Danger-associated molecular patterns
DCs	Dendritic cells
DMSO	Dimethyl sulphoxide
DRiPs	Defective ribosomal products
dsDNA	Double-stranded DNA
E	Early
EBERs	EBV-encoded small RNAs
EBNA	EBV nuclear antigen
EBV	Epstein Barr Virus
ELISA	Enzyme linked immunosorbant assay
ER	Endoplasmic reticulum
ERAAP	ER aminopeptidase-associated with antigen processing
ERAD	ER-associated protein degradation

FasL	Fas-ligand
FcR	Fc receptors
FCS	Foetal calf serum
Flt3L	Flt3 ligand
FRET	Fluorescence resonance energy transfer
Gly-Ala	Glycine-alanine
GM-CSF	Granulocyte/macrophage colony-stimulating-factor
HC	Heavy chain
HCMV	Human cytomegalovirus
HD	Healthy donor
HDACs	Histone deacetylases
HHV	Herpesvirus
HLA	Human leukocyte antigens
HRP	Horse-radish peroxidase
HS	Human serum
HSV	Herpes simplex virus
ICAM	Inter-cellular adhesion molecule
IE	Immediate early
IEDB	Immune epitope database
IFN	Interferon
IL	Interleuken
IM	Infection mononucleosis
IMGT	ImMunoGeneTics
IRF	Interferon regulatory factor
KSHV	Kaposi's sarcoma
L	Late
LCL	Lymohoblastoid cell lines
LCs	Langerhan cells
LFA	Lymphocyte function-associated antigen

LMP	Latent membrane proteins
LPS	Lipopolysaccharide
LRR	Leucine-rich repeats
MCMV	Murine cytomegalovirus
MCP	Major capsid protein
mCP	Minor capsid protein
mDCs	Myeloid DCs
MDP	Macrophage DC progenitor
MHC	Major histocompatibility complexes
MIIC	MHC class II late endosomal compartment
miRNAs	MicroRNAs
moDCs	Monocyte-derived dendritic cells
MTP	Major tegument protein
MyD88	Myeloid differentiation primary response gene 88
NBD	Nucleotide binding domain
NFkB	Nuclear factor-kB
NK	Natural killer
NLRs	NOD-like receptors
NOD	Nucleotide oligomerisation domain
NOX2	NADPH-oxidase 2
OriP	Origin of viral replication
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDCs	Plasmacytoid dendritic cells
PFA	Paraformaldehyde
PHA	Phytohaemagglutinin
PLC	Peptide loading complex

pMHC	Peptide-MHC
PMNs	Polymorphonuclear leukocytes
Poly(I:C)	Polyinosinic–polycytidylic acid potassium salt
PRR	Pattern-recognition receptors
PTLD	Post transplant lymphoproliferative disorder
rhLCV	Rhesus lymphocryptovirus
RIG-I	Retinoic acid-inducible
RLRs	RIG-I- like receptors
ROS	Reactive oxygen species
RR	Ribonucleotide reductase
SM	BSLF2-BMLF1 spliced gene
SR	Scavenger receptors
TAP	Transporter associated with antigen processing
TCR	T cell receptors
TIR	Toll/Interleukin-1 receptor
TLR	Toll-like receptors
TNFR	Tumour necrosis factor receptor
TOP	Thimet oligopeptidase
TPA	12-O-tetradecanoylphorbol-13-acetate
TPPII	Tripeptidyl peptidae II
TRAIL	TNF-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adapter-inducing interferon-γ
Ub	Ubiquitin
vGPCR	Viral G-protein coupled receptor
VV	Vaccinia viruses
VZV	Varicella zoster virus
XLP	X-linked lymphoproliferative
ZREs	Z-responsive element

CHAPTER 1

Introduction

1.1. The immune response to pathogens

Many microorganisms including viruses, bacteria or parasites require a specific niche for them to survive and to proliferate and these niches can include colonisation of host animals. This colonisation can be symbiotic with both the microbes and the host benefiting as is the case with gut flora. However this colonisation can also lead to disease particularly with newly invading microorganisms that can proliferate uncontrollably and cause damage to tissues prior to host control. To prevent disease the human immune system has evolved, which serves to recognise and counter the threat. Likewise microorganisms have evolved mechanisms to help evade these defences in what has been described as an 'arms race' between host and pathogen.

The first line of the immune system is the innate response that serves to provide rapid, non-specific protection against invading microbes. The innate immune system is comprised of circulating and tissue resident phagocytes such as dendritic cells (DCs) and macrophages, natural killer cells (NK), polymorphonuclear leukocytes (PMNs) including neutrophils, eosinophils, basophils and mast cells, and complement. These respond through germline-encoded pattern-recognition receptors (PRRs) that are capable of binding certain highly conserved and microbial components called pathogen-associated molecular patterns (PAMPs). Examples of PAMPs include microbial carbohydrates, lipopolysaccharides, proteins and viral RNA or DNA. Recognition of different microbial ligands by specific PRRs

leads to downstream signalling resulting in production of proinflammatory cytokines and chemokines. These signals by the innate response alert the adaptive immune system to danger.

The adaptive immune response is characterised by being highly antigen-specific, distinguishing 'self' from 'non-self' and by developing 'memory' to future challenges by a pathogen. Cells that comprise the adaptive immune response are the T and B lymphocytes whose antigen-specificity is generated by gene rearrangements to create unique T cell receptor (TCR) and immunoglobulin genes respectively. Both T and B lymphocytes undergo central tolerance with cells that are non-responsive to or that recognise self-antigen being removed or having their receptor edited. B lymphocytes that have been activated by binding of their B cell receptor (BCR) to its cognate antigen are capable of producing antibodies that help neutralise or opsonise extracellular antigen (humoral response). T lymphocytes in comparison recognise their cognate antigen from proteins processed within cells and displayed in a complex with major histocompatibility complex (MHC) molecules on the cell surface (cell-mediated response). Once activated and with the appropriate signals T and B lymphocytes can differentiate into memory cells that persist and circulate throughout the body.

The immune system has evolved to be immensely complex to deal with the huge range and diversity of potential pathogens. How the body recognises, processes and responds to immunogens requires a great deal of study. One such phenomenon that is studied is immunodominance. This feature describes how, despite the complexity of many pathogens, the humoral and cell-mediated responses of the adaptive immune system appear to focus on only a small portion of the antigenic features of the pathogen. Understanding how and which of these antigens generates an immune response can help inform the rules governing epitope selection and stimulation of responses. This can also be used to investigate how specific

microbes have evolved to evade the immune system and therefore provide a rationale for vaccine targets.

One such complex pathogen includes the human herpesvirus- Epstein-Barr virus (EBV) that has evolved to persist in its host despite a functioning immune system and can be used as an investigative model. Particularly relevant is how in cases of some immunodeficiency or suppression EBV infection can manifest as distinct malignancies. Therefore investigating the phenomenon of immunodominance in relation to EBV infection can help inform our understanding of how this virus can be controlled.

1.2. Immunodominance

As mentioned above, immunodominance is a feature of the immune system where only a restricted range of T cell specificities are detected to numerous potential epitopes from an antigen source. Hierarchies of magnitude or prevalence of T cell responses that particular antigens evoke can be measured. Those responses that drive the greatest magnitude are labelled as immunodominant whereas responses that are found across many individuals or restrictions are labelled as immunoprevalent. Responses that are present but at a lower frequency to these above are labelled as subdominant (Yewdell, 2006).

How the immune system recognises and responds to foreign antigens, particularly in the context of virus infection will be discussed below with a particular emphasis on how these relate to immunodominance. A comprehensive review by Yewdell (1999) discusses the factors determining immunodominance (Tscharke et al., 2015, Yewdell, 1999). Largely, the key determinants that influence the antiviral CTL response are thought to include: the number of and repertoire of naïve T cells, the affinity of the TCR binding to peptide-MHC complexes,

the interactions between the T cells and target cell regarding co-stimulation and also the nature and abundance of viral antigens or epitopes that can be presented by the target cell (Tscharke et al., 2015).

1.3. The Innate Immune response to viruses

In comparison to the large repertoire of rearranged specific receptors utilised by the adaptive immune system the repertoire of PRRs is relatively limited. These receptors are expressed constitutively by cells of the innate immune system and react with specific PAMPs but can be upregulated under inflammatory conditions. PRRs that are involved in the recognition of viruses include a number of membrane-associated Toll-like receptors (TLRs) and cytoplasmic nucleotide binding oligomerisation domain-like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (Thompson et al., 2011).

TLRs were initially identified in fruit flies (Anderson et al., 1985) and later identified to have a role in immune defence in drosophila (Lemaitre et al., 1996) with a human homologue identified a year later (Medzhitov et al., 1997). TLRs found on the cell surface include TLR2 and TLR4 and can recognise viral ligands such as envelope or fusion proteins (Barbalat et al., 2009). Other TLRs activated by viral infection include TLR3, TLR7, TLR8 and TLR9 and recognise viral nucleic acids.

TLRs are characterised by having extracellular domains containing leucine-rich repeats (LRRs) and an intracellular Toll/Interleukin-1 receptor (TIR) domain (Takeda and Akira, 2005). Upon binding of ligands the TLRs dimerize, undergo conformational changes and recruit TIR-domain-containing adaptor proteins such as myeloid differentiation primary response gene 88 (MyD88) and TIR-domain-containing adapter-inducing interferon-γ (TRIF) among others (Akira et al., 2006). These adaptor proteins then lead to the activation of

downstream signalling pathways and activation of transcription factors like NF-κB or interferon regulatory factors (IRFs). Whilst TLRs are membrane associated, RLRs and NLRs are located in the cytoplasm (Akira et al., 2006). These PRRs are less well studied with RIG-I a member of the RLRs only identified in 2004 (Yoneyama et al., 2004). RLRs have a helicase domain that binds to dsRNA and a caspase recruitment domain (CARD) that is responsible for downstream signal transduction (Bruns and Horvath, 2012). Specific RLRs have been shown to be stimulated in different viral infections (Ng et al., 2012). NLRs contain nucleotide binding domains and LRRs that can identify ligands, a pyrin domain involved in inflammation and apoptosis and a CARD involved in signal transduction (Ting et al., 2008). Signals from RLR and NLR activation converge downstream as with TLR signalling to activate NF-κB or interferon regulatory factors (IRFs) leading to production of proinflammatory cytokines, type I interferons and additional signals to recruit and activate immune cells and induce the adaptive immune system (Rouse and Sehrawat, 2010, Kawai and Akira, 2010).

1.4. The adaptive immune response

1.4.1. Generation of the naïve T cell repertoire

The adaptive immune system is highly specific with the ability to respond to a huge diversity of antigens. This relies on the function of T and B cells which express unique surface receptors generated through somatic gene rearrangement. For T cells that recognise foreign antigen bound to self-MHC molecules this requires a diverse naïve TCR repertoire that is generated in the thymus and can ultimately help shape immunodominance patterns. Additionally extra-thymic sites have been proposed as sites for T cells development such as in the human tonsil (McClory et al., 2012). The TCR is composed of α- and β- or γ- and δ-

chains forming a heterodimer with the αβ -TCRs making up the vast proportion. Within each of the heterodimer chains are 3 complementarity-determining regions (CDRs) that act at the interface between the TCR and peptide-MHC (pMHC) molecule. The diversity of the TCR α and β chains is generated through recombination of non-contiguous gene segments V(D)J (variable, diversity, joining) and combined with a constant region (Nikolich-Zugich et al., 2004). Further diversity may be introduced through mechanisms of imprecise joining and DNA repair followed by the random pairing of α and β chains (Nikolich-Zugich et al., 2004). Altogether it has been estimated that there are more than 10^{15} possible αβ -TCRs and more than 10^{18} possible γδ -TCRs that can be formed (Davis and Bjorkman, 1988).

Within the thymus, T cells then undergo positive and negative selection that limit the diversity of possible TCRs, shaping the peripheral repertoire. Positive selection occurs in the thymus cortex where only developing thymocytes expressing TCRs with intermediate affinity and/ or avidity for self-pMHC complexes survive. Those thymocytes with TCRs that bind too strongly or weakly, are removed by clonal deletion or 'death by neglect' respectively (McGargill et al., 2000, Klein et al., 2014). The lineage direction of cells expressing either of the co-receptors CD8 or CD4 is determined during positive selection with appropriate binding to either MHC class I or MHC class II molecules respectively (Klein et al., 2014).

Following maturation in the thymus, T cells migrate to the periphery where they circulate between the blood and secondary lymphoid organs such as the lymph nodes, spleen and Peyer's patch. The number and repertoire of naïve T cell has a great effect on the primary virus-specific CTL response. Several studies have shown that in response to exposure of their cognate antigen, naïve T cells expand in proportion to their starting frequency (Jenkins and Moon, 2012, Obar et al., 2008) and that naïve T cell frequency is a determinant of CD8+ T cell immunodominance (Kotturi et al., 2008).

1.4.2. Priming T cell responses

Mature recirculating T cells in the periphery that have not encountered their cognate antigens presented by an antigen-presenting cell are known as naïve T cells. These naïve T cells can sample peptides presented on MHC class I and class II expressed on dendritic cells and other professional APCs within peripheral lymphoid organs (Nolz et al., 2011). Naïve CD8+ T cells circulate in the blood and are able to home to secondary lymphoid tissues via the expression of chemokine receptors and integrins such as CCR7 and CD62L (L-selectin) (Sallusto et al., 2004, Nolz et al., 2011). Under inflammatory conditions naïve CD8+ T cells have been shown to home to lymphoid tissues along chemokine gradients like CCL19 and CCL21 (Balkwill, 2004). These chemokines along with recruiting naïve CD8+ T cells also enhance contacts with DCs (Castellino et al., 2006). Entry into peripheral lymphoid organs via high endothelial venules (HEVs) occurs through the concerted action of chemokines and cell-adhesion molecules. These molecules initiate the rolling, activation, adhesion and diapedesis of leukocytes across the endothelial layer and into the paracortical area of the lymph node (T cell zone) (Miyasaka and Tanaka, 2004). Within the paracortex the naïve T cells encounter mature antigen presenting-dendritic cells. Here they transiently bind to each APC they encounter through cell adhesion molecules such as LFA-1 and ICAM-1 on the T cell and dendritic cell respectively (Nolz et al., 2011). Dendritic cell 'licensing' through the CD40-CD40L interaction with CD4+ T cells may enhance the activation and expansion of CD4+ and CD8+ T cell responses through the up-regulation of MHC molecules and co-stimulatory molecules (Hernandez et al., 2007). Activation of dendritic cells through this pathway may synergize with activation through TLR signalling (Ma and Clark, 2009). Activation of the T cell requires the engagement of the TCR with a pMHC complex but also from signals delivered through binding to co-stimulatory molecules and the presence of distinct cytokines. These

interactions are summarised in figure 1. Activation of the naïve T cell leads to their proliferation and differentiation into effector T cells driven by the cytokine interleukin-2 (IL-2) amongst others. Absence of co-stimulatory molecules such as B7-1 and B7-2 (CD80 and CD86) expressed on DCs can lead to T cell anergy to prevent reactivity to self-antigens whilst the removal of IL-2 can result in T cell death by apoptosis (Ropke et al., 1996). Additionally, cytokines secreted by the innate immune system effectors and those released from the DC or the microenvironment can polarise activated T cells to acquire different effector functions which is particularly important in driving different subsets of CD4+ T cells and determining the type of adaptive immune response (Zhu et al., 2010).

1.4.3. CD8+ T cell effector function

Upon activation CD8+ T cells differentiate into CD8+ cytotoxic T cells or CTLs. These cells are able to kill cancerous or infected cells without the requirement for co-stimulation and are particularly important in the response against viruses. CTLs can interrogate cells through binding of its TCR to a pMHC complex on the target cell. This results in the reorganisation of its cytoskeleton and the formation of an immunological synapse (Dustin, 2014). Here lytic granules are released that contain the cytotoxic proteins perforin and granzyme (Bolitho et al., 2007, Voskoboinik et al., 2015). Perforin is maintained in a monomeric inactive form in acidic conditions within the granule but oligomerises in the neutral pH of the immunological synapse to form transmembrane pores in the membrane of the target cell. Subsequently serine proteases such as granzymes A and B can enter the target cell, either through the formed pores or other means, and induce apoptotic cell death (Bolitho et al., 2007, Voskoboinik et al., 2015). Alternatively CTLs can upregulate the expression of Fas Ligand (FasL) or TRAIL, which can bind the death receptor Fas on the surface of the target cell and induce apoptotic

cell death (He et al., 2010). Asides from the methods of cytotoxicity above, effector CD8+ T cells are capable of releasing inflammatory cytokines such as IFNγ, IL-2 and TNFα amongst others that are important in cell-mediated immunity and help to reduce the burden of a viral infection.

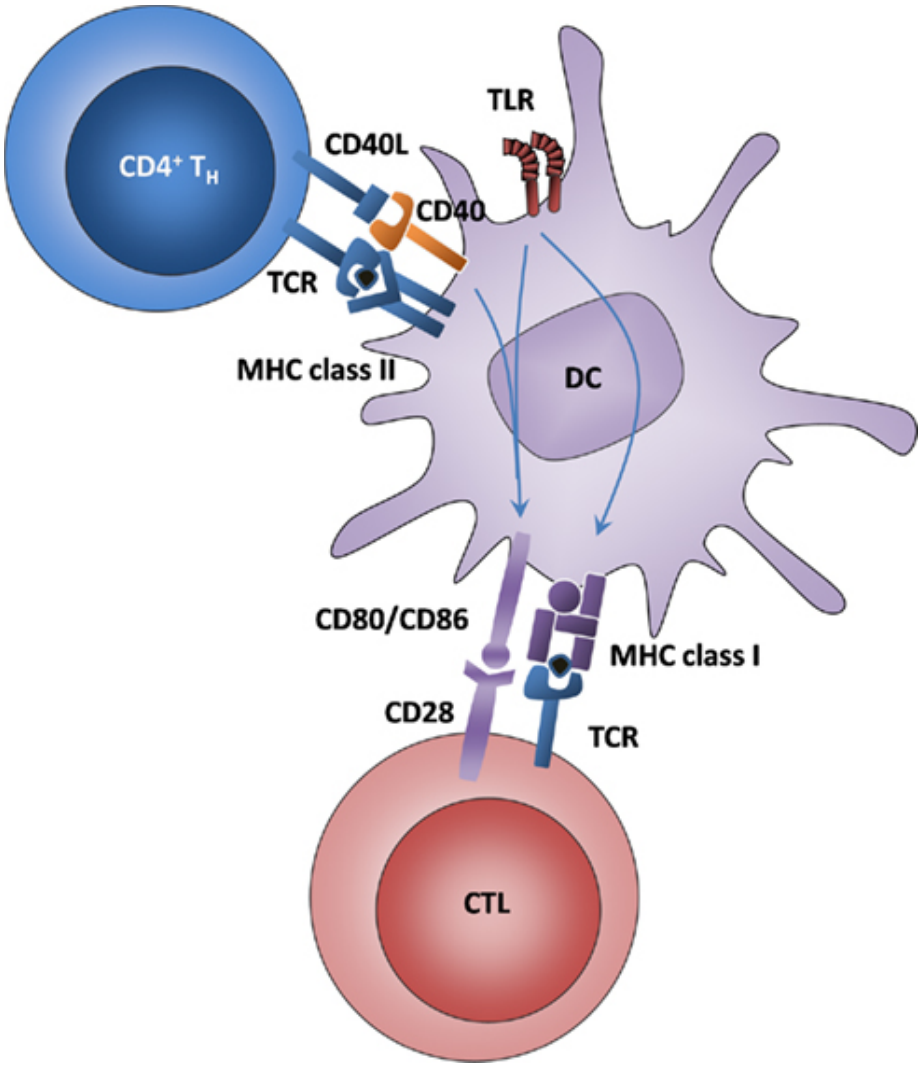


Figure 1.1. Interaction between a naïve CD8+ T cell and a professional APC.
DCs interact with CD4+ T cells via antigen presented on MHC class II molecules and via CD40-CD40L interactions. Downstream signals from CD40 and from TLR ligation result in the up-regulation of MHC class I molecules and co-stimulatory molecules CD80 and CD86. This enables the DC to interact with naïve CD8+ T cells, resulting in the activation of the CD8+ T cell. Adapted from (Thaiss et al., 2011).

1.5. The immunobiology of dendritic cells

1.5.1. Dendritic cell lineage and subsets

Dendritic cells, named for their many processes, are potent antigen presenting cells and unique in the ability to induce primary immune responses (Banchereau et al., 2000). First identified in 1974 by Steinman and Cohn (Steinman and Cohn, 1974) much work has since been done to show their unique properties in priming naïve T cell responses.

DCs exhibit a large amount of heterogeneity but do show some similarity between mice and humans. Typically identified for their lack of lineage markers (CD3, CD19, CD20 and CD56) and high levels of expression of MHC class II, DCs also show high endocytic capability (Levine and Chain, 1992). Many different subsets arise from different developmental pathways in response to external signals and the plasticity of subset differentiation is crucial in balancing immunity against pathogens and tolerance against self-antigens. A number of recently identified surface markers can be used to identify DCs and their precursors in the blood and at different anatomical locations. However surface markers are not always sufficient to separate subsets due to the plasticity of DCs under steady state and inflammatory conditions. Therefore a functional-anatomical classification has been developed and can be used to discriminate between migratory and non-lymphoid tissue-resident DCs (Collin et al., 2013, Shortman and Naik, 2007). Additionally transcriptional profiling of DC subsets in context with the hematopoietic lineage has been used to identify unique populations (Collin et al., 2013, Lindstedt et al., 2005, Robbins et al., 2008, Crozat et al., 2010) and to identify homologous subsets between mice and humans. Identifying specific DC markers through transcriptional profiling and immunohistochemistry has aided adoptive transfer experiments, which seek to identify function and anatomical locations of DCs in mice (Lundberg et al., 2013). Conditional knockouts of specific DC subset markers and transcription factors have

helped elucidate functions and anatomical locations of DC subsets in mice (summarised in (Merad et al., 2013) and can then be used to infer the functions and localisation of human DC subsets.

DCs differentiate from bone marrow-derived precursors and home to specific peripheral tissues via the blood where they take up residence. Macrophages and monocytes are thought to branch off from DCs at a common precursor called the macrophage DC progenitor (MDP). Recruitment of circulating DC precursors to peripheral tissues can be rapid and likely brought about by local inflammation and the production of chemokines (Banchereau et al., 2000, McWilliam et al., 1994). Unlike other APCs such as macrophages and B cells, DCs are also enriched in lymphoid organs where naïve T cells are activated. A requirement for continual replenishment of tissue-resident DCs from blood precursors has been shown in individuals who lack tissue resident DCs such as those undergoing hematopoietic stem cell transplants or those with immunodeficiencies in the mononuclear phagocyte compartment (Haniffa et al., 2009, Collin et al., 2011). These subsets and anatomical locations in humans will be briefly described below.

Dendritic cells are split into two lineages, myeloid and plasmacytoid. Myeloid DCs (mDCs) typically express the antigens CD11c, CD33 and CD11b and in humans include the CD1c+, CD141+ and CD14+ DC subsets (Collin et al., 2013). Plasmacytoid DCs (PDCs) typically lack myeloid markers and are so named for their resemblance to plasma cells. Plasmacytoid DCs are characterised by the markers CD123, CD303 and CD304 (Collin et al., 2013, Reizis et al., 2011). CD1c+ and CD141+ mDCs share homology with the mouse DC subsets CD11b and CD8+/CD103+ subsets respectively. CD14+ mDCs, also known as interstitial DCs are

found in most tissues and are thought to arise from monocytes (Collin et al., 2013). Another DC subset include the Langerhans cells (LCs) that were first identified by Paul Langerhans in 1868 and were later identified as sharing immunogenic properties with DCs (Schuler and Steinman, 1985). Langerhans cells are a subset of self-renewing DCs and can account for up to 5% of cells in the epidermal layer of the skin (Merad et al., 2008).

Within the blood there are mDCs, monocytes and PDCs that are likely the precursors of the DCs in tissues and lymph nodes. These myeloid DCs and monocytes (but not PDCs) can migrate from the blood into tissues in steady state but also particularly in response to inflammatory signals. Within tissues 'migratory' or 'tissue' DCs are in an immature form and are capable of scavenging antigens and then migrating through afferent lymphatics in a CCR7-dependent manner to lymph nodes where they can present antigens to naïve T cells. Blood-derived 'Resident' DCs can be found in secondary lymphoid organs such as the spleen, Peyer's patches and tonsils and consist mainly of CD1c+ and CD141+ DCs (Joffre et al., 2012). These DCs differentiate within the lymphoid tissue but do not migrate out. Therefore by having DCs in immature forms within lymphoid and non-lymphoid tissues antigens can be sampled from both tissues and blood.

1.5.2. In vitro derived DCs

Dendritic cells are relatively difficult to isolate due to their low frequency in peripheral blood and that they don't proliferate in culture. Manipulation *in vitro* may also affect the phenotype and functional properties of the isolated DCs. There are two *in vitro* models of DC generation predominantly using the cytokines Flt3 ligand (Flt3L) or granulocyte/macrophage colony-stimulating-factor (GM-CSF). By culturing CD34+ hematopoietic progenitor cells with GM-CSF and TNFα, Caux and colleagues (Caux et al., 1992) were able to produce DCs that

closely resembled LCs and were capable of stimulating allogeneic CD4+ T cell responses. In addition CD14+ monocytes were shown by Sallusto and Lanzevecchia (Sallusto and Lanzavecchia, 1994) to be able to be differentiated into monocyte-derived DCs (moDCs) under the presence of the cytokines IL-4 and GM-CSF. Further, PDCs, CD1c+ and CD141+ mDCs were shown to be generated *in vitro* using Flt3L and thrombopoietin (Chen et al., 2004). Of these approaches, differentiating monocytes into moDCs has been the most widely utilised and is the subject of several immunotherapy trials (Banchereau et al., 2001, Palucka and Banchereau, 2012).

Generating immature moDCs *in vitro* using GM-CSF and IL-4 typically takes up 7-8 days and then requires a further 2 days for maturation. This is very time consuming and unlikely to represent what happens physiologically in response to a viral challenge. Further work has shown that moDCs can be generated much more rapidly in the presence of IFNγ and that this produces semi-mature DCs that are capable of priming CD8+ T cells against exogenously derived viral antigens (Spadaro et al., 2012, Lapenta et al., 2006).

1.5.3. Antigen capture by DCs

Exogenous antigen at peripheral sites can be transported to secondary lymphoid organs in two ways (Norbury, 2006). If the concentration of the antigen in question is particularly high at a peripheral site, then it can drain through lymphatics directly to the lymph nodes and the spleen for it to be sampled by resident DCs. Alternatively exogenous antigen may be internalised at the peripheral site by DCs and then transported within the DC to the secondary lymphoid organs for presentation to T cells.

Dendritic cells are capable of sampling their environment through a number of different endocytic pathways that can be either receptor-mediated or receptor-independent. Such

endocytic pathways include macropinocytosis, clathrin-mediated endocytosis and phagocytosis. Upon internalisation, antigen is transported within endosomal vesicles that eventually fuse with late endosomes and lysosomes. These endosomal compartments become increasingly acidic and contain proteolytic enzymes for the degradation of antigen that can then be transported into antigen presentation pathways, as discussed later.

As mentioned previously, DCs in an immature state tend to have greater endocytic capacity compared to mature DCs, however the previously held dogma that mature DCs are incapable of endocytosis appears to be incorrect (Garrett et al., 2000, Platt et al., 2010). Additionally different DC subsets and different APCs express a wide range of cell surface receptors that allow for uptake of a number of exogenous antigens. These different routes of antigen internalisation can have an effect on how that antigen is processed and presented to the adaptive immune system. Therefore these pathways have an influence on immunodominant responses dependent upon the nature of the antigen.

Immature mDCs are constitutively able to internalise large quantities of exogenous solute through macropinocytosis, observed by the use of fluid phase markers such as Lucifer yellow or dextran (Sallusto et al., 1995, Liu and Roche, 2015). However in other cell types this process usually requires signalling by growth factors (Lim and Gleeson, 2011). This process in DCs is receptor-independent and involves actin-mediated ruffling of the plasma membrane. Vesicles formed at the plasma membrane can be relatively large, up to 5μM in diameter, allowing the DC to internalise large quantities of solute that leads to a high turnover of cell surface glycoproteins. An example of this high solute uptake was shown by Sallusto and colleagues (Sallusto et al., 1995) to have a rate of 1100mm^3 /hr in moDCs. However upon the delivery of a maturation stimulus, such as through TLR signalling, this process of

maropinocytosis ceases. Concomitantly with delivery of a maturation signal, there is a transient burst (up to 1 hour) of increased macropinocytic activity (West et al., 2004) followed by its cessation. Other endocytic processes unlike macropinocytosis involve the expression of cell surface receptors. Many of these receptors have been investigated and their selective expression by different APCs and DC subsets may allow for directed targeting of antigens for immunotherapy reviewed in (Apostolopoulos et al., 2013, Tel et al., 2013a). Such membrane bound receptors include C-type lectin receptors (CLRs), scavenger receptors (SR) and Fc receptors (FcR) amongst others. Each of these classes of receptors binds to different ligands found on yeast, bacteria and viruses, with CLRs recognising carbohydrates, SRs recognising low-density lipoproteins and FcRs recognising antibody-complexed antigens. These receptors can cluster in clathrin-coated pits that can transport bound antigen to late endosomes/ lysosomes for antigen processing and presentation. Unlike macropinocytosis that is downregulated upon maturation, endocytosis in clathrin-coated pits has been shown to be unaffected by DC maturation (Garrett et al., 2000). Alternatively binding of large particulate antigens by these receptors can mediate phagocytic uptake and internal delivery to lysosomes, forming phagolysosomes (Norbury, 2006, Apostolopoulos et al., 2013, Roche and Furuta, 2015). The nature of the receptor-ligand interaction can affect the destination of the endocytosed antigen with some antigens being maintained in early endosomes that have little degradative activity whilst others are transported to late endosomes/ lysosomes (Joffre et al., 2012, Blum et al., 2013).

1.5.4. Antigen presentation through MHC molecules

MHC molecules present peptide antigens that are the products of proteolysis within the cell (discussed below). In humans MHC molecules are known as human leukocyte antigens

(HLAs). As mentioned previously CD8+ T cells recognise their cognate antigens in complex with MHC class I molecules and CD4+ T cells theirs in complex with MHC class II molecules. MHC class I molecules are expressed on all cells in the human body whereas MHC class II molecules are constitutively expressed on APCs of the immune system. All vertebrates contain a large multigenic region called the MHC on chromosome 6 that contains genes for MHC molecules and for proteins essential for antigen presentation (Blum et al., 2013). In humans this region is immensely gene dense and contains more than 200 genes (Williams, 2001).

In humans there are three genes encoding classical MHC class I called HLA-A, B and C as well as three genes encoding MHC class II molecules called HLA-DR, DP and DQ. Other non-classical MHC molecules that are structurally similar are also involved in host immunity but will not be addressed here. The genes encoding for the MHC molecules are some of the most polymorphic in the human genome with new alleles being constantly identified. This huge allelic variation is most likely driven by infection with pathogens. An online database - the IMGT/HLA (http:www.ebi.ac.uk/imgt/hla)- contains a regularly updated compilation of HLA gene sequences along with designated nomenclature for describing the alleles. HLA-B is the most polymorphic human MHC gene with more than 2000 alleles described (Trowsdale and Knight, 2013). The enormous amino acid variation that drives the allelic polymorphism is focused in the peptide-binding groove of the MHC molecule where the TCR interacts with it. MHC class I and II molecules exhibit a similar structure with a membrane distal peptide binding groove and a membrane proximal immunoglobulin-like constant region. MHC class I molecules are heterodimers consisting of an MHC heavy chain associated with a β2-microglobulin protein (β2M). The α1 and α2 domains of the heavy chain form the peptide-binding groove that can bind specific peptides of 8-10 amino acids. The α3 domain forms the

membrane proximal immunoglobulin-like constant region. MHC class II molecules consist of 2 heavy chains α and β with both N-termini forming the peptide-binding groove that can hold peptides typically longer than seen in MHC class I molecules and with a length of 8-15 amino acids with residues that overhang (Blum et al., 2013).

1.5.5. Antigen processing in the MHC class I pathway

Peptides that associate with the MHC class I molecule are derived from the intracellular proteolysis of proteins. There are two sources of peptides that have been proposed, either from the proteolytic turnover of functional proteins or from the rapid degradation of newly synthesized proteins before they form functional proteins (York and Rock, 1996, Yewdell et al., 1996, Neefjes et al., 2011). A high turnover of protein is essential for normal cellular function with proteins that are no longer required or are abnormal, being targeted for degradation. The half-life of functional proteins varies widely from minutes to days and this stability should be represented in the time that the proteins-derived peptide fragments are presented by MHC class I molecules. However this is not always the case with observations that peptides from some viral proteins are presented much quicker than their half-life would suggest they should be (Neefjes et al., 2011, Khan et al., 2001). Therefore an alternative notion is that the majority of presented peptides arise from newly synthesised proteins that are in some way abnormal. These products were termed ‘defective ribosomal products (DRiPs) and are caused when the translated polypeptide has terminated early due to transcriptional or translational errors (Yewdell et al., 1996). In fact Schubert *et al* (Schubert et al., 2000) showed that up to 30% of newly synthesized proteins were contributed towards DRiPs. They also postulated that the disposition of certain proteins forming DRiPs could be as a result of protein size or difficulties in folding.

One of the major pathways of protein degradation in the cell is mediated by the proteasome in the cytosol. In humans the 26S form of the proteasome is more commonly used and consists of a proteolytic central 20S core and two regulatory 19S caps. For proteins to be targeted for proteasomal degradation they first require a molecular tag in the form of the small peptide ubiquitin. Proteins are ubiquitylated through a cascade of enzymatic activity mediated by the enzymes E1, E2 and E3 (Adams, 2003). Sequential action by these enzymes creates a polyubiquitin chain that 'flags' the protein for degradation. The 19S caps have deubiquitinase activity and are required for the cleaving and unfolding of polyubiquitinated proteins so that the denatured polypeptide can enter the proteolytic 20S core. The 20S core is composed of 4 heptameric stacked rings made up of α and β subunits in a α7 β7 β7 α7 orientation (Maupin-Furlow, 2012). Under inflammatory conditions in the presence of IFNγ or TNFα an alternative proteasome complex can be formed, predominantly in immune cells termed the immunoproteasome (Blum et al., 2013, Neefjes et al., 2011, Sijts and Kloetzel, 2011). This complex has alternative β subunits that have substituted the constitutive β subunits. These new immunoproteasome subunits contain alternative catalytic sites leading to an altered peptide repertoire available for presentation (reviewed in Yewdell, 2005). This altered peptide pool can thus alter CD8+ T cell immunodominance (Zanker et al., 2013, Chen et al., 2001). In the presence of IFNγ an alternative regulator called 11S or PA28 can bind irreversibly to the 20S core, replacing the 19S subunit on at least one side (Sijts et al., 2002). This new subunit, that has increased constitutive levels in APCs, may enhance the uptake of substrates into or the release from the proteasome and thus lead to an expanded peptide pool in times of immune stress (Neefjes et al., 2011, Sijts et al., 2002).

Once peptides have been produced from the proteasome they may undergo further trimming in the cytosol from various aminopeptidases such as tripeptidyl peptidase II (TPPII), thymet

oligopeptidase (TOP), and neurolysin (Groothuis and Neefjes, 2005). Peptides then need to gain access to the endoplasmic reticulum (ER) where they can be loaded onto MHC class I molecules. This translocation is predominantly mediated by the ER membrane-spanning protein TAP (transporter associated with antigen processing). TAP belongs to the ATP-binding cassette (ABC) family of transporters and is made up of two subunits TAP1 and TAP2. Studies of cells deficient in TAP showed decreased levels of MHC class I expression on the cell surface, thus highlighting the importance of TAP in antigen processing and presentation (Salter et al., 1985). Translocation of peptides across TAP is ATP-dependent and is selective for peptides between 6 and 16 amino acids with efficiency falling for longer peptides (York and Rock, 1996, Neefjes et al., 2011). Each molecule of TAP can associate with a molecule of tapasin, an ER chaperone protein that recruits MHC class I molecules. Additionally two other chaperone proteins calreticulin and ERp57 that help stabilise the partially folded MHC class I molecule, associate with tapasin and TAP to form the peptide-loading complex (PLC). Peptides that are too long for loading onto MHC class I molecules may be further trimmed by the ER aminopeptidase-associated with antigen processing (ERAAP) or may be actively transported out of the ER, back into the cytosol by the ER-associated protein degradation (ERAD) system (Neefjes et al., 2011). The above description of antigen processing in the MHC class I presentation pathway is shown in figure 2. These systems can influence the availability of peptides that can bind MHC class I molecules in the ER. Once a peptide has bound to the peptide-binding groove of the MHC class I molecule, this stabilises the complex and allows the translocation of the pMHC complex to the cell surface for presentation. However expression levels of different HLA molecules at the cell surface can vary with HLA-A and HLA-B molecules typically expressed more highly than HLA-C molecules (reviewed in (Neefjes et al., 2011, Akram and Inman, 2012)). All these

factors can have an influence in shaping the CD8+ T cell response to viruses as shown in studies on human-immunodeficiency virus (HIV) (Bihl et al., 2006, Friedrich et al., 2011).

1.5.6. Antigen processing in the MHC class II pathway

Peptides bound to MHC class II molecules are typically derived from proteins found extracellularly. Antigens can be internalised by cells (as described above) and then transported through an endosomal network that gets progressively more proteolytic until it reaches the MHC class II late endosomal compartment (MIIC). This increased proteolytic capability is mediated by an increase in luminal acidity and the fusion with vesicles derived from the trans-Golgi network that contain proteolytic enzymes called cathepsins (Turk et al., 2012). The expression of different cathepsins in different APCs can lead to production of unique peptides and whilst macrophages contain high numbers of lysosomal proteases, DCs and B cells contain relatively few (Delamarre et al., 2005). The maturation of endosomes to become more proteolytic can be regulated through signalling such as through TLRs as discussed later.

MHC class II molecules are assembled in the ER and associate with the invariant chain (Ii) that blocks the peptide-binding groove. This Ii-MHC class II complex is transported from the ER into the MIIC where the Ii chain is partially digested leaving a residual class II peptide (CLIP) within the peptide-binding groove. A separate protein, HLA-DM, is required for the removal of CLIP in exchange for peptides generated in the MIIC. This activity requires an acidic environment that is found in late endosomes. HLA-DM may also aid the removal of low affinity peptides from the peptide binding groove and repetitive action leads to the association of high-affinity peptide binding with MHC class II molecules. Once peptide is

bound stably to MHC class II molecules the pMHC-II complex can be translocated to the cell surface for presentation to CD4+ T cells (Neefjes et al., 2011).

1.5.7. Cross-presentation

Directing antigen into either MHC class I or class II pathway is not completely discrete and antigens destined for one pathway may be processed in the other. The processing of antigens derived from an extracellular source, which enter the MHC class I processing pathway and are presented by these molecules, is termed cross-presentation. Antigens derived from within the cell may enter the MHC class II pathway through autophagy or 'self-eating'. Autophagy will not be described in detail here but can arise through stress or starvation conditions on the cell that requires the production of energy from cell-derived proteins.

Cross-presentation occurs predominantly in DCs but has been shown in other APCs like B cells and macrophages though at lower efficiencies (Heit et al., 2004, Debrick et al., 1991, Hon et al., 2005). When CD11c+ cells were selectively depleted in mice the CD8+ T cell response to cell-associated antigens from *Listeria monocytogenes* or recombinant vaccinia virus was abrogated (Jung et al., 2002, Heipertz et al., 2014). Initial studies by Bevan's group and others showed that CD8+ DCs in mice were the predominant cross-presenting subset *in vivo* (den Haan and Bevan, 2002, Schnorrer et al., 2006). Later studies showed that this DC subset corresponded to the CD141+ DC subset in humans (Jongbloed et al., 2010). However further studies by Tel and colleagues showed that all human DC subsets, including PDCs were capable of cross-presentation (Tel et al., 2013a). This ability of DCs to cross-present is primarily thought to be due to their very low proteolytic activity in their endosomal compartment compared with other phagocytes like macrophages or neutrophils. This is due to the low levels of lysosomal proteases and their decreased activity within lysosomes as a result

of a high lysosomal pH regulated by ROS (reactive oxygen species) production by NADPH-oxidase 2 (NOX2) (Joffre et al., 2012). This low proteolytic activity favours retention of the internalised antigen within this endosomal compartment potentially allowing time for their translocation into the MHC class I processing pathway. External signals can then act on DCs to enhance proteolysis, which favours antigen presentation. The use of LPS in a study by Trombetta *et al* to mature DCs resulted in the enhanced degradation of HRP (horse-radish peroxidase) within lysosomes (Trombetta et al., 2003). This enhanced proteolysis was shown to be due to increased acidity within these lysosomal compartments upon maturation. This increased acidity favoured the generation of mature proteases from their proenzyme state and provided optimum pH for efficient proteolysis. Other proinflammatory signals such as TNFα may also promote this lysosomal proteolysis (Wagner et al., 2013). However the mechanism for how antigen can 'escape' the endosomal compartment and be presented by MHC class I molecules remains under investigation.

Currently there are two main pathways for exogenous antigens to enter the MHC class I pathway; the 'cytosolic' pathway and the 'vacuolar' pathway (shown in figure 1.2.) (Lin et al., 2008, Joffre et al., 2012, Segura and Amigorena, 2014, Fehres et al., 2014). The cytosolic pathway is sensitive to proteasomal inhibitors and therefore reasons that endocytosed material must be able to translocate out of the endosomal compartment and into the cytosol. Once in the cytosol the antigen may be processed in the proteasome and then gain entry into the MHC class I processing pathway as described above. How this translocation is achieved is unclear but may involve components of the ERAD machinery found in the ER and recruited to phagosomes (Blum et al., 2013). Evidence for the 'cytosolic' pathway comes from the use of cytochrome C that acts in the cytosol of cells to induce apoptosis. Intravenous addition of this molecule induced apoptosis in DCs and therefore indicates that it could be transported out of

the endosomal compartment and into the cytosol (Lin et al., 2008). Other studies have shown that a FRET (fluorescence resonance energy transfer)-labelled cytosolic probe could be cleaved by an exogenous enzyme and that this method could be used to quantify transport into the cytosol from endosomal compartments (Joffre et al., 2012).

Alternatively the 'vacuolar' pathway is sensitive to inhibitors of lysosomal proteolysis such as chloroquine but is not sensitive to proteasomal inhibitors and is TAP-independent (Joffre et al., 2012, Lin et al., 2008). Therefore antigens are thought to be processed into peptides and loaded onto MHC class I molecules within the endosomal compartment. In this pathway MHC class I molecules are thought to be recycled from the plasma membrane in endosomes with peptide exchange occurring in lysosomes under acidic conditions. This is supported by evidence suggesting that a conserved tyrosine residue in the cytoplasmic domain of MHC class I molecules that promotes internalisation is crucial in cross-presentation (Lizee et al., 2003). In support of the cytosolic pathway, transport of newly synthesized MHC class I molecules from the ER to the cell surface by CD74 has also been shown to be crucial for cross-presentation (Basha et al., 2012). An addendum to the 'vacuolar' pathway involves fusion of the ER with endosomes or phagosomes as evidenced by the presence of ER-resident proteins in these compartments (Joffre et al., 2012, Mantegazza et al., 2013). A study by Spadaro *et al* using IFNγ to generate fast moDCs showed intracellular compartments with strong co-localisation of MHC class I molecules, calnexin, the early endosome marker EEA-1 along with internalised viral protein (Spadaro et al., 2012).

The ability of DCs to cross-present is influenced by many factors such as local inflammatory mediators or stimulatory factors, nature of the antigen or nature of expressed endocytic receptors and these factors are now being investigated for their role in DC-mediated immunotherapy (Nierkens et al., 2013). Antibody-complexed antigens have also been shown

to be efficiently endocytosed by different DC subsets and that opsonisation of the antigen may protect said antigen from lysosomal proteases and thus aid cross-presentation (Platzer et al., 2014, Baker et al., 2011). Apoptotic cell debris has been shown to be particularly immunogenic to DCs and may enhance cross-presentation ability through the presence of DAMPs (Danger-associated molecular patterns) (Albert et al., 1998, Brusa et al., 2008). The use of adjuvants or inflammatory cytokines in DC maturation has also been shown to enhance cross-presentation activity (Le Bon et al., 2003, Lorenzi et al., 2011). Many of these adjuvants mimic PAMPs (Nierkens et al., 2013, Fehres et al., 2014) and can activate TLRs and other PRRs. However the timing of these signals is important as they can result in the shutdown of endocytic mechanisms but also the production of highly proteolytic endosomal compartments that do not favour cross-presentation (Wagner et al., 2013). Therefore the two pathways seem to require antagonistic processes with one favouring antigen retention in low proteolytic compartments whilst the other favours high proteolytic activity and endosomal sorting of antigens into compartments containing MHC class I molecules. Cross-presentation thus acts to complement conventional antigen processing in stimulating antiviral and antitumoral immunity.

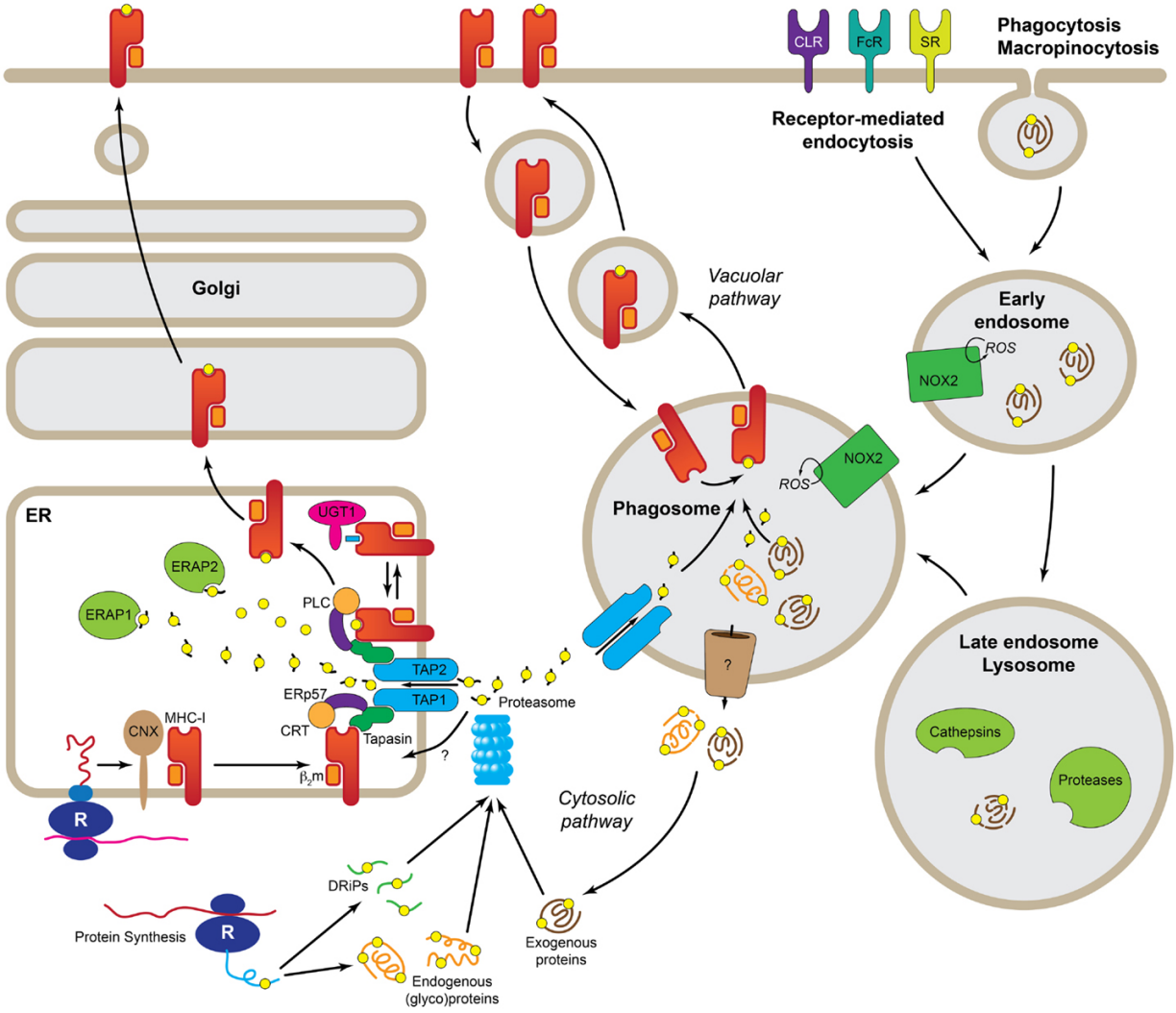


Figure 1.2. Antigen processing in the MHC class I pathway
Newly synthesized MHC class I molecules are formed in the ER through associations with calnexin (CNX), calreticulin (CRT) and ERp57 and are recruited to form a peptide-loading complex (PLC) with TAP and tapasin. Cytosolic proteins and defective ribosomal products (DRiPs) are degraded into short peptides by the proteasome and translocated into the ER lumen by TAP where peptides of a suitable length can be loaded onto appropriate MHC class I molecules. The peptide-MHC complex can then be transported through the Golgi apparatus to the cell surface for presentation to CD8+ T cells.
Alternatively exogenously derived antigen can enter the MHC class I pathway through either of two putative pathways, the vacuolar and cytosolic pathways (red boxes). Dendritic cells can endocytose exogenous antigen through receptor-mediated endocytosis, phagocytosis or macropinocytosis. The antigen is delivered into the endolysosomal pathway and is proteolytically degraded. In the cytosolic pathway, degraded protein products may be transported out of the endocytic pathway and into the cytosol where it can enter the MHC class I processing pathway described above. In the vacuolar pathway, recycling of MHC class I molecules from the cell surface may bind peptides in the endosomes and then be transported back to the cell surface. Adapted from (Fehres et al., 2015).

1.6. Epstein-Barr virus

Epstein-Barr virus (EBV) is a human herpesvirus that is ubiquitous in the human population. Typical of all herpesviruses, EBV establishes a lifelong infection in its host. Herpesviruses are subdivided into three subfamilies; α-, β-, and γ-herpesviruses of which EBV is part of the latter. Other significant human herpesviruses include herpes simplex virus type-1 (HSV-1) that is a member of the α-herpesviruses and cytomegalovirus (CMV) that is a member of the β-herpesviruses. In humans there are eight known herpesviruses that can cause a spectrum of diseases ranging from mild fevers to severe malignancies.

1.6.1. Structure and genome

In the virion, EBV contains a linear double-stranded DNA (dsDNA) genome of approximately 172kbp encoding for more than 80 gene products (Callan, 2003, Baer et al., 1984, Werner et al., 2007). This DNA genome is contained within an icosahedral capsid and is surrounded by a matrix of tegument proteins and an envelope membrane. The virus envelope is derived from the plasma membrane of an infected cell that the virus acquires when budding from the cell. Within the viral envelope are various virus glycoproteins that are principally involved in attachment and entry into cells.

The genome was cloned and sequenced using an EBV DNA BamHI fragment-cloned library (reviewed in (Young and Murray, 2003). The specific BamHI sites are used as the basis for describing ORFs, transcription sites and RNA processing sites. The BamHI fragments were labelled A-Z according to decreasing size with lower case letters designating the smallest fragments (figure 1.3.). The first letter of the described ORF refers to BamHI and the second refers to the fragment label A-Z as previously stated. This is then followed by a description of the ORFs orientation and whether it is leftward (L) or rightward (R) reading on the standard

genome map and finally the F and number designate a reading frame (Baer et al., 1984) (Werner et al., 2007, Young and Murray, 2003).

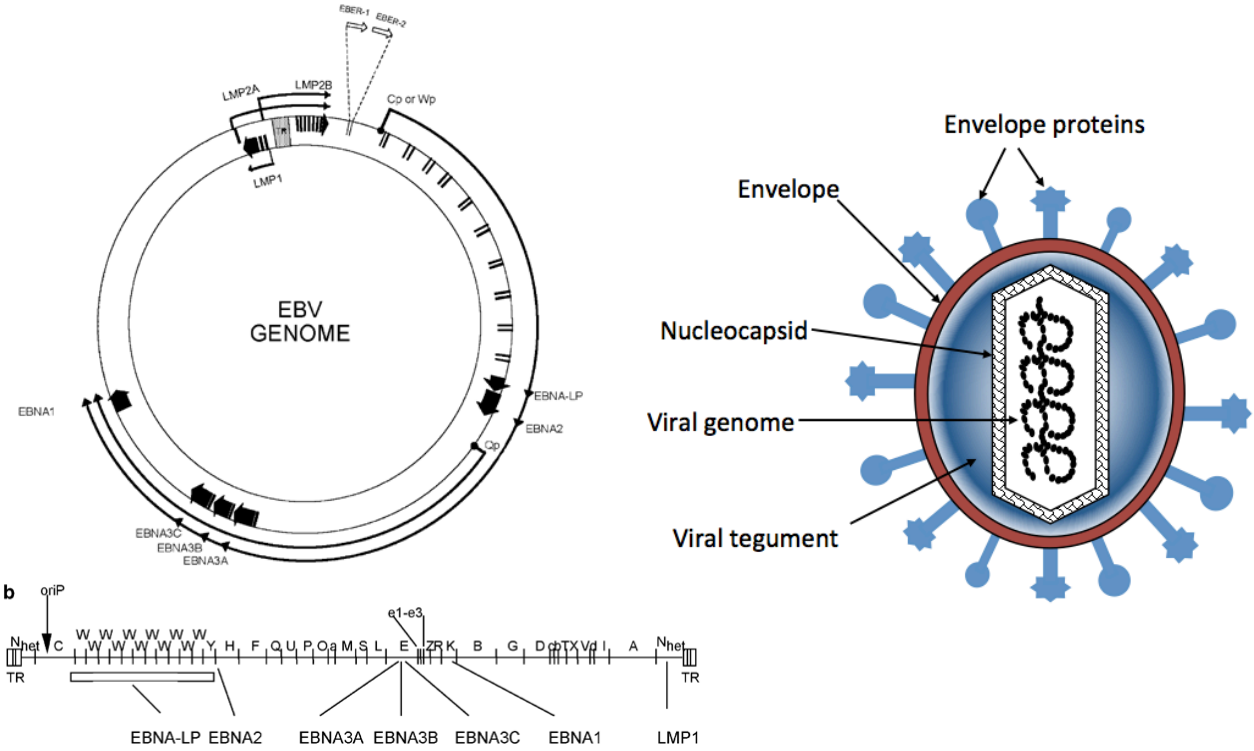


Figure 1.3. EBV virion structure and genome
(Left) A diagram of the circular prototype B95.8 EBV genome with the location of the open reading frames below (image adapted from Young and Murray, 2003). The BamHI fragments are named alphabetically according to size with the largest denoted by A. Lowercase letters denote the smallest fragments. Latent proteins and promoters are labelled. (Right) A diagram of the EBV virion with the various structures labelled.

1.6.2. EBV infection

EBV has a prevalence of more than 90% of the human population worldwide, typically being acquired in early childhood with little associated morbidity. However when primary infection is delayed until adolescence, it can be associated with the self-limiting disease infectious mononucleosis (IM), also known as glandular fever. IM is characterised by the following symptoms: fever, pharyngitis, malaise and lymphadenopathy and also by the expansion of antiviral CD8+ T cells (Callan, 2003, Balfour et al., 2015).

EBV is transmitted in saliva and upon transmission is thought to infect squamous epithelial cells or infiltrating B cells within the oropharynx (Dunmire et al., 2015, Rickinson et al., 2014). To infect B cells the viral glycoprotein gp350/220 binds to CD21 (also known as the complement receptor 2-CR2) and another glycoprotein gp42 binds to MHC class II molecules. This process triggers endocytosis of the virus particle through the recruitment of a number of other glycoproteins (gHgL and gB) that mediate fusion between the virus and endosomal membranes, permitting the tegumented nucleocapsid to be released into the cytoplasm (Hutt-Fletcher, 2007). In contrast, virus entry into epithelial cells does not require gp350 or gp42 and soluble gp42 has actually been shown to inhibit virus entry into epithelial cells (Kirschner et al., 2007). However the fusion complex of gHgL and gB is essential for virus entry (Hutt-Fletcher, 2007). A separate glycoprotein, BMRF2 has been shown to be critical for virus entry into oral epithelial cells through the binding of cell surface integrins (Tugizov et al., 2003, Xiao et al., 2008). This switch in tropism is enabled by the sequestering of gp42 by cellular HLA class II molecules in B cells leading to the release of virions with lower levels of gp42 in their envelopes and an increased ability to infect epithelial cells (Connolly et al., 2011, Borza and Hutt-Fletcher, 2002).

Infection of infiltrating B cells in the tonsils can lead to the establishment of a latent infection in these B cells where the linear genome is circularised and maintained as an episome tethered to the host genome. Different patterns of EBV latent gene expression results in different latency states, types I, II and III, that can be expressed at different times of infection or anatomical location and can be associated with distinct malignancies. The different latency proteins provide the necessary signals to enable B cell transformation, survival and differentiation into memory B cells that enables the virus to persist for the lifetime of the host.

Initial infection is thought to result in a growth-transformation of B cells and the expression of latency type III that drives B cell activation and proliferation. This programme leads to activation of the Wp and Cp promoters and the expression of 6 nuclear antigens (EBNA-1, 2, 3A, 3B, 3C and –LP), 3 latent membrane proteins (LMP1, LMP2A/B), 2 small noncoding RNAs (BARTs), EBV-encoded small RNAs (EBERs) and various microRNAs. This pattern is best observed during IM, post-transplant lymphoproliferative disease (PTLD) and in the EBV-transformed B lymphoblastoid cell line (LCL) (Tierney et al., 2015) (Taylor and Blackbourn, 2011).

Subsequently infected B cells are thought to differentiate into memory B cells and undergo a more limited pattern of gene expression termed latency I/II gene pattern with latency I expressing, EBNA-1, LMP2A/B, EBERs and BARTs, and latency II expressing EBNA-1, EBNA-2, LMP2A/B, LMP1 EBERs and BARTs (Kang and Kieff, 2015). Latency type I occurs in Burkitt Lymphoma (BL) whilst latency type II occurs in Hodgkin lymphoma (HL) and nasopharyngeal carcinoma (NPC). Ultimately latency type 0 pattern of gene expression occurs where EBV protein expression is silenced and there is only expression of the noncoding elements and microRNAs. Memory B cells recirculate through the blood and oropharyngeal lymphoid tissues with occasional virus reactivation possibly as a result of local

mucosal signals or from differentiation into plasma cells. Virus reactivation is then thought to seed infection in oral epithelium resulting in a large production of virus and shedding in saliva (Niedobitek et al., 1997, Thorley-Lawson, 2001, Dunmire et al., 2015, Rickinson et al., 2014).

EBNA-1 is involved in the maintenance of the EBV genome within proliferating cells. It accomplishes this by tethering the viral episome via the plasmid origin of replication (OriP) site in the episome to the host chromosomes. This allows replication of the viral episome during cellular replication ensuring a copy of the EBV genome is delivered to daughter cells. Expression of EBNA1 is found in all EBV-associated tumours. EBNA-LP and EBNA-2 are the first two proteins produced during EBV infection and serve to promote B cell transformation and proliferation through the activation of several cellular and viral genes. Such genes that are upregulated include EBNA-1, CD21, CD23, c-fgr and c-myc that are important for B cell growth and proliferation. EBNA-2 is a phosphoprotein that activates the viral latency promoter Cp, the bidirectional promoter and the LMP2A promoter (Peng et al., 2004) through the interaction with the DNA binding protein CBF-1 (Cp binding factor-1) and the recruitment of the basal transcription machinery (Young and Murray, 2003). This makes EBNA-2 a potent transactivator of latent viral gene expression. Similarly to EBNA-2, the three EBNA-3 proteins (A, B and C) also up-regulate the expression of cellular and viral genes with EBNA-3A and -3C required for B cell transformation. The EBNA-3 proteins also bind to CBF-1 but with stronger affinity than EBNA-2 does and therefore they inhibit EBNA-2s activity. The EBNA-3 proteins are also able to repress transcriptional activity through the interaction with several cellular factors including histone deacetylases (HDACs). This is important as EBNA-2 has been shown to block B cell differentiation, which is required for the virus to survive in the memory B cell compartment (White et al., 2010). EBNA-3A and -3C are capable of regulating the cell cycle through the disruption of G1, G2 and M checkpoints

whilst EBNA-3C has additional functions in being able to induce upregulation of cellular CD21 and viral LMP (Krauer et al., 2004, Young and Murray, 2003). LMP1 is required for B cell transformation and is a functional homologue of a constitutively active form of CD40. CD40 is expressed on the B cell surface and is a member of the tumour necrosis factor receptor (TNFR) family. The binding of TNFR-associated factors to CD40 results in B cell activation and proliferation. Similarly LMP1 can bind to these TNFR-associated factors with similar outcome. Additionally LMP1 can protect infected B cells from apoptosis through upregulation of the pro-survival, bcl-2 family of proteins. The LMP2 proteins, like LMP1, are also constitutively active. LMP2 acts to prevent EBV reactivation into lytic cycle and to promote B cell survival through mimicking BCR signalling (Thorley-Lawson, 2001).

1.6.3. Productive/lytic infection

The lytic form of EBV infection is required for the production of virus progeny, essential for transmission. Upon primary EBV infection of B cells there can be partial activation of lytic cycle genes in a pre-latency phase before the cells enter a latent state. The other main cell types that EBV infects are epithelial cells where it undergoes lytic replication. Following a latent state in the B cell compartment, reactivation into lytic cycle is required and is dependent on signals from the host cell's environment. These signals may arise from stress responses or from B cell transition through germinal centres and differentiation into plasma cells (Lieberman, 2013). Studies of EBV lytic replication *in vitro* are difficult as there are no *in vitro* systems that are permissive for 'efficient' EBV lytic replication. Therefore studies have focused either on spontaneous reactivation or on the use of broad chemical inducers of lytic cycle in latently infected cells such as LCLs. Such chemical inducers include phorbol esters like 12-0-tetradecanoyl phorbol-13-acetate (TPA), sodium butyrate, azacytidine and

calcium ionophores (Kenney and Mertz, 2014). However these agents usually don't induce more than 10% of latently infected cells into lytic cycle. Anti-IgG cross-linking of the BCR in the Akata cell line is a more physiological model of lytic induction and has proven to be an effective mechanism with a high degree of lytic reactivation (Yuan et al., 2006).

EBV lytic protein expression follows a temporal and sequential cascade beginning with two immediate early (IE) proteins followed by the expression of up to 30 early (E) proteins then the expression of up to 30 late (L) proteins. IE genes are expressed early after induction in the presence of protein synthesis inhibitors such as cycloheximide. There are two IE proteins, BZLF1 (also known as Zta or ZEBRA) and BRLF1 (also known as Rta). These two proteins act as transcriptional activators of the viral E genes and both have been shown individually to mediate E gene expression (Guo et al., 2010). However in many cell lines BZLF1 is more effective than BRLF1 in inducing lytic replication and in some cell lines only BZLF1 is required. BZLF1 is a member of the bZIP family of proteins and shares homology with the AP1 site binding proteins c-Jun and c-Fos. BZLF1 binds as a homodimer to Zta-response elements (ZREs) that are similar to AP1-related sequences and are found in many E EBV gene promoters and in the promoters of the two IE gene promoters (Adamson, 2005). BZLF1 protein may facilitate the switch from latency to lytic cycle through the inhibition of the Cp promoter (Lieberman, 2013). BZLF1 is also able to activate the EBV origin of lytic replication (OriLyt) possibly through the recruitment of other E-expressed viral replication proteins. The E genes can be differentiated from L genes by their synthesis in the presence of inhibitors of viral DNA synthesis such acyclovir (ACV). Viral DNA replication involves the formation of an initiation complex at the oriLyt composed of six viral replication proteins (Fixman et al., 1995). These proteins are BALF5 (viral DNA polymerase), BMRF1 (DNA polymerase processivity factor), BALF2 (ssDNA-binding protein homolog), BSLF1 (primase

homolog), BBLF2/3 (helicase-primase homolog) and BBLF4 (helicase homolog). Other E proteins important for viral DNA replication are the ribonucleotide reductase (RR) proteins BORF2 and BaRF1, the thymidine kinase protein (BXLF1) and the uracil DNA glycosylase BKRF3 (Cohen, 2006). Aside from their role in viral replication E proteins are also involved in protecting the cells from apoptosis (BHRF1 and BALF1) and also some involved in modulating the immune response to the virus infection which will be discussed further below. The L expressed genes largely encode the structural proteins including the capsid proteins, tegument proteins and glycoproteins. During lytic replication EBV is thought to express 12 glycoproteins with the majority involved in cell attachment and virus entry. Two of these glycoproteins have a role in immune evasion with BILF1 expressed as an E protein and BDLF3 expressed as a L protein (Hutt-Fletcher, 2015, Quinn et al., 2016). EBV entry into B cells requires five glycoproteins, gp350 which can attach to CD21 and CD35, and gB, gHgL and gp42 which mediate fusion of the virus envelope with the cell membrane. Gp350 is also being investigated as a promising vaccine candidate particularly in preventing IM (Cohen, 2015). EBV entry into epithelial cells requires attachment via BMRF2 and fusion with gB, and gHgL but not gp42. Two of the other glycoproteins, gM and gN are involved in the envelopment of the virus (Hutt-Fletcher, 2015). The virus capsid is composed of the major capsid protein (MCP), a small capsid protein (sCP) and two triplex proteins (BORF1-mCP binding protein and BDLF1-mCP). Virus proteins involved in capsid assembly include the scaffold protein BdRF1 and maturation protease BVRF2 (Henson et al., 2009). The virus tegument proteins and tegument binding proteins have been shown to have a variety of functions including immune evasion (van Gent et al., 2014), virus reactivation (Liu and Cohen, 2016), and activation of viral early gene expression (Tsai et al., 2011). Once the replication cycle has finished the virus particle is formed. The nucleocapsid is formed within

the nucleus and then traffics through the ER and trans-Golgi network, acquiring an envelope and other tegument proteins. During this process it has been shown that the autophagic membrane label LC3B-II is incorporated into mature virus particles, implicating the autophagy pathway in the exocytosis of EBV particle (Nowag and Munz, 2015). Exocytosis of the virus particle is achieved through budding from the cell membrane releasing an enveloped virus particle.

1.6.4. The host immune response to EBV infection

The host-pathogen relationship of EBV infection continues from the time of primary infection for the lifetime of the host. The virus has evolved many different strategies to enable its survival in the host under strong evolutionary pressures from the host immune system. Windows into this interplay can be best studied when the host-pathogen balance becomes perturbed and disease results. Such perturbations include the acute immunopathologic disease IM but it also includes cases of immune disruption or dysregulation that can lead to EBV-associated malignancies. These features have made EBV a useful model for investigating the antiviral immune response in humans.

The cell-mediated immune response to EBV is crucial in controlling viral replication and expansion of EBV-infected cells. This is shown dramatically in individuals undergoing IM who have a tremendous expansion of their CD8+ T cell compartment. This expansion has been shown to be EBV-specific and not just a result of bystander activation by inflammatory cytokines or an EBV-superantigen (Callan et al., 1996). The development of MHC class I tetramer technology in the 1990's along with *ex vivo* IFNγ-capture ELIspot and T cell cloning assays has enabled researchers to identify individual epitope responses and to enumerate frequencies in the blood of IM patients (Callan et al., 1998, Hislop et al., 2002b, Hislop et al.,

2007). Analysis of the CD8+ T cell specificities during IM showed that responses were against both latent and lytic antigens though the highest frequencies were against lytic antigens (Steven et al., 1996, Woodberry et al., 2005). This is thought to reflect the high levels of lytic replication in B cells and the high amounts of virus shedding in oral tissues. Through the investigation of a limited number of lytic antigens expressed during replication, the CD8+ T cell response appears to be focused against epitopes derived from the IE and some E expressed antigens. Responses against these proteins can be particularly abundant with one epitope derived from the IE protein BZLF1 accounting for up to 44% of the entire CD8+ T cell compartment in one donor (Callan et al., 1998, Hislop et al., 2002b). Responses to the few L antigens that have been studied show that responses to these proteins appear to be rare and at very low frequencies (Pudney et al., 2005). CD8+ T cell responses to EBV latent proteins in IM are smaller (1-5%) than responses to lytic proteins but also show an immunodominance hierarchy typically focused against the EBNA3A, 3B and 3C proteins with subdominant responses against EBNA2, LMP1 and LMP2 proteins. Few responses are typically seen against the other latent proteins and initially none were found against EBNA1. In some individuals rare HLA class I allotypes can lead to differences in the immunodominance hierarchies (Hislop et al., 2007, Chapman et al., 2001, Lee et al., 1997). A recent study (Brooks et al., 2016) showed that dominant CD8+ T cell responses could be seen in certain HLA contexts against epitopes from EBNA2, EBNA-LP and BHRF1 within a few days following EBV primary infection of B cells. Similarly disparate HLA-restricted responses to EBNA1 can also be observed in IM patients despite the presence of a glycine-alanine repeat (GAr) that limits presentation through the MHC class I pathway (Sharipo et al., 1998). These patterns of immunodominance in the latent proteins can be partly explained by the fact that the EBNA3 proteins comprise around 60% of the unique amino acid sequences

from the latent proteins but also that most studies have used Caucasian donors that have a range of common HLA allotypes that the responses to the EBNA3 proteins are restricted through (Brooks et al., 2016).

Following resolution of IM there is a rapid contraction of EBV-specific T cells through apoptosis as antigen availability becomes limited (Hislop et al., 2002b, Hislop et al., 2007). This contraction can also lead to the apparent loss of particular responses (Hislop et al., 2002b). EBV-specific memory CD8+ T cells account for a high proportion of the memory CD8+ T cell compartment in healthy virus carriers. Responses have largely been studied in healthy virus carriers with no history of IM and who undergo low-level virus shedding. In these donors CD8+ T cell responses to lytic proteins typically account for up to 2% of the total CD8+ T cell pool and show a similar immunodominance hierarchy. However frequencies can be higher in some donors and memory inflation has also been observed with responses to a single lytic epitope reaching more than 14% of the total CD8+ T cell compartment (Hislop et al., 2007, Khan et al., 2004, Hislop et al., 2002b). Memory CD8+ T cell responses to some latent proteins have been shown to develop slowly post-IM but are broadly similar to those responses seen during IM (Hislop et al., 2002b, Woodberry et al., 2005).

The observed immunodominance hierarchy of CD8+ T cell responses against lytic proteins is thought to reflect the efficiency of how successively expressed antigens are presented by infected B cells. A study by Pudney *et al* (2005) analysed CD8+ T cell responses from IM patients to 2 IE, 11 E and 10 L and showed that responses consistently targeted epitopes from IE and some E proteins. This study then went further and generated CD8+ T cell clones to representative IE, E and L proteins and assayed for direct recognition of lytic LCL targets. These findings correlated with the IM patient screens and showed that the LCL targets were

most efficiently recognised by CD8+ T cell clones specific for IE proteins with decreasing recognition by CD8+ T cell clones specific for E proteins and L proteins respectively (Pudney et al., 2005). However IM responses were only tested against a limited number of lytic proteins and only a small number of T cell clones that were deemed representative were tested for recognition of lytic LCL targets. This hierarchy therefore implied that there was impairment in antigen processing and presentation with transition through the lytic cycle. This impairment has been linked to the presence of several EBV-encoded immune evasion genes that will be discussed later (Ressing et al., 2015). Memory CD8+ T cell responses in healthy virus carriers are thought to follow the same immunodominance hierarchy to that observed during IM with responses to L proteins being rare. However a study of macaques infected with the related Rhesus lymphocryptovirus (rhLCV) identified that CD8+ T cell responses specific for L proteins could be detected at frequencies comparable to responses for IE and E proteins and that the development of these responses were associated with increasing duration of infection (Orlova et al., 2011). The authors went further; screening LCL-expanded T cell effectors from healthy virus carriers against a panel of L antigens expressed from recombinant vaccinia viruses (VV), with all 5 donors tested showing some reactivity. A subsequent study used overlapping peptide panels derived from the 2 IE proteins, 6 E and 7 L proteins of EBV to screen for memory CD8+ T cell responses in healthy virus carriers (Abbott et al., 2013). They revealed that CD8+ T cell responses against some L proteins could be detected but that these responses were subdominant to those found against IE and E proteins, which appeared to match the previous finding by Pudney and colleagues (Pudney et al., 2005).

During IM there is little increase in the absolute numbers of CD4+ T cells though EBV-specific reactivity can be detected through the use of MHC class II tetramers (Long et al., 2013). However the individual responses to each latent protein tends to outnumber those

responses against each lytic protein. Similarly to CD8+ T cells there is also a contraction of the EBV-specific CD4+ T cell response following resolution of IM. Interestingly however, the CD4+ T cell response to EBNA1 shows different kinetics and often doesn't appear until months after IM has resolved and this is also reflected in the serological response to EBNA1 that is probably linked to the absence of CD4+ T cell help (Rickinson et al., 2014, Long et al., 2013). Memory CD4+ T cell responses, however, were shown in one study by Mautner and colleagues (Adhikary et al., 2007) to primarily target late expressed structural proteins in LCL expanded CD4+ T cell lines. These structural proteins were shown to access the MHC class II presentation pathway through release of virus particles from lytically infected LCLs that could then be taken up by receptor-mediated endocytosis in neighbouring semi-permissive LCLs. However a subsequent study showed that memory CD4+ T cell responses also target non-structural lytic proteins and that they appeared to be distributed broadly across the whole lytic proteome with no clear immunodominance hierarchy. These responses were shown to be independent of virus production and presenting a pattern driven by cross-presentation of antigen (Long et al., 2011).

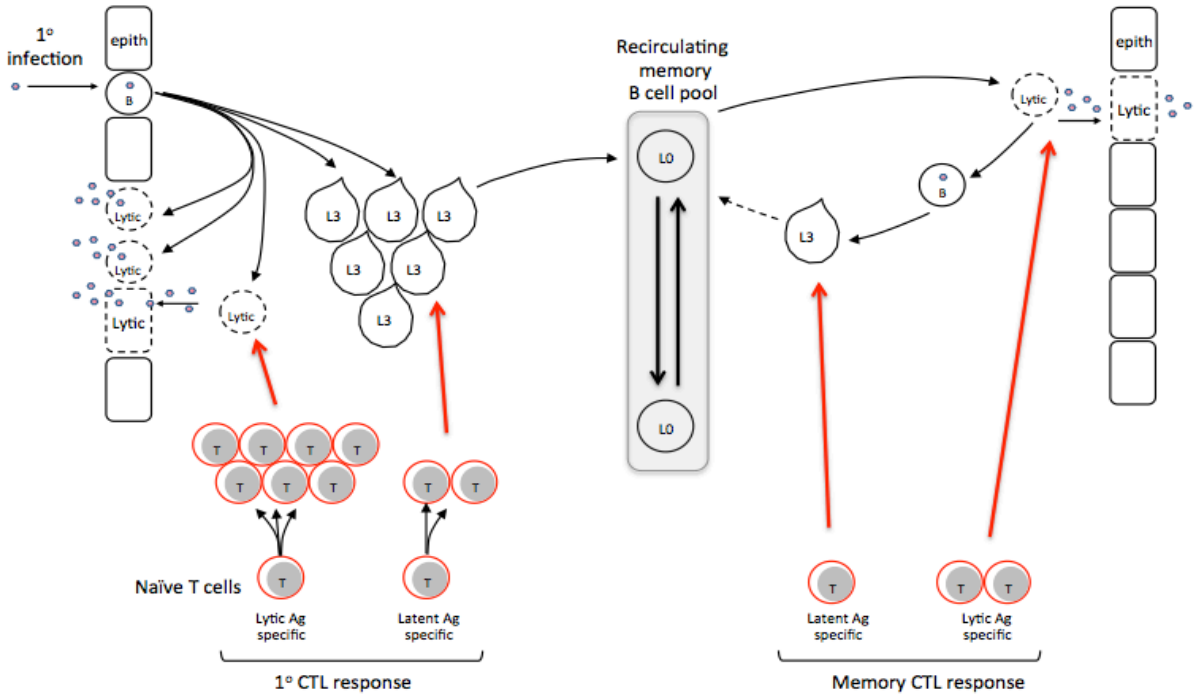


Figure 1.4. EBV infection and CTL immune response.
Oral transmission of EBV leads to lytic infection of permissive squamous epithelial cells and infiltrating B cell within the oropharynx. This leads to high levels of virus shedding within the throat. Whether newly infected B cells support lytic replication immediately or after growth transformation is unknown. EBV then colonises the B cell system establishing a growth-transformation latent infection known as latency 3 (L3). This is followed by the down regulation of latency genes to establish an 'immunologically silent' latent infection with little or no gene expression known as latency 0 (L0). This form of latent infection is found predominantly in the memory B cell pool with cells circulating between the blood and oropharyngeal lymphoid tissues. Occasional reactivation of latently infected B cells into lytic cycle leads to the production of virions, seeding low-level virus shedding in the throat or new infection of adjacent B cells. In the immunocompetent host, these processes can be subject to immune controls. During primary EBV infection as seen in patients with infectious mononucleosis (IM) there is a large expansion of EBV-specific CD8+ T cells with dominant specificity against lytic cycle antigens. Following resolution of IM, these expanded T cells are culled to leave lower numbers of antiviral memory T cells in the blood. (Adapted from (Rickinson et al., 2014).

1.6.5. Immune evasion by EBV

As a result of the immune pressures applied upon them, viruses have evolved many different strategies to subvert or evade immune responses against them. Different virus families have different constraints upon them and different immunological pressures depending upon the niche they occupy. As such the mechanisms of immune evasion vary between different families of viruses. However common immunological pathways are typically targeted.

EBV has coevolved with humans for millennia and evolved several evasion mechanisms relevant to the different phases of infection it undergoes. Like the other herpesviruses EBV undergoes lifelong persistence in its host and has to maintain a balance between viral replication and immune control.

As mentioned earlier, EBV undergoes a growth transforming latent phase within B cells before down-regulation of viral gene expression establishing a Latency type 0 phenotype thereby evading cellular immune controls. However during productive replication, up to 80 proteins are expressed, potentially providing a large number of peptide determinants that can be presented to the immune system. With this large coding potential it has been estimated that over 50% of the viral genome is dedicated to modulating host antiviral responses (Ressing et al., 2015).

Upon EBV infection, innate immune sensors detect the virus and initiate antiviral immunity and signal the adaptive immune response. These innate sensors have thus been recognised as frequent targets of viral immune evasion mechanisms. The EBV host shutoff protein BGLF5 is expressed during the early stage of the lytic cycle and this alkaline exonuclease functions to down regulate the expression of many cellular immune genes to various degrees. Amongst those proteins affected are the innate sensors TLR2 and TLR9, HLA class I and class II molecules as well as the co-stimulatory molecules CD80 and CD86 (van Gent et al., 2015).

There is also a general interference with the activation of IRF and NFkB signalling pathways by a number of lytic proteins including BRLF1, BZLF1, BGLF4 and BPLF1 and a number of latent proteins EBNA1, LMP1 and LMP2 (Ressing et al., 2015). These actions can result in the inhibition of type I IFNs and TNFα that overall has an effect to dampen down inflammation and leads to a reduction of cell surface HLA expression. In combination with this EBV encodes soluble factors BARF1 and BCRF1, which encode a soluble form of the colony-stimulating factor-1 (CSF-1) receptor and the vIL-10 respectively (Cohen and Lekstrom, 1999, Salek-Ardakani et al., 2002, Ressing et al., 2015). CSF-1 acts to stimulate macrophage differentiation and IFNγ secretion and this effect is neutralised by the BARF1 encoded CSF-1R. Further work in a Rhesus macaque model showed that infection with recombinant lymphocryptovirus containing a defective form of BARF1 that couldn't block CSF-1 mediated signalling resulted in decreased virus loads. Additionally, in persistence these macaques also had a lower frequency of virus-infected cells (Ohashi et al., 2011). Virus IL-10 is an anti-inflammatory cytokine that can modulate CD4+ T cell priming and effector functions and inhibit DC maturation. The expression of four immune evasion proteins during lytic cycle, BNLF2a, BILF1, BGLF5 and BDLF3 exert an effect to reduce the level of viral epitopes expressed on the surface of the infected cell. BNLF2a is unique to the family of lymphocryptoviruses and is expressed early during the lytic cycle. Its expression prevents the binding of ATP and peptides to TAP and thereby reduces peptide loading of MHC class I molecules and recognition by CD8+ T cells (Jochum et al., 2012a). BILF1 is an early expressed gene and encodes a viral G-protein coupled receptor (vGPCR) that has been shown to physically associate with MHC class I molecules on the surface of infected cells and this enhances their endocytosis for lysosomal degradation (Zuo et al., 2009). As well as inducing endocytosis of MHC class I molecules, this protein also diverts the exocytosis of newly

synthesized pMHC complexes to the cell surface (Zuo et al., 2011). However BILF1 only appears to target HLA-A and B molecules and not HLA-C, likely to avoid the NK cell mediated response. BDLF3 encodes the glycoprotein gp150 and is a newly identified immune-evasin whose mechanism is still under debate; either through ubiquitin mediated degradation of MHC molecules or through action as a glycan shield that blocks antigen presentation (Quinn et al., 2016, Gram et al., 2016). BDLF3 is expressed during the late phase of the lytic cycle and it ultimately leads to decreased cell surface expression of MHC class I and II molecules and thereby impacts upon T cell recognition. These proteins act in a coordinated manner to interfere with antigen processing and presentation throughout the entire lytic cycle and the observation that the CD8+ T cell response to EBV lytic proteins is skewed towards the IE and E proteins suggests that responses are driven through B cell presentation (Quinn et al., 2014, Pudney et al., 2005).

Aside from the expression of viral proteins EBV also expresses a number of microRNAs (miRNAs) that have been shown to have an immunomodulatory effect. These miRNAs can be packaged into virus particles along with viral mRNAs and noncoding RNAs to target immune defence mechanisms during a pre-latent phase (Cullen, 2013, Jochum et al., 2012a, Jochum et al., 2012b). Packaged mRNAs include BNLF2a that can have an immediate effect on antigen processing and recent work by the Hammerschmidt group has shown that several miRNAs can modulate levels of MHC molecules, co-stimulatory molecules and production of proinflammatory cytokines as well as down-regulating expression of TAP1 and TAP2 molecules. However the expression levels of these miRNAs within the virus particle may not be sufficient to have immediate immunomodulatory effects during the pre-latent phase (Hammerschmidt, personal communications).

The combination of all these different mechanisms of immune evasion and modulation serves to demonstrate how EBV has evolved to persist in the human host under strong immune challenge. In patients with primary or secondary immunodeficiency, typically linked to defects in cellular immunity, certain malignancies can develop such as post-transplant lymphoproliferative disease (PTLD) or X-linked lymphoproliferative (XLP) syndrome amongst others (Rickinson et al., 2014, Parvaneh et al., 2013, Cai et al., 2015). By studying these EBV-associated disorders much can actually be learnt about how the immune system deals with virus infection.

1.7. Studies of immunodominance

Investigators have studied immunodominance as it is crucial in understanding: cellular immunity and immunosurveillance, immune evasion mechanisms, and for vaccine design (Chen et al., 2000). There have been many studies investigating the factors that govern immunodominance with most work being carried out in viruses with small coding capacities such as Human immunodeficiency virus (HIV) or Influenza. However recent technological advances have allowed viral immunologists to investigate large complex viruses such as poxviruses and herpesviruses. Such approaches to examine immunodominance include the construction of cDNA libraries consisting of complete gene products or viral genome fragments, *in silico* predicted synthetic peptides of optimum size for binding to MHC molecules, overlapping 15mer peptides, or the identification of MHC bound peptides by mass-spectroscopy (Yewdell, 2006). Each of these approaches will be addressed in terms of their advantages and disadvantages.

1.7.1. cDNA libraries of complete gene products

This approach uses the expression of full-length cDNA gene products in APCs to screen for T cell responses. This relatively low cost approach enables the identification of naturally processed peptide determinants, including many posttranslational modifications. However this approach may be less sensitive than others and does rely on how well these products can be transfected and expressed in APCs (Yewdell, 2006). This approach was used for vaccinia virus (VV) infection in H-2d and H-2b restricted mice with a number of determinants identified (Tscharke et al., 2005, Tscharke et al., 2006). It was observed in this study that half of the overall response to VV was directed against only a handful of determinants and that one determinant accounted for about a quarter of the overall response. These authors also identified that the route of administration of VV also had an effect on the observed immunodominance hierarchy. Later studies have also used this approach to look for responses to herpes simplex virus types 1 and 2 (HSV-1/2) and in varicella zoster virus (VZV) (Jing et al., 2012, Jing et al., 2016). This first study used a novel approach to expand up memory CD8+ T cell responses to HSV-1/2 using moDCs to cross-present apoptotic infected HeLa cells. The following day, activated CD8+ T cells were enriched for the expression of CD137 and then bulk stimulated *in vitro*. The expanded CD8+ T cell lines were then screened against cDNA expression constructs across the whole HSV-1 genome. From this they were able to identify a large number of determinant antigens that were broadly taken from across the virus genome. The author's later work showed a substantial amount of cross-reactivity between responses to HSV-1/2 and to VZV.

1.7.2. cDNA libraries of virus genome fragments

Instead of complete gene products, smaller gene fragments can be utilized instead. The advantages with this approach over the one above are that responses to overlapping fragments can aid the identification of the peptide determinant. Some viruses also encode gene products through alternative reading frames, which could be detected in this approach. The disadvantages are similar to the above approach in terms of transfection efficiency and expression in APCs but by having gene fragments instead of whole gene products, posttranslational modifications will be missed. This approach was used by Jing *et al* (2005) using 300bp fragments covering the VV genome with a depth of about 50x thereby enabling detection of specific determinants (Jing et al., 2005). The authors found immunodominant determinants from 15 gene products with 10 of these belonging to early expressed gene products, 1 to a late expressed product and 4 that were unknown.

1.7.3. *In silico* prediction of peptide determinants

There are a number of online databases online that allow for the prediction of MHC restricted peptide determinants from inputted protein sequences. These include SYFPEITHI and the Immune epitope database (IEDB) and they utilise different algorithms for peptide prediction based on factors such as binding affinity to specific MHC haplotypes, anchor positions, as well as factoring in antigen processing elements (Yanover and Bradley, 2011, Rammensee et al., 1999). Using peptides can be a more sensitive approach, however generating peptides is more expensive despite recent advances and the prediction algorithms may miss many naturally processed peptides that don't conform to motifs. Additionally previously identified immunogenic peptides are used to inform the prediction algorithms and therefore tend to be focused around peptides restricted through the most prevalent HLA allotypes and therefore

the algorithms tend be less robust for less prevalent HLA allotypes. Despite these issues numerous studies have used *in silico* predicted peptides to try and identify determinants. One study by Oseroff *et al* (2005) used more than 6000 *in silico* predicted peptides spanning 258 VV antigens to identify CD8+ T cell responses restricted through disparate HLA-A and B molecules (Oseroff et al., 2005). From this approach they identified 48 peptides from 35 VV antigens that were immunogenic, with the majority of these antigens expressed in the early phase of infection. There were dominant responses identified with one HLA-A*02.01 restricted epitope identified in 4 of 6 individuals that was greater than the other A*02.01 restricted responses in those individuals. However, broadly, individuals that shared some HLA alleles, recognised different epitopes showing the breadth of the immune response to VV.

1.7.4. Overlapping 15mer peptides

Instead of attempting to predict immunogenic peptides another approach is to generate a vast array of overlapping 15mer peptides. The benefits of this approach is that it covers all MHC class I and II allotypes however the number of peptides required makes this approach very expensive and any immunogenic peptide still needs to be verified that it is processed naturally. For individual virus antigens or viruses with small coding capacities this approach is ideal, however for viruses with large coding capacities the cost is often prohibitive. Even so this approach has been adopted in many studies. This approach may be insensitive if the frequency of virus reactive T cells is low. However HCMV generates large and sustained T cell responses and so Sylwester and colleagues (2005) could use 13,687 peptides covering all 213 ORFs of the CMV genome to identify CD8+ and CD4+ T cell responses from 33 healthy virus carriers (Sylwester et al., 2005). The authors identified responses to 151 CMV ORFs

with no particular trend in regards to the function or kinetics of expression. Many studies of T cell responses to HCMV have focused on responses to pp65 and IE-1 on the basis that they were immunodominant and representative of the total T cell response to HCMV. However this study by Sylwester *et al* has shown that the T cell response is broader than previously thought and also that HCMV infected individuals devote a median of 10% of their total memory CD8+ T cell repertoire to a media of 21 CMV ORFs. This was an exhaustive study that identified the frequency and size of responses to peptide determinants from across the CMV genome but also used this information to examine the relationship of these responses with the abundance, kinetics and function of the proteins they were derived from.

1.7.5. Peptide identification by mass-spectroscopy

Continuing advances in mass-spectroscopy is enabling both the relative and absolute quantification of pMHC complexes on the surface of cells (reviewed in (Tscharke et al., 2015)). This approach identifies eluted peptides from the surface of cells, which can then be crosschecked against genomic sequences and fitted to individual HLA allotypes. However this approach is technically demanding and may struggle to detect low abundance peptides. Additionally the presentation of a peptide does provide any information on its immunogenicity. A study by Ternette *et al* (2016) used this approach to identify peptides eluted from HIV-1 infected cells and was able to identify 75 peptides (Ternette et al., 2016). Of these 75 peptides, a third of them had not previously been reported and 83% had not been restricted to a single HLA allotype. Other studies have also used this approach to identify patterns in the restricting HLA allotype with some showing a tendency for CD8+ T cell responses to be restricted through HLA-B alleles (Schellens et al., 2014).

1.7.6. Immunodominance summary

These different approaches to examine immunodominance all have their advantages and disadvantages but have all helped to identify novel epitopes and increase our understanding of antigen processing and presentation. The identification of immunogenic epitopes can be used to design vaccines that elicit T cell responses that can sufficiently control infection and reduce the chances of viral escape variants. Further to this is the notion that though immunodominant determinants may account for the majority of the T cell response to a virus infection, subdominant determinants may be critical and help sustain durable immunity (Friedrich et al., 2007, Ruckwardt et al., 2010). Continuing advances in fields such as mass-spectroscopy and MHC multimers will enable better identification of T cell responses to different pathogens and help develop our understanding of disease correlates.

1.8. Scope of thesis

Previous findings of an immunodominance hierarchy amongst the EBV lytic proteins during IM has shown that CD8+ T cell responses appear to be focused against antigens expressed at the early stages of the lytic cycle with only rare responses to L expressed proteins. Subsequent studies, whilst largely reaffirming previous observations, have also highlighted that differences may be present in the different disease states. One study showed that memory CD8+ T cell responses could be readily detected against L lytic proteins in humans though these responses were subdominant to IE and E expressed proteins. A separate study using macaques infected with a related herpesvirus showed that memory CD8+ T cell responses equivalent in size to those against IE and E expressed proteins could also be readily detected against L lytic proteins and an increased response size was associated with increased duration

of infection. All these studies examined only a fraction of the EBV lytic proteome and therefore responses against a large number of proteins would have been missed.

This research project aims to expand on previous studies using new tools to examine immunodominant responses across the entire lytic proteome of EBV during both primary and persistent disease states. To expand EBV-specific memory CD8+ T cell responses in healthy virus carriers a novel system involving the cross-presentation abilities of dendritic cells was optimised. Comparisons between different disease states could provide answers into the biology of the virus and how T cell responses are primed, maintained and diversified during the lifetime of the host.

CHAPTER 2

METHODS AND MATERIALS

2.1. TISSUE CULTURE

2.1.1. Tissue culture media and reagents

RPMI-1640 supplemented with 2mM L-glutamine (Sigma). Stored at 4°C.

Opti-MEM- (Gibco)

Foetal calf serum (FCS) (PAA) aliquoted and stored at -20°C.

Human serum (HS) (PAA) derived from a male type AB. Aliquoted and stored at -20°C.

Penicillin-streptomycin (Gibco) containing 500 IU/ml penicillin and 5000µg/ml streptomycin. Aliquoted and stored as 100x stock and stored at -20°C.

Trypsin-express (Gibco) was used according to manufacturer's recommendations.

Phosphate buffered saline (PBS) was made by dissolving 1 tablet (Oxoid) per 100ml of filtered-deionised water (sterile-distilled water SDW). 50ml and 500ml aliquots were then sterilised by autoclaving for 20 minutes at 121°C.

Recombinant interleukin-2 (IL-2) (PeproTech). Supplied as lyophilised powder and reconstituted in PBS to give a concentration of 10^5 IU/ml and stored as 200ul aliquots at -20°C.

Recombinant interleukin-3 (IL-3) (PeproTech). Supplied as lyophilised powder and reconstituted in PBS + 0.1% BSA to give a concentration of 50µg/ml. Aliquoted and stored at -80°C.

Recombinant interleukin-4 (IL-4) (PeproTech). Supplied as lyophilised powder and reconstituted in PBS+ 0.1% BSA to give a concentration of 50µg/ml. Aliquoted and stored at -80°C.

Recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (PeproTech). Supplied as lyophilised powder and reconstituted in PBS+ 0.1% BSA to give a concentration of 50µg/ml. Aliquoted and stored at -80°C.

Recombinant tumour necrosis factor α (TNFα) (PeproTech). Supplied as lyophilised powder and reconstituted in PBS+ 0.1% BSA to give a concentration of 50µg/ml. Aliquoted and stored at -80°C.

Recombinant interleukin-6 (IL-6) (PeproTech). Supplied as lyophilised powder and reconstituted in PBS+ 0.1% BSA to give a concentration of 50µg/ml. Aliquoted and stored at -80°C.

Recombinant interleukin-1β (IL-1β) (PeproTech). Supplied as lyophilised powder and reconstituted in PBS+ 0.1% BSA to give a concentration of 50µg/ml. Aliquoted and stored at -80°C.

Imidazoquinoline compound-Resiquimod (R848) (InvivoGen). Supplied as lyophilised powder and reconstituted in DMSO to give a concentration of 5mg/ml. Stored at -20°C.

Polyinosinic–polycytidylic acid potassium salt (Poly(I:C)) (Sigma). Supplied as lyophilised powder and reconstituted in SDW to give a concentration of 5mg/ml. Stored at -20°C.

CpG oligonucleotide ODN2216 (InvivoGen). Supplied as lyophilised powder and reconstituted in SDW to give a concentration of 1mg/ml. Stored at -20°C.

Monkey Leukocyte antigen-144 supernatant (MLA-144). Supernatant is derived from cultured MLA-144 cells and filtered through a MilliporeSteritop^{TM} 0.22µm vacuum-driven disposable bottle top filters. Stored in 60ml aliquots at -20°C.

Lymphoprep (PAA).

Dimethyl sulphoxide (DMSO)

Lipofectamine 2000 (Invitrogen**)**

2.1.2. Peptides

Peptides were synthesised by either Peptide 2.0 or Alta biosciences and dissolved in DMSO to obtain a concentration of 5mg/ml and stored at -20°C. To identify restricting peptide epitopes for T cell responses, a combination of online prediction software was used (SYFPEITHI- www.**syfpeithi**.de and IEDB- www.**iedb**.org).

2.1.3. Mycoplasma testing

All cell cultures were routinely tested for the presence of mycoplasma by using a Mycoalert kit (Cambrex), as per manufacturer's protocol.

2.1.4. Cryopreservation

Cells to be preserved were pelleted and re-suspended in freezing media (RPMI-1640 + 10% DMSO + 20% FCS) and transferred into sterile 1ml cryovials (Nunc). These were then stored

in a 'Mr Frosty-containing isopropanol' at -80^{o}C overnight, allowing for a gradual decrease in temperature (1ºC/minute). The next day, cryovials were transferred into liquid nitrogen freezers.

2.1.5. Revival of cryopreserved cells

To revive cells, 1ml cryrovials were placed into a 37^{o}C water bath for thawing. Following this cells were washed with standard media (RPMI-1640 + 10% FCS), re-suspended in appropriate media and transferred into an appropriate plate or flask, for culturing in a 37ºC, 5% CO2 incubator.

2.1.6. MLA-144

The gibbon cell line MLA-144 is an established line derived from a spontaneous lymphosarcoma of gibbon and is used to stimulate T cell growth. Cells were maintained in standard media in 150cm^2 flasks for 2 weeks without feeding, after which supernatant was harvested and filtered, for use as T cell feeding media.

2.2. CELL CULTURE

2.2.1. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was collected from consenting donors into a syringe with heparin to give a final concentration of 1000IU/ml, or into vacutainers and then diluted 1:1 with warm RPMI-1640. Blood/RPMI 1640 was then layered onto 15ml lymphoprep and centrifuged at 1600rpm with no brake. Mononuclear cells were isolated, washed and used immediately for

experiments or cryopreserved. All experiments were approved by the West Midlands (Black Country) Research Ethics Committee (07/Q2702/24).

2.2.2. EBV transformed Lymphoblastoid cell lines (LCLs)

Established LCLs were maintained in standard media (RPMI-1640 + 8% FCS + 50IU/ml penicillin + 50ug/ml streptomycin) and were split once or twice a week by removing half of the culture and replacing with fresh standard media.

2.2.3. B95.8 virus producing cell line

B95.8 cells were maintained in 10ml of standard media. Cells were split two times per week.

2.2.4. MJS, COS-7, HEK293 and HEK293-p2089 cells

MJS, HEK293 and HEK293-p2089 cells were maintained in 75cm2 flasks in 18ml of (RPMI-1640 + 8% FCS + 50IU/ml penicillin + 50ug/ml streptomycin), with the addition of geneticin for HEK293-p2089 to maintain the p2089 bacmid (Delecluse et al., 1998). Cells were split as required by removing media and washing with 10ml PBS. Following this 2ml of trypsin express was added and incubated with cells for 1-5 minutes at room temperature. Following this the relevant amount of standard media was added and cells were split as required.

2.2.5. Isolation human of CD14+ monocytes

CD14+ monocytes were isolated from donor PBMCs using positive selection CD14+ microbeads (Miltenyi) according to manufacturer's guidelines. Briefly, donor PBMCs were

re-suspended in 80µl MACS buffer per $1x10^7$ cells and cultured with 20µl paramagnetic CD14 microbeads per $1x10^7$ cells. Labelled cells were incubated at 4°C for 15 minutes before being washed in MACS buffer and then applied to a magnetic column with the non-labelled cells washed through the column. The column would then be removed from the magnetic field and the labelled cells would be flushed out with MACS buffer.

2.2.6. Differentiation of monocytes into monocyte-derived dendritic cells (moDCs)

Isolated CD14+ monocytes were washed once in standard media and then re-suspended to $1x10^6$ cells/ml standard media containing 10% FCS. Depending on recovered cell numbers, $25cm^2$ or $75cm^2$ flasks were used and flasks were laid flat for 2 hours at 37^oC, 5% CO_2 to allow cell adherence. After 2 hours, 50ng/ml GM-CSF and 50ng/ml IL-4 were added. Cells were cultured for 4 days at 37^oC, 5% CO_2 to yield immature moDCs.

2.2.7. Isolation of plasmacytoid dendritic cells (PDCs)

PDCS were isolated from donor PBMCs using positive selection CD304+ microbeads (Miltenyi) as detailed for CD14+ cells above. Isolated PDCs were maintained overnight in DC media containing 10% FCS at a cell density of $1x10^6$ cells/ml. Media was supplemented with 10ng/ml IL-3 (Bratke et al., 2011).

2.2.8. Antigen loading immature moDCs or PDCs

Immature moDCs and isolated PDCs were harvested from flasks by gentle washing and scraping. Cells were washed once in standard media, counted and re-suspended to $4x10^6$

cells/ml standard media containing 10% FCS. Antigen in the form of cell lysates were added to the DC cultures at a ratio of 1:1 (cell lysates prepared from stock number of cells/ml) or peptide 5μg/ml. DC cultures were then incubated at 37ºC, 5% CO_2 for 2 hours. After 2 hours the TLR agonists R848 and Poly(I:C) were added as a maturation stimulus for moDCs and the TLR agonist ODN2216 as a maturation stimulus for PDCs. DC cultures were placed back at 37º. After 16-20 hours the DC cultures were washed twice with RPMI-1640 and plated out at a cell density of $1x10^6$/ml.

2.2.9. T cell stimulation and generation of polyclonal T cell lines using antigen-loaded moDCs

Antigen-loaded moDCs described above were used as targets for either T cell clones or autologous CD8+ T cells at a target: effector ratio of 10:1. Autologous CD8+ T cells were cultured with the antigen-loaded moDCs for 3-4 days in standard media and then stained with a LIVE/DEAD dye, CD3, CD8 and CD137 antibodies (as described below) for cell sorting by flow cytometry. Typical yields of CD8+ T cells recovered numbered about 20-50,000. Recovered cells were sorted into T cell cloning media, pelleted and then seeded into 1 well of a 96 well U bottom plate containing γ-irradiated and PHA- allogeneic feeder PBMCs (as described above). Cells were then given a mitogenic stimulus of 30ng/ml anti-CD3 (OKT3) and the polyclonal was allowed to expand with T cell cloning media replaced when required. After 2 weeks the majority of cells were either used or frozen at -80ºC whereas a small proportion of cells were expanded further as described previously. These expanded cells were then frozen at -80ºC. Typical expansions after 2 weeks yielded cell numbers of $1-2x10^8$.

2.2.10. Generation of Polyclonal lines from IM patients

Numerous samples of IM patient blood have been collected and the PBMCs isolated and banked in the Cancer Sciences Institute in line with ethics. IM PBMCs were chosen based on their HLA class I restriction for which we had the relevant expression constructs for a number of alleles. PBMCs were thawed and immediately re-suspended in standard media containing 100IU IL-2. PBMCs were counted and washed and then re-suspended to $1x10^6$ cells /ml in T cell cloning media. Cells were then stimulated using irradiated and PHA-treated allogeneic PBMCs along with 30ng/ml OKT3 as described above and allowed to grow for 2 weeks yielding $1\text{-}2x10^8$ cells. Expanded polyclonal lines were then depleted of CD4+ T cells (Dynabeads) and used immediately or frozen at -80ºC until use.

2.2.11. Cell transfection of plasmid DNA

To ectopically express protein expression constructs, HEK293 cells, MJS cells, or HEK293-p2089 cells were washed and re-suspended in standard media containing no antibiotics. Cells were then seeded into plates or flasks at the appropriate cell number to be 80% confluent the following day. The next day, lipofectamine 2000 (Invitrogen) and plasmid DNA were diluted in OPTI-MEM to the appropriate amounts depending on the number of cells to be transfected (according to manufacturer's instructions). Lipofectamine/ DNA mixes were incubated at RT for 30mins. Supernatant from the cultured cells was then removed and the appropriate amount of the Lipofectamine/ DNA mix was added onto the cell layer. Cell cultures were incubated at 37ºC, 5% CO_2 for 4 hours before being topped up with standard media containing no antibiotics. Cell cultures were then incubated at 37ºC, 5% CO_2 for up to 48hours depending on use.

2.2.12. Cell lysate

For each sample, cells were counted, washed and pelleted in RPMI-1640 by centrifugation and then re-suspended to $4x10^6$ cells/ml in RPMI-1640. Lysates then underwent 5 freeze-thaw cycles in a dry ice-ethanol bath before then being sonicated for 30s. Samples were stored at -80ºC.

2.3. IMMUNOLOGICAL ASSAYS

2.3.1. Interferon gamma- (IFNγ) capture T cell cloning

To generate CD8+ T cell clones of known specificities, IFNγ capture cloning was employed. PBMCs from healthy or IM patient donors were thawed and stimulated with the appropriate peptides, corresponding to the clone specificities required, for 4 hours at 37ºC, 5% CO_2. Alternatively COS-7 cells ectopically expressing the relevant EBV antigen and HLA class I molecule were used to re-stimulate polyclonal T cell lines overnight at 37ºC, 5% CO_2. Following this, cells were washed with cold MACS buffer (PBS with 0.5% (w/vol) BSA and 2.5mM EDTA). Cells were then re-suspended in 80µl of cold standard media (RPMI-1640 + 10% FCS) and 20µl of PE labelled-IFNγ-catch reagent (Miltenyi Biotec). From here, the manufacturer's protocol was followed. Collected cells were then plated out using limiting dilution.

2.3.2. Limiting dilution of T cells

Antigen-specific T cells, isolated using the IFNγ-capture kit above, were re-suspended in T cell cloning media (RPMI-1640 + 10% FCS + 1% HuS + 30% MLA + 50IU/ml penicillin +

50ug/ml streptomycin + 5IU/ml IL-2). They were then seeded into 96 well round bottom plates (corning) at 0.3, 3 or 30 PBMCs per well in the presence of irradiated (4000 rads) allogeneic feeder PBMCs (10^5/ml). Feeder cells were generated from mixed PBMC preparations from blood donors (Birmingham national blood service) and then activated with phytohaemagglutinin (PHA 10ug/ml) overnight in standard media. Mixed with the feeder cells were irradiated (4000 rads) autologous LCLs (10^4/ml). Plates were incubated at 37ºC, 5% CO_2 for one week, after which they were given fresh T cell cloning media. One week after this, any clones that grew were expanded further in to 24- well plates (Corning), in the presence of γ-irradiated autologous LCLs (10^5) and PHA treated allogeneic feeder PBMCs (10^6). After this, established T cell clones were maintained by feeding when required with fresh T cell cloning media by removing 1ml of media and replacing with fresh media. If clones needed to be restimulated, 200,000 T cells were removed from culture and mixed with PHA treated, γ –irradiated allogeneic feeder PBMCs (10^6) and autologous LCLs (10^5) in T cell cloning media in one well of a 24 well plate. After one week these were fed with 1ml media and maintained as normal.

2.3.3. IFNγ enzyme linked immunosorbant assay (ELISA)

To test the ability of CD8+ T cell clones to recognise targets, an IFNγ-capture ELISA was used. Firstly, target LCLs were sensitised with appropriate peptide, washed twice in standard media and seeded at a density of $5x10^5$ cells / 100µl of RPMI-1640 media per well of a v-bottom 96-well plate (target to effector ratio of 10:1). Effector CD8+ T cells were similarly washed in RPMI-1640 media to give $5x10^4$ cells/ 100µl and added to target cells to give a final volume of 200µl. All target to effector combinations were carried out in duplicate or

triplicate dependent on the assay as described in the figure legend. As negative controls, IFNγ release by T cells was measured in the absence of target cells (Effector only) and additionally T cells were challenged with non-antigen loaded cells. As a positive control target LCLs were sensitised with 5µg/ml of peptide for 1 hour and then washed three times in standard media, before adding to 96-well plate. Cells were then incubated overnight at 37ºC, 5% CO_2. MaxiSorp 96-well plates (Nunc) were coated with 50µl per well of anti-human IFNγ antibody (0. 75µg /ml) (Thermo Scientific) in coating buffer (0.1M Na_2HPO_4, pH 9) and incubated overnight at 4ºC.

The following day MaxiSorp plates were washed with wash buffer (PBS-0.05% tween-20) and then blocked with 200µl of blocking buffer (PBS-0.05% tween-20 + 10% BSA) for 1 hour at room temperature. Following this, plates were then washed six times with wash buffer and then 50µl of cell supernatant from the overnight culture assay was harvested and added to the MaxiSorp plates. To quantify IFNγ production, parallel assays were conducted where 50µl of an IFNγ standard was added in duplicate over doubling dilutions from 20000pg/ml to 312.5pg/ml of recombinant IFNγ (Peprotech). Plates were then incubated at room temperature for 3 hours after which, plates were washed six times with wash buffer. Biotinylated anti-human IFNγ (Thermo Scientific), diluted in blocking buffer to 0.75µg/ml was added in 50µl to each well and plates were incubated for 1 hour at room temperature. Following this, plates were washed six times in wash buffer and then 50µl of streptavidin-peroxidase (ExtraAvidin-Peroxidase, Sigma), diluted 1/1000 in blocking buffer, was added to each well and plates were incubated at room temperature for 30 mins. Plates were then washed 8-times in wash buffer and 50µl of peroxidase substrate (3, 3', 5, 5'-tetramethylbenzidine (TMB) solution, Tebu-bio Laboratories) was added and the plates incubated for 30 minutes to allow for colour development. To stop the reaction, 50µl of 1M

hydrochloric acid was added, resulting in a soluble yellow product. Plates were then read using dual wavelengths of 450nm and 695nm. Using the results from the IFNγ standards, the level of IFNγ release could be calculated as IFNγ release in pg/ml.

2.3.4. IFNγ enzyme linked immunospot (ELIspot) assay

To test the number of IFNγ-secreting T cells an IFNγ-ELIspot was used according to manufacturer's guidelines (MABTECH- Human IFN-γ ELISpotBASIC and as described previously (Abbott *et al*, 2013)). Briefly, filtered solutions of PBS, 50% ethanol, and standard medium were prepared. The 96 well PVDF-plate membrane was washed with 50% ethanol for no longer than 1 minute and then washed 5x with sterile water before coating with anti-IFNγ antibodies and incubated overnight at 4°C. Replicate wells were seeded with T cell effectors in filtered standard medium along with peptides at a final concentration of 20μg/ml, or as a positive control, PHA at a final concentration of 1mg/ml, or, as a negative control an equivalent volume of DMSO. After incubation at 37°C for 16 hours, the cells were discarded and the captured IFNγ detected with a biotinylated anti-IFNγ antibody followed by streptavidin-Alkaline phosphatase and TMB chromogenic substrate solution. The spots were then counted on an automated plate reader.

2.3.5. Screening polyclonal T cell lines for reactivities to EBV lytic gene products.

MJS cells or COS-7 cells were used as target cells in recognition assays. These were grown in bulk and seeded at a density of 15,000 cells/ well of a 96 well flat bottom plate to be 80% confluent the following day. Sufficient wells were required for 80 EBV-lytic protein expression constructs against both HLA-A, B, or C allele constructs all in duplicate (i.e.

(80x3) x2= 480 wells / polyclonal line). The following day media was removed and a transfection mix containing 0.3µl lipofectamine 2000, 150ng EBV-lytic antigen/ GFP negative control, 50ng HLA- A, B or C alleles was added per well in 40µl OPTI-MEM. Transfected cells were incubated for 4 hours at 37ºC, 5% CO_2 before 100µl standard media containing no antibiotics was added. Cell cultures were then incubated at 37ºC, 5% CO_2 overnight. Following this, media for each well was harvested and plated into a well of a 96 well V bottom plate. Trypsin was added to the transfected cells and incubated at RT for 1-5mins. The harvested supernatant was added back to the relevant wells and the entire cell resuspension was seeded into the 96 well V bottom plate. The plates were then centrifuged at 1800 rpm for 5mins to pellet the cells. The supernatant was then flicked off and 100µl fresh standard media was added per well to these targets.

Expanded polyclonal lines from either IM donors or healthy virus carriers were thawed and resuspended in standard media to a cell density of $1x10^6$ cells /ml. For each large proteome screen $48x10^6$ cells were required with cells were seeded at $1x10^5$ / well of a 96well V-bottom plate in 100µl. The culture was then incubated at 37ºC, 5% CO_2 overnight. The following day 50µl supernatant from each well was used in an IFNγ-capture ELISA, described above.

2.4. FLOW CYTOMETRY

2.4.1. Cell surface and viability staining

Cells were counted and seeded into 3ml FACS tubes and were washed by centrifugation at 1600rpm for 5mins with MACS buffer (Miltenyi). The supernatant was decanted and the cells were then stained with a viability dye (LIVE/DEAD stain Invitrogen) according to manufacturer's instructions. Cells were then washed once as previous, supernatant decanted

and relevant cell surface antibodies were added. Preparations were incubated on ice for 30mins at room temperature before two further washes and then cells were re-suspended in 100-150µl MACS buffer depending on number of cells. Samples were run on an LSR-II (BD biosciences) and then analysed using FlowJo (Tree Star).

2.4.2. MHC class I Tetramer staining

Cells were counted and seeded $5x10^5$ cells/ FACS tube. Cells were pelleted and then washed once with 0.5ml HS and 2mls MACS buffer. MHC class I tetramers were centrifuged briefly and then added at a pre-titrated concentration. Samples were incubated for 30mins at 37ºC, 5% CO_2 and then washed once in MACS buffer. Viability and surface antibodies were then stained as above.

2.4.3. Intracellular cytokine staining

Cells were counted and seeded $1x10^6$ into 3ml FACS tubes and were washed by centrifugation at 1600rpm for 5mins with MACS buffer (Miltenyi). The appropriate stimuli were added of peptide (final concentration peptide 1ug/ml), DMSO (negative control) or PMA/Ionomycin (Positive control-10ng/ml and 1ug/ml) and then the cells were incubated for 1 hour at 37ºC followed by the addition of Brefeldin A (BFA-10mg/ml final conc.) and incubation overnight at 37ºC. After 20 hours the cells were washed and then stained with the viability dye followed by surface antibodies as previously done. Cells were then washed, fixed with 1% Paraformaldehyde (PFA), permeabilized with 0.5% saponin and then stained with cytokine antibodies. Samples were re-suspended in100µl MACS buffer and then run on an LSR-II (BD biosciences) and then analysed using FlowJo (Tree Star).

2.5. MOLECULAR TECHNIQUES

2.5.1. Media and buffers

LB media- LB (Luria Broth) was prepared by dissolving 20g/L of LB powder (Invitrogen) in sterile distilled water (SDW). This was then sterilised by autoclaving at 121ºC for 20 minutes at 15psi.

LB agar- LB agar was prepared by dissolving 20g/L of LB agar powder (Invitrogen) in SDW and sterilising by autoclaving at 121ºC for 20 minutes at 15psi.

Antibiotics- Ampicillin was made up as a 1000x stock at 100mg/ml in distilled water and stored at -20ºC.

Bacterial strains- For the maintenance of plasmids, Top10 competent bacteria were used.

2.5.2. RNA extraction

HLA typed donor LCLs were counted and $1x10^6$ cells were pelleted and RNA was extracted according to the NucleoSpin RNA isolation kit (Macherey-Nagel). RNA was eluted in RNAse-free water and kept on ice. Extracted RNA was quantified using a Nanodrop machine measuring A260/A280 (Thermo Scientific). The RNA was either frozen at -80ºC or was used immediately to generate cDNA.

2.5.3. cDNA synthesis of HLA class I molecules

Extracted RNA was used immediately after isolation or was thawed on ice after storage at -80ºC. Primers for generic HLA class I molecules were generated for initial experiments with later more specific primers used for specific HLA allotypes. RNA was reverse transcribed

into cDNA using the SuperScript III reverse transcriptase kit (Invitrogen) and according to their instructions. Specific 3' HLA primers were used with 1µg RNA sample. Generated cDNA samples were treated with RNAse H (Invitrogen) for 20mins at 37ºC to remove RNA template and were then quantified using a Nanodrop machine measuring A260/A280 (Thermo Scientific). Samples were then frozen at -20ºC until use.

2.5.4. Cloning EBV lytic genes

Primers for the remaining EBV lytic genes were designed and ordered from Sigma and used to amplify lytic genes from p2089 bacmid (Delecluse et al., 1998), kindly provided by Dr Claire Shannon-Lowe.

2.5.5. Gel purification

PCR products were electrophoresed on a 1% agarose gel with ethidium bromide (EtBr) to separate fragments. Either 1kb or 100bp ladders (Invitrogen) were used as a measure of product size. Bands of the correct size were excised under UV light for minimal time and placed into nuclease free 1.5ml tubes. DNA samples were then purified using the NucleoSpin gel and PCR clean up kit (Macherey-Nagel). Purified samples were eluted in nuclease-free water and quantified. Samples were either frozen at -20ºC for long-term storage or kept at 4ºC for short-term use.

2.5.6. Generation of plasmid DNA by bacterial transformation

Purified PCR products were cloned into the pcDNA3.1-V5/His-TOPO vector according to manufacturers instructions (Invitrogen). The vector map is shown below in Fig. 2.1. Briefly A-tails were generated on the purified PCR products by a 20min PCR cycle with Taq polymerase (Invitrogen). A cloning mix containing the TOPO vector, salt solution and PCR product was then prepared and incubated at RT for 5-30mins. During this time competent TOP10 bacteria were thawed slowly on ice followed by the addition of 2µl of the cloning mix to 50µl competent bacteria in a 1.5ml tube and incubated on ice for 30 minutes. Bacteria were then heat shocked for 45 seconds at 42ºC, placed on ice for at least 1 minute and then 250µl SOB media (supplied by Invitrogen) was added and samples were incubated in a shaking incubator at 37ºC for one hour. Bacteria were then pelleted and 150µl supernatant was removed. The bacteria were then resuspended and 100µl spread onto agar plates. Plates were then incubated overnight at 37ºC.

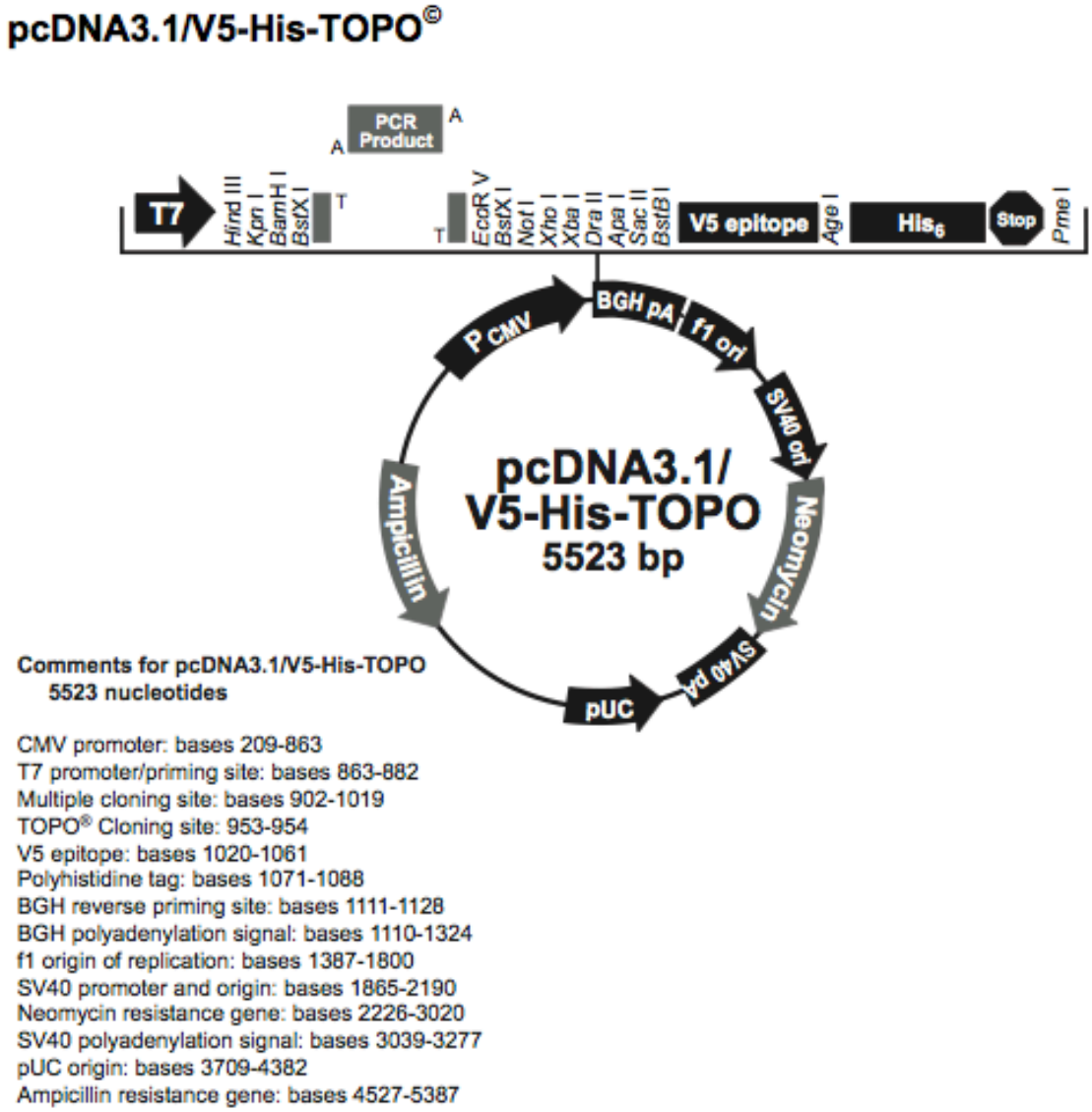


Figure 2.1. Schematic of pcDNA3.1/V5-HIS-TOPO vector with multiple cloning site.
Taken from the guidelines for the pcDNA(TM)3.1/V5-His TOPO® TA Expression Kit (Invitrogen- cat. K4800-01, K4800-40).

2.5.7. Purification of plasmid DNA

Bacteria colonies that had grown from the overnight incubation were picked and inoculated into a universal tube containing 3ml of LB broth, containing the appropriate antibiotic. Tubes were incubated in a 37ºC shaker overnight and the next day, samples were harvested and plasmid DNA extracted using a QIAgen Mini Prep kit according to manufacturers instructions (QIAgen). DNA was eluted in 50µl of nuclease free water and then quantified using a Nanodrop machine (Thermo Scientific). Newly generated expression constructs were then sent for DNA sequencing in the Life and Environmental Sciences (LES) department at the University of Birmingham. Reported sequence reads were analysed using Seq Scanner 2 software and aligned to the human herpesvirus 4 complete wildtype sequence AJ507799 or to HLA sequence reads on the ImMunoGeneTics (IMGT) website (http://www.ebi.ac.uk/ipd/imgt/hla/).

To generate a stock plasmid DNA, 1ml of the 3ml transformed bacteria culture was inoculated into 200ml of LB broth, containing the appropriate antibiotic, in a 1L conical flask. This was incubated overnight in a 37ºC shaker. The following day, the bacteria culture was transferred into large containers and pelleted by centrifugation. Following this, plasmid DNA was extracted using the QIAgen Maxi Prep kit, according to manufacturers instructions (QIAgen). The DNA was eluted in 200µl of nuclease free water, quantified and stored at -20ºC.

2.5.8. Glycerol stocks

A 3ml culture of transformed cells was generated as above and was mixed with glycerol to form a 70% glycerol: 30% bacterial culture prep. This prep was then frozen quickly in dry ice

and transferred to -80ºC. A small scraping from the glycerol stock using an inoculation loop was used to seed fresh cultures

CHAPTER 3

Developing a model of dendritic cell cross-presentation for the stimulation and expansion of EBV lytic antigen specific CD8+ T cell populations.

EBV is a B-lymphotropic virus and preferably replicates within B cells and epithelial cells, where driving its lytic phase leads to the expression of more than 70 lytic proteins. During primary infection there is a large expansion of antiviral CD8+ T cells largely directed against these lytic proteins, which as this primary infection is resolved, leaves these effectors highly sensitive to apoptosis and as such they undergo considerable contraction. This leaves only a small population of 'memory' cells that provide protection against lifelong persistence of EBV. The frequency of EBV-specific memory CD8+ T cells in the blood varies with reactivity against any single epitope, potentially being as large as 10% of the CD8+ T cell pool for the most dominant responses but being on the limits of detection for other responses. Therefore to identify the global repertoire of EBV-specific memory CD8+ T cell responses in donors with persistent infection, methods to identify or amplify all responses in a non-selective way are required.

Previous studies that have investigated CD8+ T cell responses to EBV lytic antigen have primarily used LCL restimulation to generate polyclonal lines. However direct stimulation of EBV-specific CD8+ T cells is problematic due to the low number of cells undergoing lytic replication as well as the presence of several immune evasion proteins expressed by the virus during the lytic phase. During EBV replication there is a temporal and sequential cascade of gene expression beginning with two immediate early (IE) proteins followed by the expression of up to 30 early (E) proteins then the expression of up to 30 late (L) proteins. These stages

have been separated on the use of protein synthesis inhibitors and inhibitors of viral DNA synthesis that serve to inhibit E gene expression and L gene expression respectively. E proteins are important for viral DNA replication but are also involved in protecting the cells from apoptosis and in modulating the immune response to the virus infection. The immune evasion proteins serve to decrease the pool of virus-derived peptide-epitopes available for presentation by MHC class I molecules (Ressing et al., 2015). Additionally other viral proteins such as the vIL-10 may have an immunomodulatory role that will affect antiviral T cell function (Jochum et al., 2012a). Therefore using lytic LCLs as an antigen source could lead to bias in the responses that can be identified with CD8+ T cell responses concentrated against the virus immediate early (IE) and early (E) proteins. This is observed by the decreasing efficiency of recognition of lytic LCL targets by CD8+ T cell clones specific for representative IE, E and L proteins (Pudney et al., 2005, Quinn et al., 2014).

Alternative strategies for examining CD8+ T cell responses to EBV lytic proteins have used overlapping peptides to screen for memory responses from a subset of IE, E and L proteins (Abbott et al., 2013). This study identified the presence of memory CD8+ T cell responses to IE, E and L proteins with the responses to L proteins as subdominant. However this strategy is insensitive to low frequency responses and only focused on a limited subset of lytic proteins. To examine the global repertoire of responses to EBV an alternative approach was developed based on a similar study on HSV-1 (Jing et al., 2012). This study used monocyte-derived dendritic cells to stimulate autologous HSV-1 specific CD8+ T cell responses that could then be enriched, expanded *in vitro,* and screened against a genome wide HSV-1 cDNA library. However differences in the virus biology between HSV-1 and EBV infection meant that this system had to be adapted for use in stimulating and enriching EBV-specific CD8+ T cell responses. To do this, different subsets of dendritic cells were examined for their ability to

cross present an EBV infected cell lysate to stimulate lytic-specific CD8+ T cell responses. The kinetics of activation and expansion of EBV-specific responses could then be tracked using MHC class I tetramer technology.

3.1. *In vitro* generation of monocyte-derived dendritic cells

In the HSV-1 study by Jing *et al* (Jing et al., 2012), monocyte-derived dendritic cells (moDCs) were loaded with apoptotic HSV-1 infected cells and used to stimulate HSV-1 specific memory CD8+ T cell responses. Therefore a similar method was attempted here. The *in vitro* generation of moDCs from blood monocytes was identified 20 years ago by Sallusto and Lanzevecchia (Sallusto and Lanzavecchia, 1994) and has been well documented for its use in studying DC immunobiology.

To enrich CD14+ monocytes from peripheral blood mononuclear cells (PBMCs) two different methods have been used; plastic adherence of monocytes with gentle washing to remove other cell populations or the magnetic capture of CD14 expressing cells. The plastic adherence method is useful for the crude enrichment of monocytes however initial attempts at this led to high contaminating levels of T cells that could interfere with downstream assays (data not shown). Therefore the alternative method of magnetic separation (Miltenyi) was used. Magnetically labelled cells can be bound to a column whilst the unlabelled fraction is washed through the column. This method can produce a highly enriched CD14-expressing population, the vast majority of which will be monocytes with few T cell contaminants (Fig 3.1).

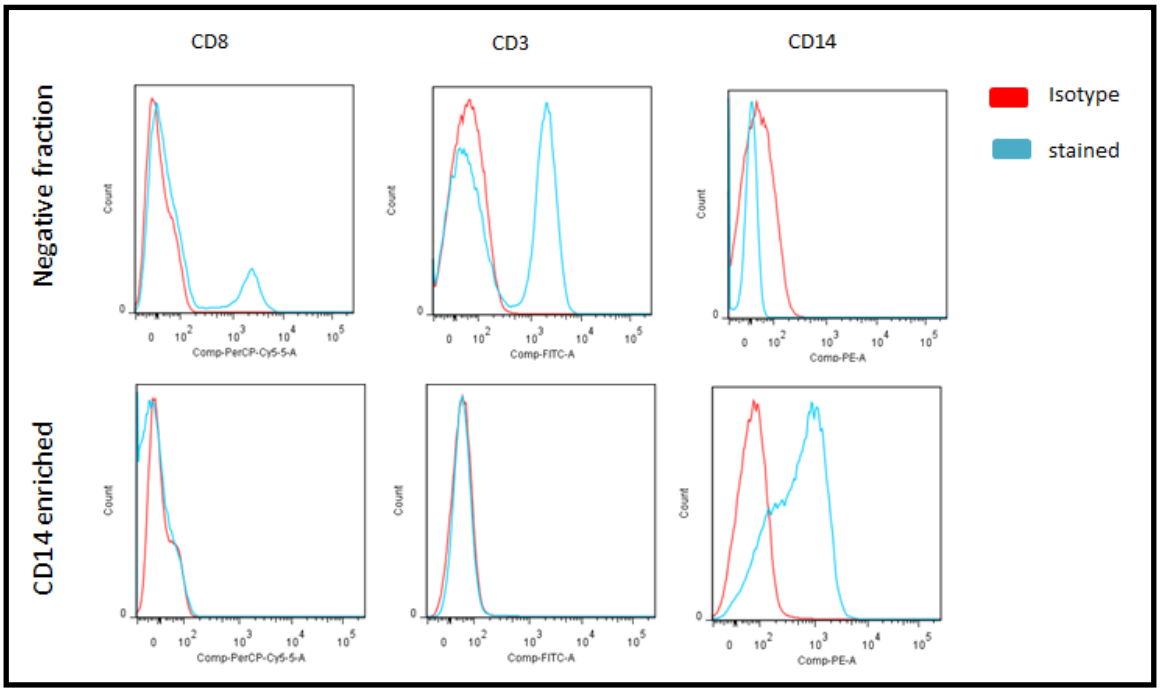


Figure 3.1. Enrichment of CD14+ PBMCs.
CD14+ expressing monocytes can be enriched from PBMCs by magnetic bead separation. The CD14+ expressing and negative fractions were collected. These fractions were analysed by flow cytometry for the expression of CD3, CD8 and CD14 to show the amount of contaminating T cells.

Having established that CD14+ monocytes can be enriched to a high purity, methods to differentiate and mature these into moDCs were optimised. Firstly, enriched CD14+ cells were plated out at a density of $1x10^6$/ ml in 10% FCS-RPMI and given the cytokines GM-CSF and IL-4 to differentiate the monocytes over 4-5 days into immature moDCs using previously described protocols (Sallusto and Lanzavecchia, 1994, Jing et al., 2012). The immature moDCs are then further differentiated into mature moDCs by treatment with a maturation stimulus consisting of either inflammatory cytokines (IL-6, IL-1β and TNFα) or TLR agonists polyinosinic-polycytidylic acid (Poly(I:C) and R848 (resiquimod) (Tel et al., 2013b).

Parallel cultures of immature moDCs were treated with either of these stimuli and the efficiency of maturation was measured by analysing expression of cell surface markers. These included CD14, which, after differentiation from monocytes, is lost from these cells. Upon differentiation of immature moDCs to mature moDCs, markers associated with antigen uptake such as CD209 are down regulated whilst the expression of co-stimulatory molecules CD83 and CD86 as well as MHC molecules are upregulated. Fig 3.2 shows a representative plot for the surface phenotype of immature and mature forms of moDCs using TLR agonists as the maturation stimulus. A similar phenotype was observed when using inflammatory cytokines as the maturation signal. TLR agonists were subsequently used for all further assays due to studies that have shown that DC maturation cocktails containing TLR agonists rather than inflammatory cytokines are capable of driving potent TH1 responses that can aid CTL expansion (Langenkamp et al., 2000, Jensen and Gad, 2010).

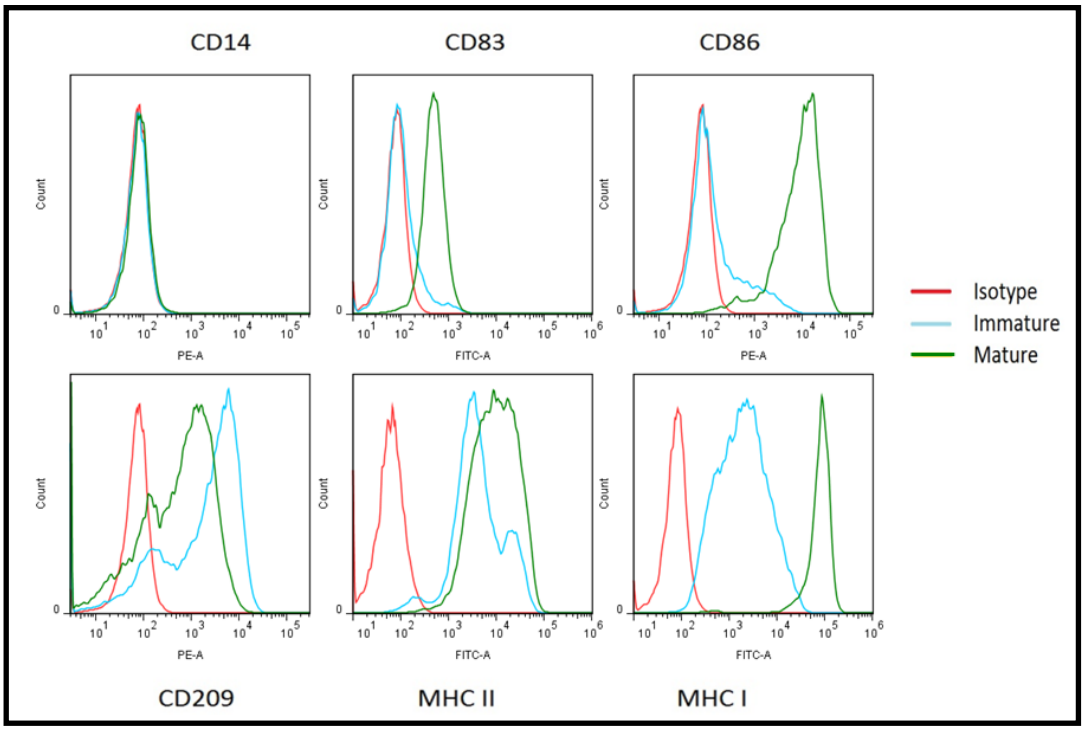


Figure 3.2. *In vitro* differentiated moDC phenotype.
Dendritic cells were differentiated from CD14 expressing monocytes and treated with a maturation stimulus of either inflammatory cytokines or TLR agonists. Immature and mature forms of moDCs were stained using markers of antigen uptake (CD209) or antigen presentation (CD83, CD86, MHC I, or MHC II) and analysed by flow cytometry. Immature moDCs (blue), mature moDCs (green) and isotype control (red).

3.2. Recognition of antigen-pulsed DCs by T cell clones.

As a protocol to efficiently select and mature moDCs had been developed a method was developed using moDCs to process and present viral antigens to PBMC-derived CD8+ T cells and measuring T cell effector function as a readout. As an initial experiment we first determined if moDCs could present exogenous antigens to CD8+ T cell clones. Here a model was devised where moDCs were pulsed for 5 hours with two concentrations of a lysate of CMV-infected cells and then challenged with a CD8+ T cell clone specific for the CMV encoded pp65 epitope RPH restricted through HLA B*07.02. The ability of the clone to recognise the antigen-loaded cells was assessed by measuring IFNγ secretion and used as a measure of the DCs ability to process and present antigen. As a positive control the moDCs were sensitised with the clones' synthetic peptide-epitope and as a negative control moDCs were challenged with lysates from mock-infected cells. The moDCs were also challenged with a CD4+ T cell clone in parallel that was specific towards the peptide-epitope LLQ from pp65 to confirm that the moDCs were capable of endocytosing antigen. Figure 3.3 shows representative results of three assays. Here challenge of moDCs sensitised with synthetic peptides induced strong responses from both CD4+ and CD8+ clones. When pp65-specific CD4+ T cells were incubated with CMV-antigen loaded DCs these T cells secreted IFNγ suggesting the DC took up this antigen and processed and presented it. However no response was seen from the CD8+ T cells specific for the pp65 peptide-epitope RPH when challenged in parallel.

As individual dendritic cell subsets in the blood have been shown to possess different antigen-processing and presentation abilities a different DC subset was tested to see if it could present epitopes to CD8+ T cell clones. Here plasmacytoid dendritic cells (PDCs), a subset recently shown to be able to cross-present soluble antigen (Tel et al., 2013b) were used. This subset

was enriched from donor PBMCs by magnetic bead sorting to a relative purity of above 95% (Fig 3.4) as analysed using several different markers specific to PDCs. The PDCs were maintained in media supplemented with IL3 before being pulsed with antigen as described previously for moDCs. Concomitantly with antigen delivery, a maturation stimulus ODN2216 was also used to stimulate maturation through TLR9 expressed by PDCs. Again a pair of CD4+ and CD8+ T cell clones were used to assess antigen presentation by the PDC. Here, due to donor HLA restriction, an alternative CD4+ T cell clone was used with specificity for the pp65 peptide-epitope AGIL.

Similarly to moDCs, CMV-antigen loaded PDCs stimulated CD4+ T cells to secrete IFNγ suggesting they were able to process and present exogenously-derived antigen (Fig 3.5. data from two experiments). Testing RPH-specific CD8+ T cells in parallel did induce a response by the CD8+ T cell clone that was not significantly different in the first assay (Fig. 3.5. Top) but in the second did reach significance (Fig. 3.5. Bottom). This suggests that PDCs are capable of processing and presenting antigen to CD4+ and less efficiently to CD8+ T cells. The moDCs, however, were only capable of re-stimulating the CD4+ T cell response and not the CD8+ T cell response in this assay.

These experiments suggested that PDCs at least were capable of stimulating CD8+ T cell responses but that this experimental model of cross-presentation using CD8+ T cell clones was not sufficiently sensitive. Therefore an alternative method was developed to examine expansion of EBV-specific CD8+ T cell responses after stimulation by antigen-loaded moDCs and PDCs. MoDCs were run along-side PDCs due to the difficulty in isolating sufficient numbers of PDCs for these experiments as 60mls of peripheral blood from a healthy individual typically yields only $5x10^5$ PDCs.

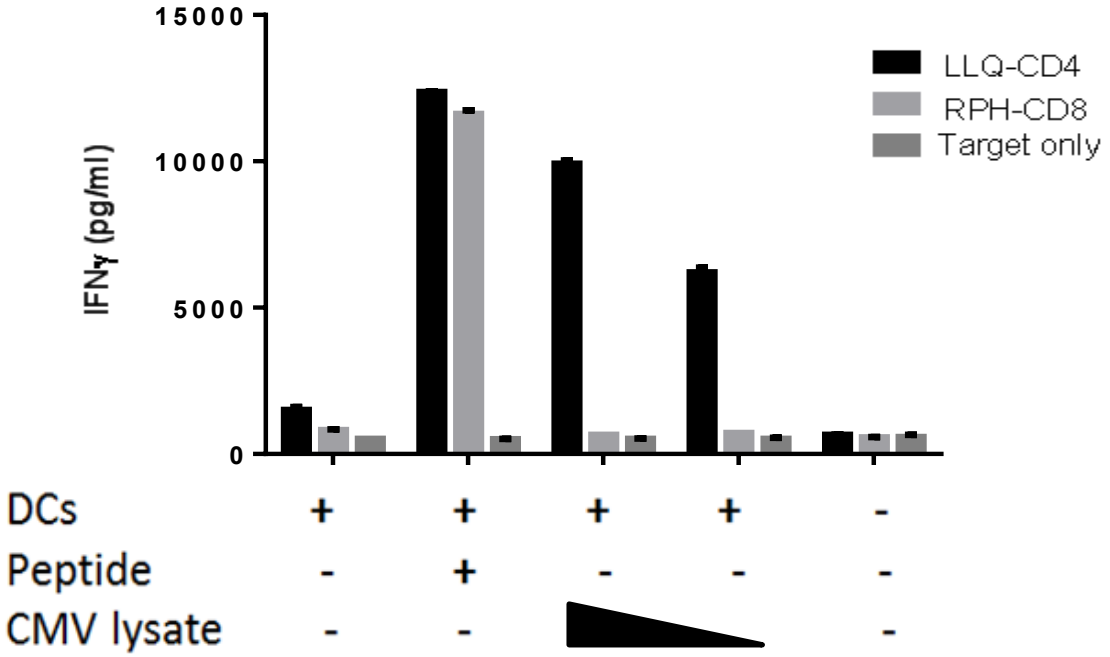


Figure 3.3. T cell Recognition of moDCs pulsed with CMV antigens.
moDCs were pulsed with whole CMV-infected cell lysate or CMV specific peptides derived from the protein pp65- RPH (CD8) and LLQ (CD4). IFNγ secretion was used as a measure of T cell recognition.

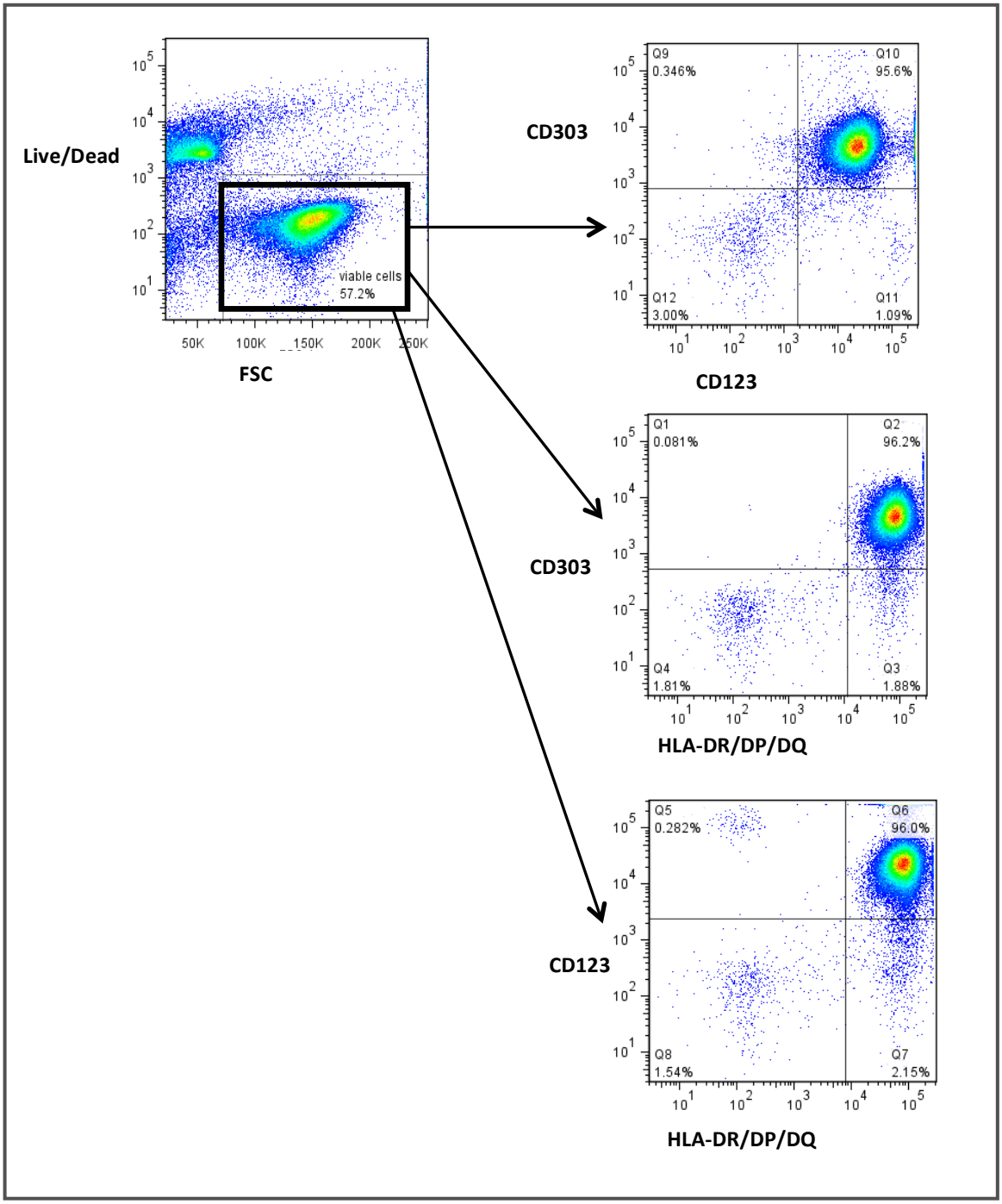


Figure 3.4. Analysis of enriched plasmacytoid dendritic cells.
PDCs were enriched by positive selection (Miltenyi) of CD304+ cells from peripheral blood. PDC markers (CD303 and CD123) along with MHC class II were used to examine the relative purity of the magnetically bound cells.

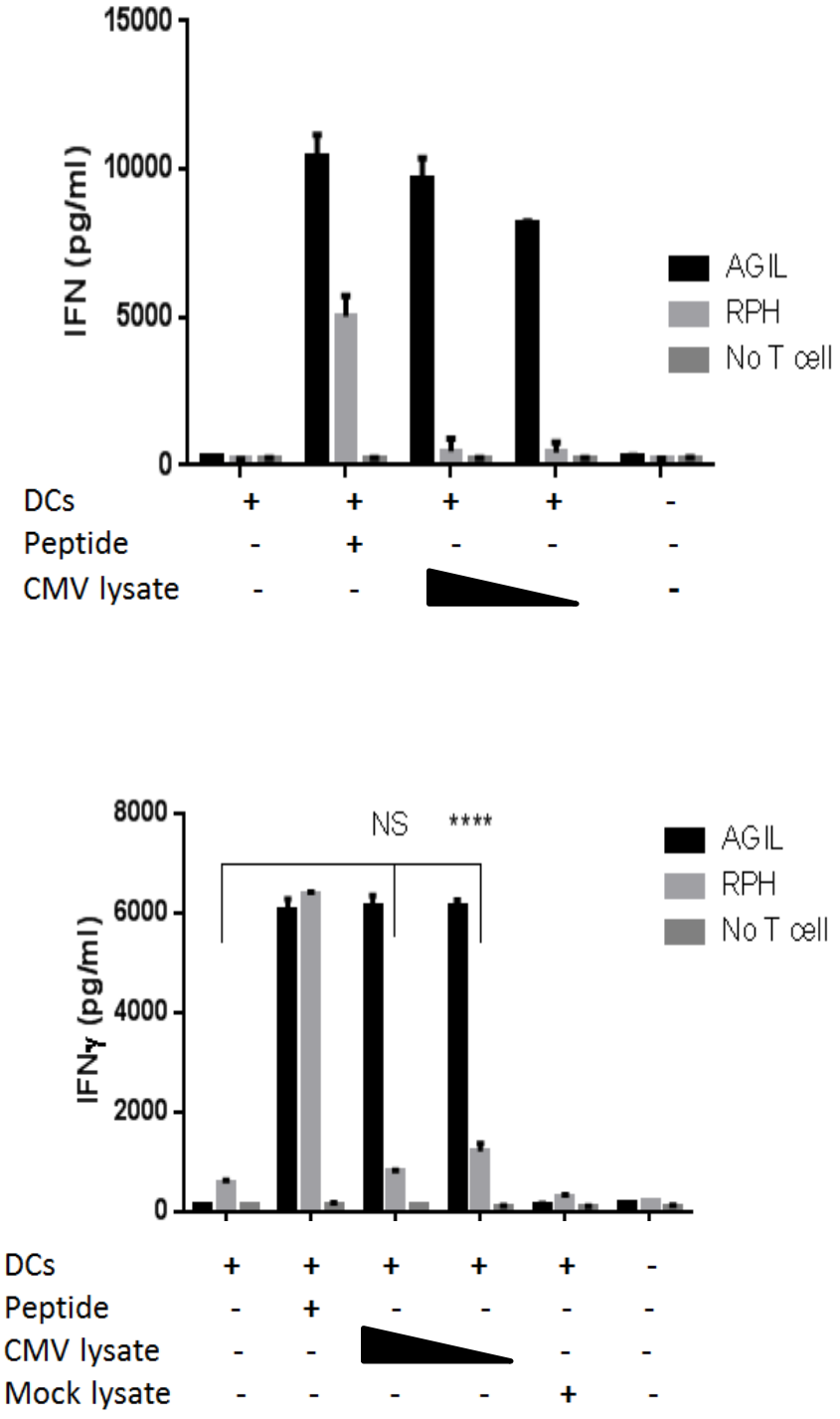


Figure 3.5. T cell Recognition of PDCs pulsed with CMV antigens.
PDCs were pulsed with whole CMV-infected cell lysate or CMV specific peptides derived from the protein-pp65 RPH (CD8) and AGIL (CD4). IFNγ secretion was used as a measure of T cell recognition. Results of two independent assays shown. Wilcoxon matched pairs analysis was used to test significance.

3.3. Expansion of CMV-specific CD8 T cells through priming by moDCs and PDCs

As weak responses were seen using the CD8+ T cell clones to probe the epitope-specific presentation on DCs, a second more sensitive approach was developed to detect presentation by measuring antigen-specific outgrowth of PBMC-derived CD8+ T cell reactivities. Here PBMC-derived moDCs and PDCs were loaded overnight with either a lysate containing CMV antigens, a lysate of mock infected cells, or the DCs were pulsed with synthetic CMV-epitope peptides and then matured using TLR agonists. These TLR agonists were chosen due to studies showing that they have potent Th1 polarizing ability when used to mature DCs (Langenkamp et al., 2000, Jensen and Gad, 2010). Autologous CD8+ T cells were incubated with the antigen-loaded DCs and the outgrowth of CMV-specific CD8+ T cell responses was followed using MHC I tetramers and intracellular cytokine secretion assays (Figs 3.6 and 3.7 respectively).

After 8 days outgrowth (Fig 3.6) the CD8 T cells in the polyclonal lines were screened using MHC class I tetramers that contained either the ELR-peptide from the CMV-protein IE-1 or the VTE peptide from the CMV-protein pp50. The negative control using DCs loaded with a mock lysate showed little if any expansion of ELR and VTE responses whereas the positive control using the ELR or VTE peptides induced a large expansion of around 30 fold. We observed expansion of both ELR and VTE responses in cultures stimulated with either moDCs or PDCs pulsed with the CMV-lysate. Compared to *ex-vivo* responses this expansion was a 2-5 fold increase. These polyclonal lines were cultured for a further 7 days and analysed using the same tetramers with further expansion of ELR and VTE responses seen (data not shown). These data suggest that both moDCs and PDCs are capable of processing and presenting exogenously-derived virus antigen which can stimulate the expansion of virus-specific CD8+ T cells within a polyclonal population of cells.

To determine whether this protocol expanded additional CMV specific responses for which tetramer reagents were not available the lines were stimulated with CMV-specific peptides and intracellular cytokine production examined (Fig. 3.7.). As controls, polyclonal lines were left unstimulated (-ve) or were stimulated non-specifically using PMA/ionomycin (+ve). All polyclonal lines had a large proportion of cells producing IFNγ upon the non-specific stimulus whereas the unstimulated lines had only a low proportion of responding cells. The polyclonal line generated against DCs pulsed with the mock lysate showed only low background of responding cells when re-stimulated using each of the CMV-specific peptides asides from the VTE challenge. This may reflect the relatively large *ex vivo* level of VTE responses found in PBMCs from this donor (Fig 3.6). When assessing the expansion of responses using the polyclonal line generated with the ELR and VTE loaded DCs, cells in the line responded when re-challenged with the ELR and VTE peptides but not with the other peptides (Light grey bars), showing specific expansion of ELR-and VTE-specific T cells in this culture.

However when we challenged the polyclonal generated with the CMV-lysate loaded DCs, additional responding specificities were detected. This was particularly evident in cultures using moDCs as the APC with responding cells specific for ELR, VTE, QIK and RTD peptides. Similarly in the lines from PDCs we also saw responses against VTE and QIK, however the other responses were not detected. Altogether these results show that a polyclonal line containing a range of responses could be expanded when using DCs pulsed with a CMV-lysate and that moDCs appeared to better than PDCs in this approach. Due to the difficulty in isolating sufficient numbers of PDCs from donors, moDCs will now be used in all further experiments.

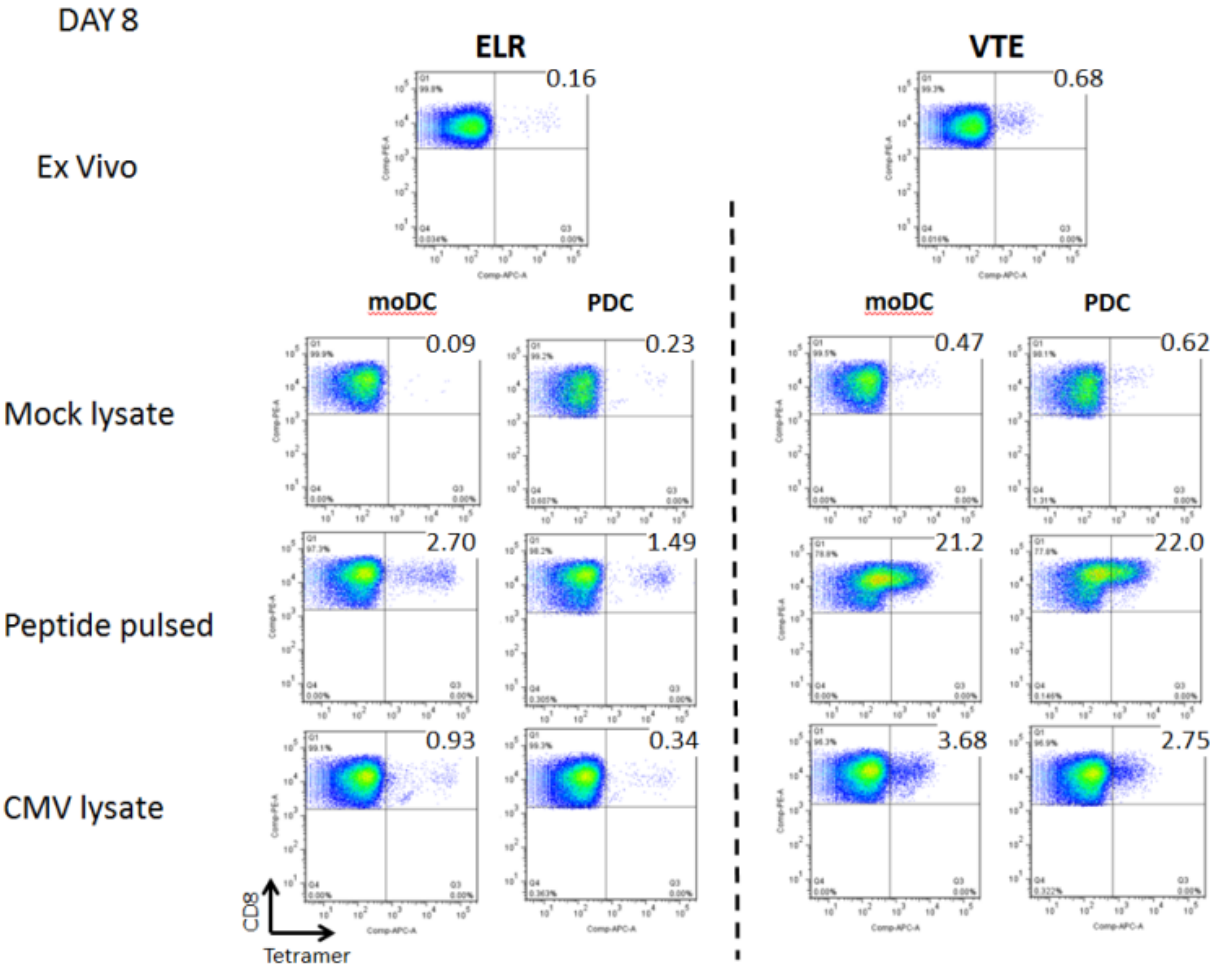


Figure 3.6. MHC I tetramer analysis of expanded CMV-specific CD8 T cell responses
Polyclonal T cell lines were generated in culture after donor CD4-depleted PBMCs were stimulated with moDCs or PDCs pulsed with either a mock cell lysate, synthetic peptides (ELR and VTE) or a CMV-infected cell lysate. 8 day expanded polyclonal lines were probed using MHC class I tetramers containing either ELR or VTE peptides. Frequencies of CD8+ Tetramer+ cells are shown in the top right hand quadrant of each graph. Donors *ex vivo* responses are used to show background response sizes.

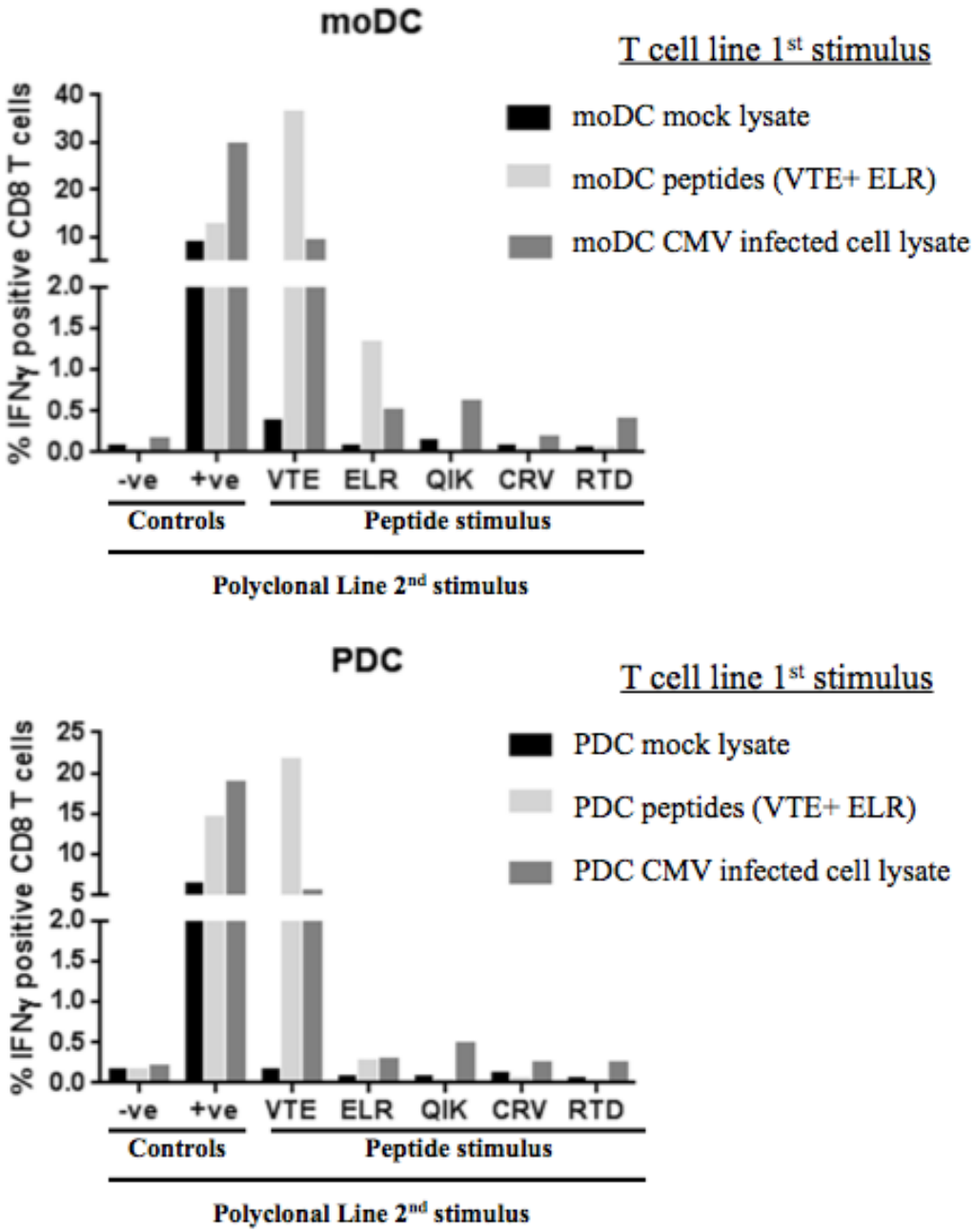


Figure 3.7. Expanded CMV-specific CD8 T cells show broad specificity.
Expanded polyclonal T cell lines were re-stimulated with CMV-specific peptides. Analysis of IFNγ production by intracellular cytokine staining was used to measure frequency of responding cells. (VTE-pp50; ELR- IE-1; QIK- IE-1; CRV- IE-1; RTD- UL69). Controls (-ve and +ve) refer to no re-stimulation and stimulation with PMA/ionomycin respectively.

3.4. Expansion of EBV-specific CD8 T cells through stimulation with EBV-antigen loaded moDCs.

As antigen loaded moDCs have been shown to be capable of expanding T cell responses against CMV antigens *in vitro*, their ability to expand EBV-specificities was now tested. Initially a panel of cell lysates containing EBV lytic antigens was generated. One such antigen source involved ectopic expression of the EBV SM protein in HEK293 cells, which were then lysed and the sample clarified by centrifugation. This antigen was chosen due to well-characterised CD8+ T cell responses to the HLA-A2 presented-peptide GLC found within the SM protein and the ability to track these T cells using GLC-specific tetramers.

A second antigen source was required that contained the complete lytic repertoire and for this we chose HEK293 cells containing the prototypic B95.8 EBV bacmid- p2089 that can be reproducibly induced into lytic cycle (Delecluse et al., 1998). Ectopic expression of the IE transactivator BZLF1 in these cells initiates the cascade of lytic gene expression which ultimately leads to virus production. Expression of the late expressed protein gp350 was used as a marker for transition through the lytic phases, with cells harvested and a stock cell-lysate generated 48 hours after transfection of BZLF1 into these cells (Fig 3.8).

We now attempted to expand GLC-specific T cells using immature moDCs pulsed with either the 2089 cell-lysate, the SM-lysate or with the GLC peptide then matured with TLR agonists. A point of contention was at what time to apply the maturation stimulus to the moDCs in conjunction with the addition of antigen (Herr et al., 2000). As such immature moDCs were loaded with the different antigens and then chased with the maturation stimulus either 2 hours or 18 hours after the addition of antigen. The moDCs were then washed 24 hours after the addition of antigen to remove any excess antigen. The loaded mature moDCs were then cultured with autologous CD8+ T cells for 14 days after which I quantified the GLC-

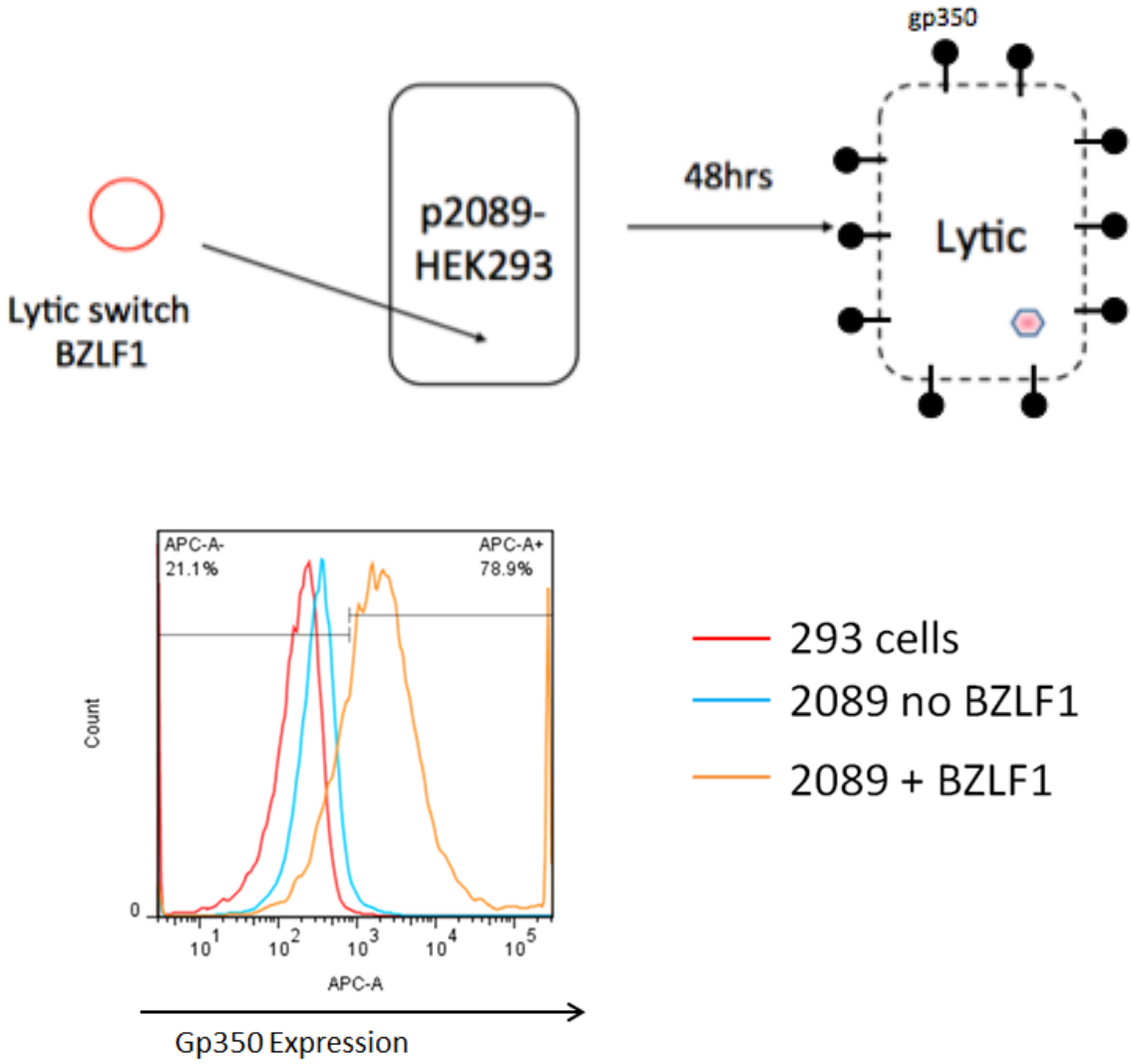


Figure 3.8. Generation of a lytic EBV antigen source
(Top) Ectopic expression of the EBV transactivator protein BZLF1 was used in *trans* to induce initiation of the lytic cycle of the p2089 EBV BACmid containing cell line p2089-293. (Bottom) percentage of cells expressing the late protein gp350.

specific CD8+ T cells using class I tetramer analysis. The background frequency of GLC-specific CD8+ T cells in this donor is around 1% as shown by the *ex vivo* response in fig. 3.9. After culture for 14 days, the two polyclonal T cell lines stimulated with the mock lysate-loaded moDCs showed no expansion of GLC-specific CD8+ T cells whilst the two lines stimulated with the peptide-pulsed moDCs showed a large expansion with around 70% of cells in the polyclonal line specific for GLC epitope. For the two lines stimulated with moDCs loaded with the 2089 lysate, there was a 3.2 fold and 4.9 fold expansion of GLC-specific CD8+ T cells respectively when the maturation stimulus was applied to the moDCs 2 hours or 18 hours after the addition of antigen. However for the two lines stimulated with the SM-loaded moDCs there was a 2-fold difference when the 2-hour time point was used (27.4% GLC-specific) over the 18-hour time point (13.7% GLC-specific). Therefore for further assays this 2-hour time point would be used.

To test the ability of this protocol to stimulate EBV-specific CD8+ T cells to a broad range of epitopes, moDCs were generated from two donors with known CD8+ T cell responses, loaded with the 2089 lysate and then used to expand autologous CD8+ T cell responses (Fig. 3.10). After 14 days expansion these cells were challenged with panels of epitope-peptides from different EBV proteins that are expressed at different phases of the lytic cycle. The responses were then measured by ELISPOT analysis. As positive controls polyclonal lines were also generated using moDCs pulsed with each of the peptides used in the ELISPOT assay, while negative controls of moDCs loaded with a mock antigen were used. These responses were all compared against the donors *ex vivo* responses.

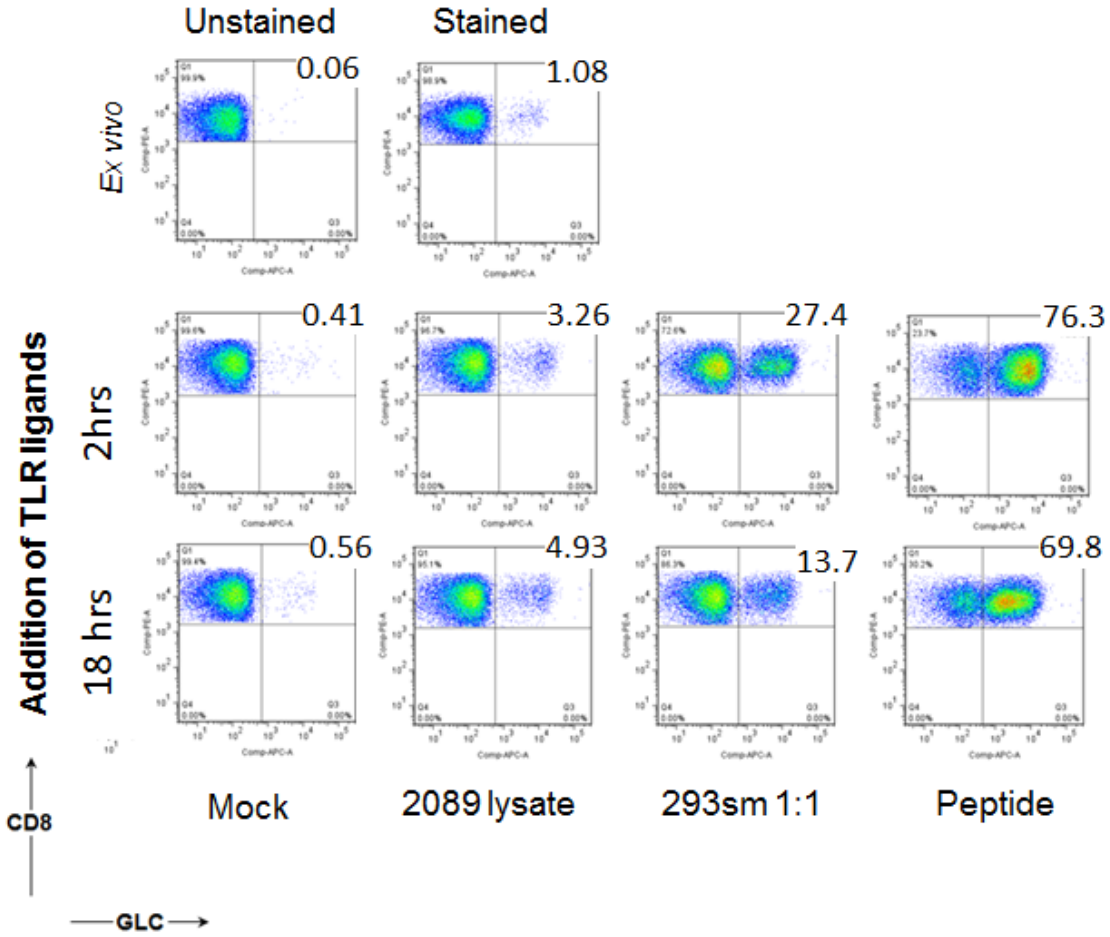


Figure 3.9. Expansion of GLC-specific T cells using moDCs matured with TLR agonists.
Polyclonal T cell lines were generated in culture after donor CD4-depleted PBMCs were stimulated with moDCs pulsed with a mock cell lysate, synthetic peptide (GLC), a lysate of SM expressing cells or an EBV-infected cell lysate. Fourteen day expanded polyclonal lines were probed using MHC class I tetramers containing the GLC peptide. Frequencies of CD8+ Tetramer+ cells are shown in the top right hand quadrant of each graph. Donors *ex vivo* responses are used to show background response sizes. TLR agonists (Poly(I:C) and R848) were added to moDC cultures 2 hours or 18 hours after the addition of antigen. Antigen pulsed cultures were washed 24 hours after the addition of antigen. Autologous CD8+ T cells were then cultured with the antigen-loaded moDCs cultures and cultured for 14 days.

For donor 1, *ex vivo* responses and responses in lines generated with mock loaded DC were barely detectable with only a small number of cells responding. In comparison clear responses were observed against the RPR, RPG and YPR (BaRF1, BNLF2b and BNRF1 respectively) peptides for both the 2089- and peptide-generated polyclonal lines suggesting that these T cells had been expanded in these lines. By contrast only a small response was seen against the TPS peptide in both the 2089- and peptide-generated polyclonal lines implying that these T cells had not been expanded, consistent with previous analyses performed in our lab that showed this donor had a small if not absent *ex vivo* response to the TPS peptide.

Donor 2 had large *ex vivo* responses to the peptide-epitopes RAK and VED taken from the IE protein BZLF1 and the L protein BKRF2 respectively. These T cells were expanded in the polyclonal lines generated from the 2089-lysate and the peptides. For the peptides TLD, FLD and WQW (BMRF1, BALF4 and BNRF1 respectively) the *ex vivo* responses were relatively small but there was a high response in the polyclonal line generated from the mock lysate. However with the 2089- and peptide-generated lines the responses to TLD and WQW appeared to have expanded.

These results show that a range of virus-specific CD8+ T cells can be expanded within a polyclonal population when using a complex antigenic preparation such as the 2089-cell lysate.

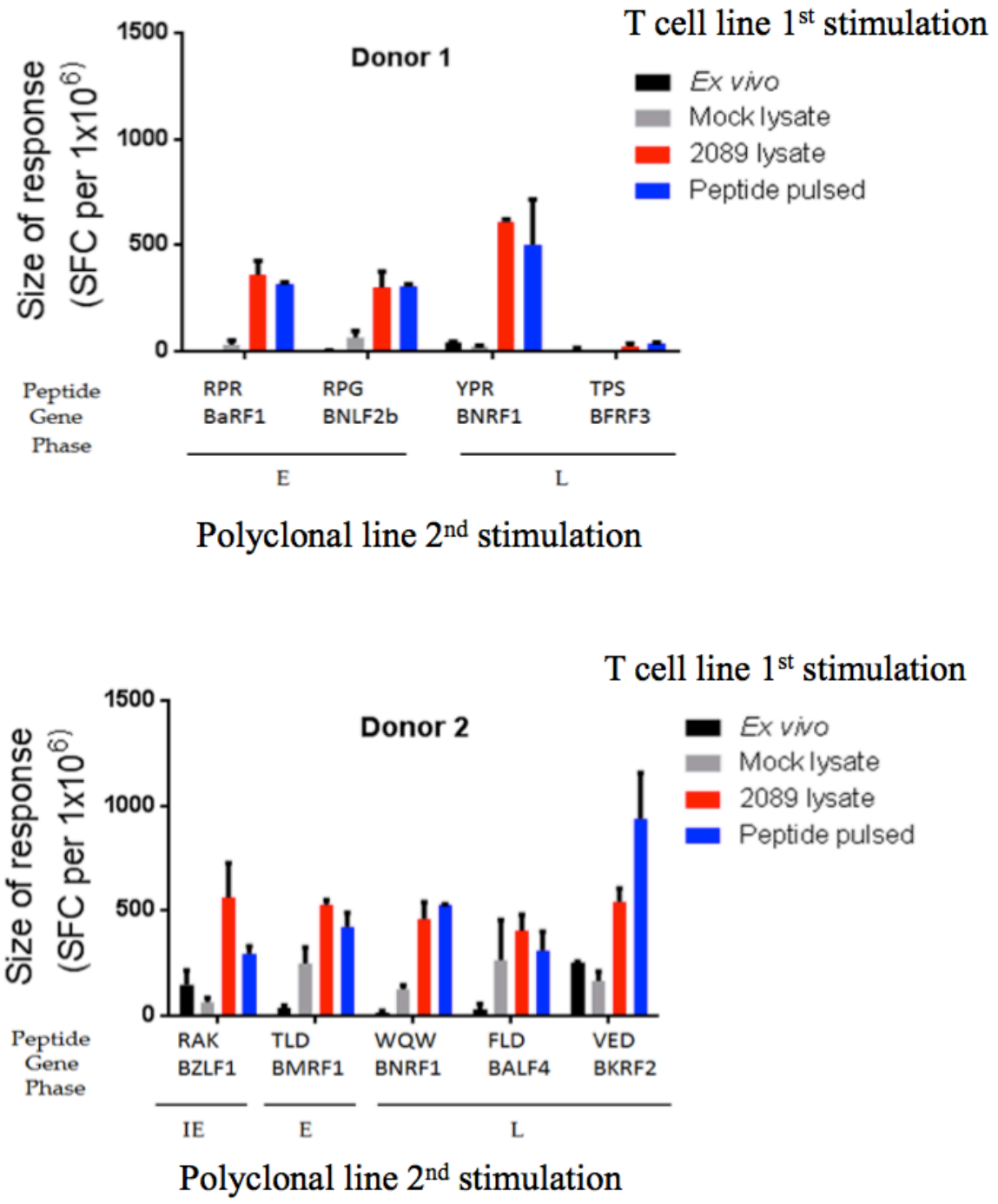


Figure. 3.10. Expansion of EBV-specific CD8+ T cells against IE, E and L antigen
Polyclonal T cell lines were generated using autologous moDCs loaded with either a mock lysate, induced 2089 cell lysate or with EBV-specific peptides (specific for each donor). Outgrown polyclonal lines were restimulated with relevant peptides and the number of responding cells measured in an IFNγ capture ELISPOT assay (SFC-spot forming colonies).

3.5. Time course for activation of EBV-specific CD8+ T cells

The previously generated polyclonal T cell lines were generated by growing out the lines in IL-2 supplemented media. It has been documented that certain specificities of T cells can out-compete others within a polyclonal mixture in a manner termed 'immunodomination' (Chen et al., 2000). Therefore to ensure there was as little bias as possible in growing out the polyclonal lines a different approach was sought. Here a similar approach to that of Jing *et al* (2012) was used where the T cells were cultured with antigen loaded moDCs and then those cells which became activated as measured by expression of CD137, would be sorted to give an enriched line that would be further expanded (Jing et al., 2012).

The kinetics of CD137 expression after challenge were then determined over six days to identify the best time to sort these cells, prior to expansion. PBMCs from a donor with a known GLC-specific CD8+ T cell response were stimulated using autologous moDCs loaded with a mock lysate (negative control), GLC-peptide (positive control), or two different amounts of the SM lysate (ratio of 1:1 or 2:1 with moDCs). The activation of GLC-specific CD8+ T cells was then tracked over several days by staining with GLC-tetramer and for CD137 expression. Fig. 3.11. (top) shows one representative result of two reporting the frequency of GLC-specific CD8+ T cells and the proportion of those cells that were expressing CD137.

No observed activation of GLC-specific T cells over the time course was seen when using moDCs loaded with a mock-lysate. In comparison when using the positive control of GLC-peptide pulsed moDCs there appeared to be an initial decrease in staining with the GLC-tetramer positive cells. This decrease in tetramer positive cells was likely due to the presence of peptide-loaded moDCs providing an overwhelming stimulus leading to the decrease in expression of the TCR on GLC-specific CD8+ T cells. However all tetramer positive cells

expressed CD137 and the frequency of dual positive cells increased over the time course though up until day 3 it was observed that the frequency of activated GLC-specific T cells did not exceed the *ex vivo* levels of around 1% as seen in Fig 3.9. By day 6 there was a clear expansion of GLC-specific CD8+ T cells exceeding 10% of the total CD8+ T cell compartment with all GLC-specific CD8+ T cells expressing the activation marker CD137.

When moDCs were loaded with a cell-lysate ectopically expressing the EBV protein-SM containing the GLC peptide-epitope, it could be seen that the frequency of activated cells increased over the time course and that this was further enhanced when increasing the amount of cell-lysate given to the moDCs. Up until day 3 no increase in the number of GLC-specific T cells was observed suggesting no substantial proliferation had occurred, however by day 6 the number of GLC-specific cells had more than doubled. This expansion continued so that by day 7 the frequency of GLC-specific CD8+ T cells had almost doubled over that observed on day 6 (Fig. 3.11. bottom).

Altogether these data show that virus-antigen loaded moDCs can stimulate virus-specific CD8+ T cells within a polyclonal culture and lead to their activation as shown by expression of the marker CD137. The frequency of activated virus-specific T cells appears to increase over time and no proliferation was seen until day 6 post-stimulation. Therefore for future assays, FACS sorting for activated CD8+ T cells will be performed around day 3, prior to any observed proliferation and so reduce the effect of immunodomination.

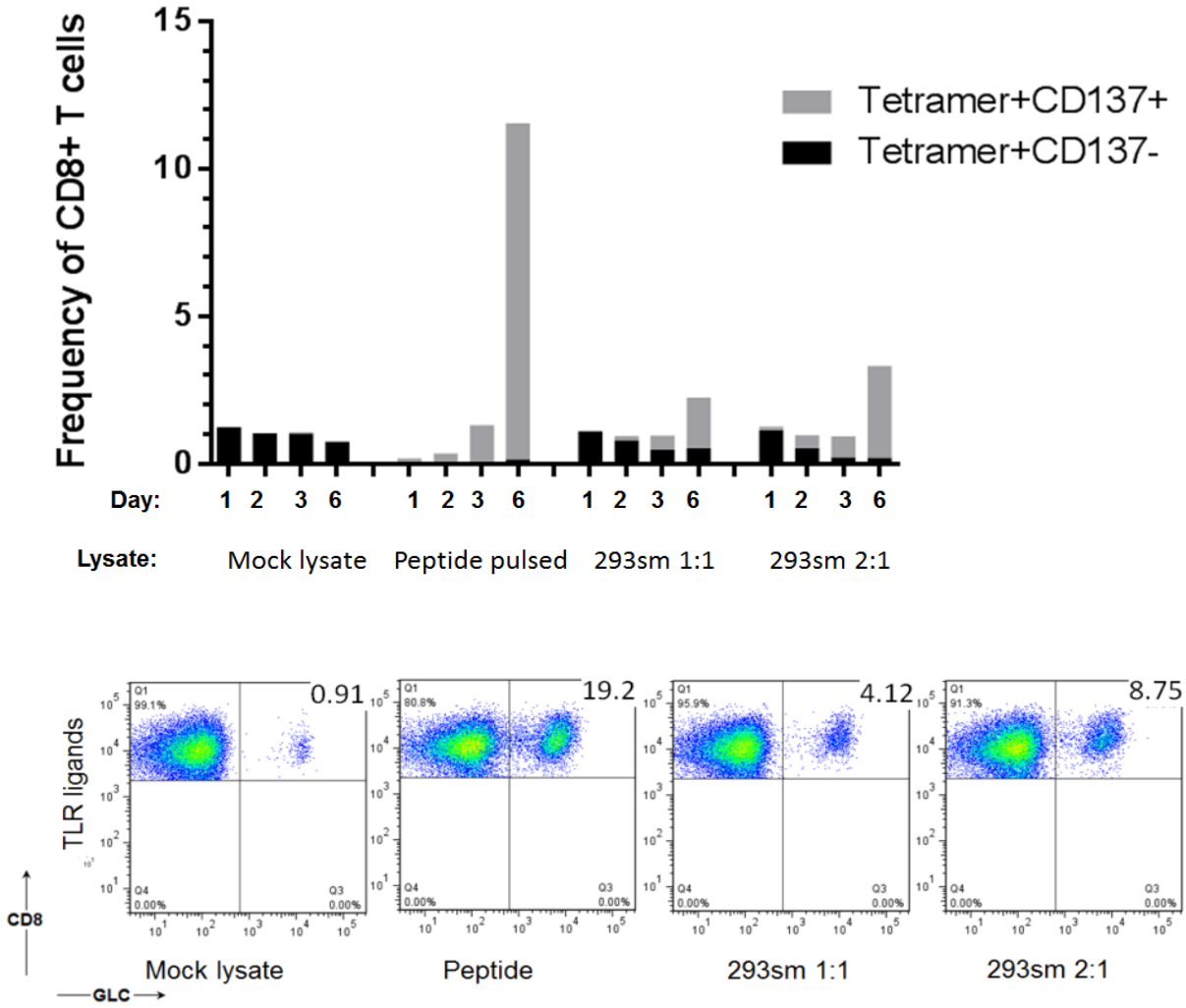


Figure. 3.11. EBV-specific T cell activation time course.
GLC-specific CD8 T cells were tracked over 6 days by co-staining with a GLC-specific tetramer and for the activation marker CD137 (Top left). After 7 days post-stimulation the frequency of GLC-specific CD8+ T cells in cultures using TLR agonists as a maturation stimulus was analysed using the GLC- specific tetramer (bottom).

3.6. Discussion

Dendritic cells have been shown in many studies to be potent antigen-presenting cells capable of stimulating both CD4+ and CD8+ T cells. Many different subsets of DCs exist within specific tissues or in peripheral blood and can have different abilities to stimulate T cell responses (Shortman and Liu, 2002, Shortman et al., 1997). Within peripheral blood there are several different subsets of DCs termed 'blood DCs' with each found at low frequencies of <1% PBMCs as discussed in chapter 1. This low frequency makes these subsets difficult to enrich and to obtain sufficient numbers for the assays intended in this study. Additionally the manipulation of these *ex vivo* DCs can affect their phenotype and function. However there are alternative methods using *in vitro*-derived DCs. Peripheral blood CD14+ monocytes can be found at a frequency of 10-15% PBMCs and were shown by Sallusto and Lanzevecchia to be capable of differentiating into moDCs using the cytokines IL-4 and GM-CSF and to become potent APCs (Sallusto and Lanzavecchia, 1994).

Therefore initial studies involved creating protocols to reproducibly isolate CD14+ expressing cells and differentiate them into moDCs. Whilst immature DCs are capable of endocytosing large quantities of exogenous antigen, mature DCs have been shown to down regulate this ability. Mature DCs have been shown instead to upregulate co-stimulatory molecules and MHC molecules to become more potent APCs. This maturation process has been shown to be driven by a number of inflammatory cytokines, TLR agonists and even by physical manipulation of DCs (Langenkamp et al., 2000, Jensen and Gad, 2010, Pierre et al., 1997, Salskov-Iversen et al., 2005). Therefore different maturation stimuli of either inflammatory cytokines or TLR agonists were tested. The enhanced APC ability was confirmed in our study by examining levels of co-stimulatory molecules and HLA class I and II expression that all increased when a maturation stimulus was delivered. Our lab traditionally used inflammatory

cytokines but this was primarily for the stimulation of CD4+ T cells. It has been shown that different stimuli can polarise T cell responses along either TH1 or TH2 pathways based on the production of inflammatory cytokines such as IL-12 (Langenkamp et al., 2000). This study also showed that the length of DC-T cell interaction could polarise T cells along TH1 and TH2 pathways.

Initial attempts at showing that moDCs could process and present exogenous antigen involved using a range of CD4+ and CD8+ T cell clones that our lab has established. These T cell clones are specific to various virus-proteins and restricted through a range of HLA alleles. All initial attempts at trying to show stimulation of CD8+ T cells using antigen-loaded moDCs were unsuccessful despite successfully showing that they were capable of processing and presenting peptides found within the same antigen to CD4+ T cell clones. Therefore a different subset of DC was tested for its ability to cross-present exogenous virus antigen to CD8+ T cell clones. Plasmacytoid dendritic cells were chosen based on a paper by Tel *et al* (2013) that showed this subset were capable of cross-presenting soluble antigen (Tel et al., 2013b). As before, responses by CD4+ T cell clones could be observed but only small responses to CD8+ T cell clones that weren't always reproducible. This can be accounted for in part through observations by Zinkernagel and colleagues (Freigang et al., 2003, Zinkernagel, 2002), showing that cross-presentation is 100,000 times less efficient than direct-presentation. Alternatively the methods used here were not optimal for cross-presentation or for stimulation of CD8+ T cells either due to the length of stimulation or the sensitivity of the readout. In the time course assay in figure 3.11 there was no observed activation of GLC-specific CD8+ T cells after 24 hours incubation with SM-loaded moDCs and it wasn't until 48-72 hours that activation could be observed. Flow cytometric analysis of surface expression markers is also more sensitive than ELISA readout of IFNγ secretion.

Therefore alternative experiments could involve examining CD8+ T cell clone activation through expression of CD137 after 48-72 hours incubation with antigen-loaded moDCs or to examine the proliferation or expansion of CD8+ T cell clones by flow cytometry.

A different assay was then chosen to examine stimulation of antiviral CD8+ T cells. This assay looked at the proliferation of virus-specific CD8+ T cells within a polyclonal culture using autologous moDCs and PDCs. Frequencies of virus-specific CD8+ T cells could be tracked using MHC class I tetramers that contained specific peptide-epitopes found within virus proteins. Alternatively a range of peptides could be used to re-stimulate virus-specific T cells within these polyclonal lines and look for intracellular production of IFNγ. These assays showed that specific antiviral CD8+ T cells could be selectively expanded from PBMCs. Further, upon re-stimulation using CMV-specific peptides, increased numbers of CD8+ T cells could be observed to produce IFNγ in the virus antigen-expanded polyclonal lines compared to a mock treated control. Other studies have examined cross-presentation through proliferation of CD8+ T cells though these studies usually focus on model antigens such as ovalbumin and well-studied OT-1 T cell responses (den Haan et al., 2000, Hirosue et al., 2014, Tel et al., 2013b). This study aims to examine wide range of CD8+ T cell responses from a complex antigenic challenge such as a whole virus cell lysate and therefore a more sensitive readout of specific T cell expansion was required.

These first studies used a lysate of CMV-infected cells due to the ease that fibroblasts could be infected efficiently and reproducibly. However an EBV antigen source was required for these studies and particularly one that primarily expressed lytic cycle antigens compared to latent antigens. The p2089-BAC expressing cell line was chosen as these cells can be maintained easily in culture and induced into lytic cycle. The 48-hour harvest time point was chosen due to studies showing that all proteins had been expressed by this time point (Yuan et

al., 2006). Additionally one focus of this thesis was to show that CD8+ T cell responses can be found against late-expressed proteins and therefore this 48 hour time point seemed appropriate despite the risk that some early-expressed proteins had been degraded (Croft et al., 2009). After 48 hours the lytic cells were harvested and a cell lysate was prepared by repetitive cycles of freeze-thawing, sonication and clarification. A proportion of cells were taken and stained for the presence of a L lytic antigen to confirm the number of cells that had transitioned through lytic cycle. This antigen source could be consistent across all further assays due to its bulk production with aliquots frozen at -80°C. Other studies have used apoptotic cell debris as their antigen source as it has been shown to be particularly immunogenic when processed by some subsets of DCs (Jing et al., 2012, Thumann et al., 2003). However in this study this option was not pursued as the efficiency of lytic induction for EBV is highly variable and therefore the differences in antigen source could affect the quantity and quality of stimulated T cell responses downstream. Other studies have used tumour cell lysates as an antigen source and their efficacy has been tested in clinical trials with positive effects (Thumann et al., 2003, Geiger et al., 2000, Nestle et al., 1998). However whilst some studies have shown that cell-associated antigen is more efficiently cross-presented than soluble antigen (Li et al., 2001), others have shown that soluble antigen can be cross-presented efficiently at least in some DC subsets (Tel et al., 2013b). One study also showed that moDCs are superior to other DC subsets in their ability to endocytose and cross-present soluble protein (Chiang et al., 2016). Despite these conflicting views, by having a stock lysate, the antigen source would at least be reproducible between experiments.

A number of attempts using a tetramer specific for an epitope within the EBV-protein (BMLF1) showed that this specific CD8+ T cell population could be expanded *in vitro* following co-culture with antigen-loaded moDCs. By culturing the antigen-loaded moDCs

concomitantly with TLR agonists a 2-fold expansion was observed compared to the addition of TLR agonists 18 hours after the addition of the SM cell lysate. This 2-hour time point was thus chosen for all further experiments. The reason for this is unclear but may be due to the viral antigen already inducing maturation of the moDC before the addition of the TLR agonists at 18 hours and therefore the additive effect of the TLR agonists was not observed. The TLR agonists used in this study were chosen for their strong Th1 polarising ability and this may be part of the reason why their addition at 2 hours post addition of lysate provided the additive effect. Additional work on how the viral antigens were cross-presented and how the timing of TLR agonists affected the presentation abilities of the DCs would be informative but was not the aim of this study.

Now that a model could be shown to cross-present viral antigens efficiently this model was used to proceed in the aim of generating a polyclonal line against all EBV lytic antigens. By using two donors with known EBV responses, polyclonal lines could be generated using the above method with 2089-lysate loaded moDCs and then these lines probed with synthetic EBV-specific epitope-peptides. These lines were shown to have expanded responses to lytic proteins taken from each lytic phase compared to *ex vivo* responses and lines generated using a mock lysate. Therefore the 2089 lysate that had been generated contained a broad range of antigens including IE, E and L antigens.

These polyclonal lines had been generated through IL-2 expansion of stimulated T cells. The outgrowth of a polyclonal line could be affected by a phenomenon called immunodomination where particular antigen-specific T cells can out-compete other specificities (Chen et al., 2000). Immunodomination could be driven by any factor or process that affects the stimulation of an antigen-specific T cell and therefore favours its survival and proliferation (Chen et al., 2000, Akram and Inman, 2012). Therefore to avoid immunodomination and

expand EBV-specific CD8+ T cells in an unbiased way expression of CD137 was used as a marker of activation for CD8+ T cells that had been stimulated by their cognate antigen presented on the moDC. This marker, CD137, has been used in previous studies for the purpose of isolating CD8+ T cells in a polyclonal culture is the early activation marker CD137 (4-1BB) (Wolfl et al., 2008, Jing et al., 2012). The precise kinetics of CD137 expression in our assay showed some contrast to previous studies where CD137+ CD8+ T cells were found after 20 hours (Jing et al., 2012). This could be due to the route of antigen delivery as that study used apoptotic HSV-1 infected HeLa cells whilst in this study a soluble cell lysate of EBV infected cells was used. The route of delivery may ultimately determine the endocytic compartment the antigen is degraded within and may affect the kinetics with how that antigen is cross-presented (Schnurr et al., 2005). In our assays, the number of GLC-specific CD8+ T cells that were also CD137+ increased over time when stimulated using moDCs loaded with the SM-lysate but no proliferation was seen until after 6 days. This activation was specific to antigen sources containing the GLC peptide as no activation was observed in the cells stimulated with the mock lysate. Therefore activated cells would be sorted by flow cytometry on day 3 before any proliferation was observed and then the sorted cells would be given a non-specific mitogenic stimulus to expand up all sorted cells.

3.7. Summary

A novel method was optimised here for the enrichment, antigen loading and maturation of moDCs. These moDCs were then used in *in vitro* assays for stimulating autologous antiviral CD8+ T cells specific for antigens from either CMV or EBV. By loading moDCs with a whole cell lysate from CMV or EBV infected cells, CD8+ T cells specific for antigens from a broad range of lytic antigens could be detected through a number of different immunological

assays. MHC class I tetramers have been used here to efficiently measure expanded T cell populations within a polyclonal line as well as to track the kinetics of specific T cell activation in conjunction with a CD137L antibody. These approaches now allow for the generation of polyclonal lines enriched for specificities against EBV lytic antigens.

CHAPTER 4

Defining patterns of CD8+ T cell immunodominance to EBV lytic proteins in IM patients and long- term healthy carriers.

EBV is a large complex virus that expresses around 70 proteins during lytic replication. This number of proteins potentially provides a large source of peptide determinants that can be processed and presented to CD8+ T cells. Previous studies of patients undergoing symptomatic primary EBV infection (IM) show large expansions of EBV specific CD8+ T cells which when assayed for specificities against a limited panel of EBV Ag, appear to be focused against epitopes derived from IE and E proteins with few responses against epitopes found within L proteins (Pudney et al., 2005). This marked focusing of responses to a relatively low number of epitopes is also seen within other complex virus infections and is termed immunodominance.

This hierarchy of immunodominance seen in primary EBV infections is also thought be similar in the memory CD8+ T cell compartment and reflects the efficiency with how antigens are processed and presented within lytically infected B cells (Steven et al., 1997, Callan et al., 1998a, Hislop et al., 2002, Pudney et al., 2005). In lytically infected B cells, early-expressed immune evasion proteins BNLF2a, BILF1, and BGLF5 act progressively through the lytic cycle to reduce the amount of peptide that can enter the ER, bind to MHC class I molecules and be presented on the cell surface. Consequently the coordinated action of these proteins has a greater effect on the processing and presentation of peptides from late-expressed proteins, thereby accounting for the reduced CD8+ T cell response to these proteins. However a subsequent study has since shown that EBV L antigens can be targets of the antiviral CD8+ T cell response but are typically subdominant to responses against IE and

E antigens (Abbott et al., 2013). Additionally a study by Orlova and colleagues showed that in a related lymphocryptovirus infection of macaques the CD8+ T cell responses against some studied L antigens was comparable to that seen against IE and E antigens. They also suggested that responses against a number of L antigens increased with increased time of infection (Orlova et al., 2011). It is unknown what might be driving this expansion of L lytic antigens but it may be the result of viral antigens being cross-presented by dendritic cells in the blood of healthy virus carriers. To resolve these discrepancies a new approach was used to examine CD8+ T cell reactivities across the entire lytic proteome in either IM patients or in recovered IM patients and healthy virus carriers.

A number of studies have used different methods to examine CTL immunodominance to viral antigens (reviewed by (Yewdell, 2006). The different methods each have their advantages and disadvantages as discussed in the introduction. The approach in this thesis was to use a cDNA library of complete gene products from all of the EBV lytic cycle proteins to probe for responses in polyclonal CD8+ T cell lines (see section 4.2.). This method allows the identification of CTL responses to specific gene products across different HLA backgrounds. However the individual immunogenic peptide epitopes are not identified.

4.1. Generation of protein expression constructs.

EBV expresses around 70 different proteins during the lytic cycle. A large number of these proteins have previously been cloned into protein expression constructs by our lab and others (see reference in table 4.1). The remaining proteins were amplified by PCR from the p2089-bacmid and cloned into expression vectors using the pcDNA3.1/V5-HIS TOPO ® TA expression kit. Difficulties in amplifying certain genes due to size (BNRF1 and BPLF1) or

internal repeats (BHLF1 and LF3) meant different strategies had to be used. BNRF1 was amplified by PCR in 2 fragments with an overlapping central region. A pMax-Cloning expression construct containing BPLF1 was a kind gift from Dr Maaike Ressing. BHLF1 and LF3 contain several long internal repeats that caused difficulties when amplifying by PCR. These two genes were subsequently synthesized by GeneArt (Thermo Fisher Scientific) with all but one of the internal repeats removed and still leaving the sequence containing all determinants for antigen recognition by T cells. BHLF1 and LF3 were amplified by PCR from the GeneArt synthesized product and cloned into the pcDNA3.1/V5-HIS expression construct. Other expression constructs contained spliced gene products (BBLF2/3, BDRF1/BGRF1 and BSLF2/BMLF1). BBLF2/3 and BDRF1/BGRF1 constructs weren't separated but for BSLF2/BMLF1 (pCEP4-SM) there was a separate expression construct for BMLF1 and therefore any response seen to pCEP4-SM without a response seen to BMLF1 could suggest a response to BSLF2.

Healthy adult lab donors with documented persistent EBV infection and known HLA types were recruited for this study. EBV-transformed LCLs previously generated in-house from these donors were used as a source of RNA for the generation of a panel of cDNAs encoding HLA A, B and C alleles. These HLA alleles were individually cloned into the above pcDNA3.1/V5-His expression vector (table 4.2).

All newly cloned expression constructs underwent colony PCR with the 3' gene primer and the 5' T7 primer within the plasmid. Only gene fragments in the correct orientation would give a positive band when gel electrophoresed. Correct sized PCR products would then undergo digestion with appropriate restriction enzymes to yield a ladder of correctly sized fragments. Once these steps were taken, then newly cloned expression constructs were sequenced within the 'Life and Environmental Sciences' (LES) department. Reported

sequence reads were analysed using Seq Scanner 2 software (Applied Biosytems) and aligned to the human herpesvirus 4 complete wildtype sequence AJ507799 or to HLA sequence reads on the ImMunoGeneTics (IMGT) website.

Table 4.1. A list of EBV lytic genes, expression vectors, temporal expression and putative function.

Gene	Temproal Expression	Putative function	Expression vector
BRLF1	Immediate Early	R transactivator (Rta)	pcDNA3.1-IRES-GFP
BZLF1	Immediate Early	bZIP protein	pcDNA3.1-IRES-GFP
BALF1	Early	Bcl2 homologue	pcDNA3.1-IRES-GFP
BALF2	Early	ssDNA binding protein	pcDNA3.1-IRES-GFP
BALF5	Early	DNA polymerase	pcDNA3.1-V5/HIS
BARF1	Early	soluble CSF-1 receptor	pcDNA3.1-IRES-GFP
BaRF1	Early	RR (small subunit)	pcDNA3.1-IRES-GFP
BBLF2	Early	helicase-primase complex	pcDNA3.1-V5/HIS
BBLF3	Early	helicase-primase complex	pcDNA3.1-V5/HIS
BBLF4	Early	helicase	pcDNA3.1-V5/HIS
BcRF1	Early	TBP-like late protein replication	pcDNA3.1-V5/HIS
BDLF4	Early	unknown	pcDNA3.1-V5/HIS
BFLF1	Early	envelope glycoprotein	pcDNA3.1-V5/HIS
BFLF2	Early	possible role in capsid envelopment	pcDNA3.1-IRES-GFP
BFRF1	Early	Tegument protein	pcDNA3.1-V5/HIS
BFRF2	Early	unknown	pcDNA3.1-V5/HIS
BFRF3	Early	capsid protein	pcDNA3.1-V5/HIS
BGLF3	Early	unknown	pcDNA3.1-IRES-GFP
BGLF4	Early	Phosphotransferase	pcDNA3.1-IRES-GFP
BGLF5	Early	DNase	pcDNA3.1-IRES-GFP
BHLF1	Early	repeat region	pcDNA3.1-V5/HIS
BHRF1	Early	Bcl2 homologue	pSG5
BKRF3	Early	Uracil DNA glycosylase	pcDNA3.1-IRES-GFP
BLLF3	Early	dUTPase	pcDNA3.1-IRES-GFP
BMLF1	Early	IE post-transcriptional regulation	pcDNA3.1-IRES-GFP
BMRF1	Early	Processivity factor	pcDNA3.1-IRES-GFP
BNLF2a	Early	Viral immune evasion-TAP	pcDNA3.1-IRES-GFP
BNLF2b	Early	unknown	pcDNA4-IRES-GFP
BORF2	Early	RR (large subunit)	pcDNA3.1-V5/HIS
BRRF1	Early	possible activator of Zp IE promoter	pcDNA3.1-IRES-GFP
BSLF1	Early	Primase	pcDNA3.1-V5/HIS
BSLF2	Early	part of the SM complex with BMLF1	pCEP4-SM
BXLF1	Early	Thymidine kinase	pcDNA3.1-IRES-GFP
BALF3	Late	Transport protein	pcDNA3.1-V5/HIS
BALF4	Late	gB/gp110 virus entry	pcDNA3.1-IRES-GFP
BBLF1	Late	Tegument protein	pcDNA3.1-IRES-GFP
BBRF1	Late	minor Capsid protein	pcDNA3.1-IRES-GFP

BBRF2	Late	unknown	pcDNA3.1-IRES-GFP
BBRF3	Late	gM	pcDNA3.1-IRES-GFP
BcLF1	Late	Major capsid protein	pcDNA3.1-V5/HIS
BCRF1	Late	IL-10 homology	pcDNA3.1-IRES-GFP
BDLF1	Late	Capsid protein	pcDNA3.1-IRES-GFP
BDLF2	Late	unknown	pcDNA3.1-IRES-GFP
BDLF3	Late	gp150	pcDNA3.1-IRES-GFP
BDRF1	Late	DNA packaging protein	pcDNA3.1-V5/HIS
BdRF1	Late	Scaffold	pcDNA3.1-IRES-GFP
BGLF1	Late	unknown	pcDNA3.1-IRES-GFP
BGLF2	Late	unknown	pcDNA3.1-IRES-GFP
BGRF1	Late	DNA packaging protein	pcDNA3.1-V5/HIS
BILF1	Late	Probable GCR	pcDNA3.1-IRES-GFP
BILF2	Late	gp78	pcDNA3.1-IRES-GFP
BKRF2	Late	gL	pcDNA3.1-IRES-GFP
BKRF4	Late	unknown	pcDNA3.1-IRES-GFP
BLLF1a	Late	gp350 adhesion	pcDNA3.1-V5/HIS
BLLF2	Late	unknown	pcDNA3.1-IRES-GFP
BLRF1	Late	gN	pcDNA3.1-IRES-GFP
BLRF2	Late	part of the VCA complex	pcDNA3.1-IRES-GFP
BMRF2	Late	unknown, membrane associated	pcDNA3.1-IRES-GFP
BNRF1	Late	Tegument protein	pcDNA3.1-V5/HIS
BOLF1	Late	Tegument protein	pcDNA3.1-V5/HIS
BORF1	Late	Capsid assembly	pcDNA3.1-V5/HIS
BPLF1	Late	Large tegument protein	pMaxCloning-flag
BRRF2	Late	unknown	pcDNA3.1-IRES-GFP
BSRF1	Late	unknown	pcDNA3.1-IRES-GFP
BTRF1	Late	unknown	pcDNA3.1-IRES-GFP
BVRF1	Late	Tegument protein	pcDNA3.1-IRES-GFP
BVRF2	Late	Assembly protein	pcDNA3.1-IRES-GFP
BXLF2	Late	gH/gp85 (fusion)	pcDNA3.1-IRES-GFP
BXRF1	Late	fusion protein	pcDNA3.1-IRES-GFP
BZLF2	Late	gp42 signal peptide	pcDNA3.1-IRES-GFP
LF3	Early	unknown	pcDNA3.1-V5/HIS
LF2	Unknown	Binds Rta regulates viral replication	pcDNA3.1-IRES-GFP
LF1	Unknown	unknown	pcDNA3.1-IRES-GFP

Table 4.1. Highlighted panels indicate expression constructs that were newly generated in this study. The majority of other expression constructs were generated by Dr Jianmin Zuo and Dr Andrew Hislop. Expression constructs for BHLF1 and LF3 were generated using GeneArt synthesised truncated gene products. For BNRF1, two expression constructs were generated with each containing half of the complete ORF and with an overlapping region. The expression construct for BPLF1 was a kind gift by Dr Maike Ressing.

Table 4.2. Donor HLA allotypes

	HLA allele					
Donor	A		B		C	
IM84	02.01	-	15.01	44.02	03.04	05.01
IM217	01.01	03.01	07.02	08.01	07.01	07.02
IM223	02.01	24.02	08.01	40.01	03.04	07.01
IM239	01.01	02.01	08.01	51.01	07.01	-
IM243	01.01	23.01	08.01	44.03	04.01	07.01
IM249	01.01	02.01	08.01	55.01	03.03	07.01
IM269	03.01	11.01	15.01	35.01	03.03	04.01
HD1	03.01	11.01	15.01	35.01	03.03	04.01
HD2	01.01	03.01	07.02	35.01	04.01	07.02
HD3	02.01	24.02	39.01	-	06.02	07.02
HD4	01.01	02.01	39.01	40.01	03.04	12.02
HD5	02.01	11.01	35.01	44.02	04.01	05.01
HD6	01.01	02.01	44.02	57.03	05.01	07.01
MJS	01.01	-	08.01	-	07.01	-

Table 4.2. A list of all IM patients and healthy virus carriers used in this study with classical HLA class I restrictions. Each HLA class I allele was cloned into a pcDNA3.1-V5/His expression vector. The HLA class I restriction of the MJS cell line is also included as this cell line was used as a target.

4.2. Methodology for expansion of EBV-specific polyclonal CD8+ T cell lines from IM patients or from healthy virus carriers.

To screen for CD8+ T cell responses against the large number of EBV lytic proteins and across disparate donor HLA class I alleles in an IFNγ-capture ELISA, a large number of donor T cells are required. The large number of cells input into this assay also enables that low frequency responses ($1/10^5$) may be detected. The frequency of EBV-specific CD8+ T cells in the blood of healthy virus carriers is relatively low with previously identified responses to specific epitopes ranging in frequency from 0.1-2% of total CD8+ T cells. Many potential responses may actually occur at frequencies lower than this and therefore an enrichment step is first required to pull out EBV-specific responses (Fig. 4.1. Bottom). To do this, moDCs from healthy virus carriers were cultured with an EBV-infected lytic cell lysate and used to cross-present viral antigens to autologous CD8+ T cells (addressed in chapter 3). EBV-reactive CD8+ T cells were FACS sorted on the basis of the activation marker CD137 and subsequently given a general mitogenic stimulus (OKT3 and PHA-treated, γ-irradiated allogeneic feeder PBMCs). Upon cell sorting, typically between 5-10^4 cells were collected from a bulk population of around 30^6 PBMCs and expanded more than 1000 fold to give a polyclonal population that could be used in the screen described above.

Alternatively this screening procedure could also be applied to examine CD8+ T cell responses from IM patients (Fig. 4.1. Top). During IM, EBV-specific CD8+ T cell frequencies have already been enriched within peripheral blood and therefore don't require any further enrichment such as the one described above for healthy virus carriers. IM patient cryopreserved PBMCs were thawed and then given a general mitogenic stimulus and expanded in culture prior to screening.

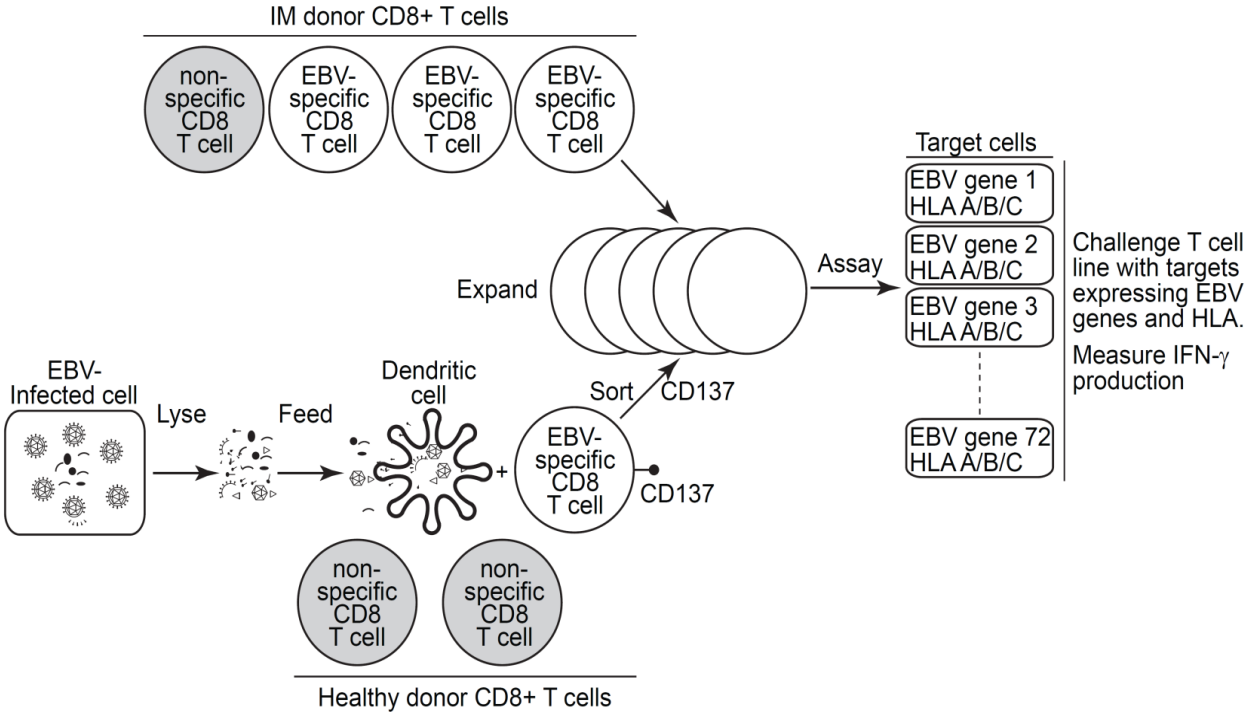


Figure 4.1. Methodology for expansion of EBV-specific polyclonal CD8+ T cell lines from IM patients or from healthy virus carriers.
EBV-specific CD8+ T cells from healthy virus carriers were enriched from PBMCs using autologous moDCs to cross-present an EBV-lytic antigen source (bottom). Reactive cells were sorted on the basis of the activation marker CD137 and expanded *in vitro* using OKT3 and PHA-treated, γ-irradiated allogeneic feeder PBMCs. Similarly PBMCs from IM patients containing a high frequency of EBV-specific CD8+ T cells were expanded as such (top). Polyclonal T cell lines cultured from either IM patients or healthy virus carriers were challenged with MJS or COS-7 cells expressing individual EBV genes across the entire EBV-lytic proteome and donor-specific HLA class I alleles. The release of IFNγ was measured in an ELISA as a readout of responding CD8+ T cells.

4.3. EBV-Lytic proteome screen of infectious mononucleosis patient PBMCs.

Models of immunodominance against human herpesvirus infections have typically examined memory T cell responses (Sylwester et al., 2005, Jing et al., 2012, Jing et al., 2016, Abbott et al., 2013). However few studies have examined T cell responses to primary infection and those that have only examined a small number of antigens (Pudney et al., 2005, Khan et al., 2007). In the study by Pudney *et al* primary CD8+ T cell responses to EBV were screened for reactivity against the 2 IE, 11 E and 10 L proteins with a number of CD8+ T cell clones also generated and screened for reactivity against lytically infected cells. This study showed that the CD8+ T cell responses to EBV appears to reflect the efficiency of antigen processing and presentation in lytically infected cells with IE and E proteins being the dominant targets. To determine whether the immunodominance hierarchy seen in the study by Pudney *et al* was accurate across the whole lytic proteome, polyclonal CD8+ T cell lines were expanded *in vitro* from IM patient PBMCs collected at the time of primary infection (see section 4.2.). These polyclonal lines were then screened against MJS targets expressing individual EBV genes along with donor HLA class I alleles.

A two week culture could result in more than a 100 fold expansion of IM donor PBMCs with expanded PBMCs depleted of CD4+ T cells and frozen in aliquots. Cells were then thawed and rested briefly before co-culture with MJS cells expressing individual EBV-lytic proteins and relevant donor HLA A, B or C alleles. An empty expression vector (IRES-GFP) was used as a negative control with the mean response plus three standard deviations used as the threshold for what was deemed a positive response shown by the horizontal black line on each graph. Some responses in the screens showed large error bars and therefore a response was only deemed positive if both duplicate wells from the assay gave values above the threshold line. Positive responses are denoted by an asterisk below the gene name on the x axis.

The MJS cell line endogenously expresses HLA A, B and C alleles (shown in table 4.2.). These HLA alleles could potentially present peptides from the ectopically expressed EBV genes however no response was typically observed unless there was co-expression of donor HLA molecules from transfected plasmids. When cell lines gave non-specific responses, particularly in cases where the donor and MJS cell line shared HLA types, a non-human cell line, COS-7, was used to separate out the restricting response by individually expressing individual HLA plasmids in the COS-7 cell line.

4.3.1. Patient IM84 (A*02.01, B*15.01, B*44.02, C*03.04, C*05.01)

A polyclonal CD8+ T cell line expanded from the PBMCs of an IM patient designated IM84 was challenged against target MJS cells expressing individual EBV genes and donor HLA A, B or C alleles with the responses shown in figure 4.2. In this polyclonal line form IM84 there were responses to 19 individual EBV proteins corresponding to both IE proteins, 11 E and 6 L proteins. The responses in this line that were the greatest magnitude were found against the E proteins BaRF1 and BBLF2/3. Other proteins eliciting clear responses were the two IE proteins, the E proteins BMLF1, BMRF1, BORF2, BRRF1 and the L proteins BcLF1 and BORF1. The response seen against BSLF2/BMLF1 is most likely attributed to BMLF1 as this response was also observed. Out of the 6 responses detected against L proteins only 3 were clear responses, whilst the remaining 3 were of low magnitude, barely above the threshold line. Of the 11 responses to E proteins, 10 were clear responses whilst the response to BGLF5 was barely above the threshold.

Donor-specific HLA A, B or C alleles were co-transfected into target MJS cells to broadly separate the HLA restrictions to the observed reactive T cells. The responses were found to be

restricted across HLA A, B and C but to identify the individual restricting allele, EBV genes and individual HLA plasmids were co-transfected into COS-7 cells and then used to challenge the polyclonal line. In this case the donor was HLA-A*02.01 homozygous and so the responses observed were attributed to this allele. After separating out the two HLA-B alleles and two HLA-C alleles (fig. 4.2. bottom), responses to HLA-B alleles appeared to be solely restricted through HLA-B*15.01 whereas responses to HLA-C alleles were restricted through either HLA-C*03.04 (BMRF1) or HLA-C*05.01 (BBLF2/3). Responses to BHRF1, BBLF4 and BDRF1/BGRF1 when retested against COS-7 cells showed no response.

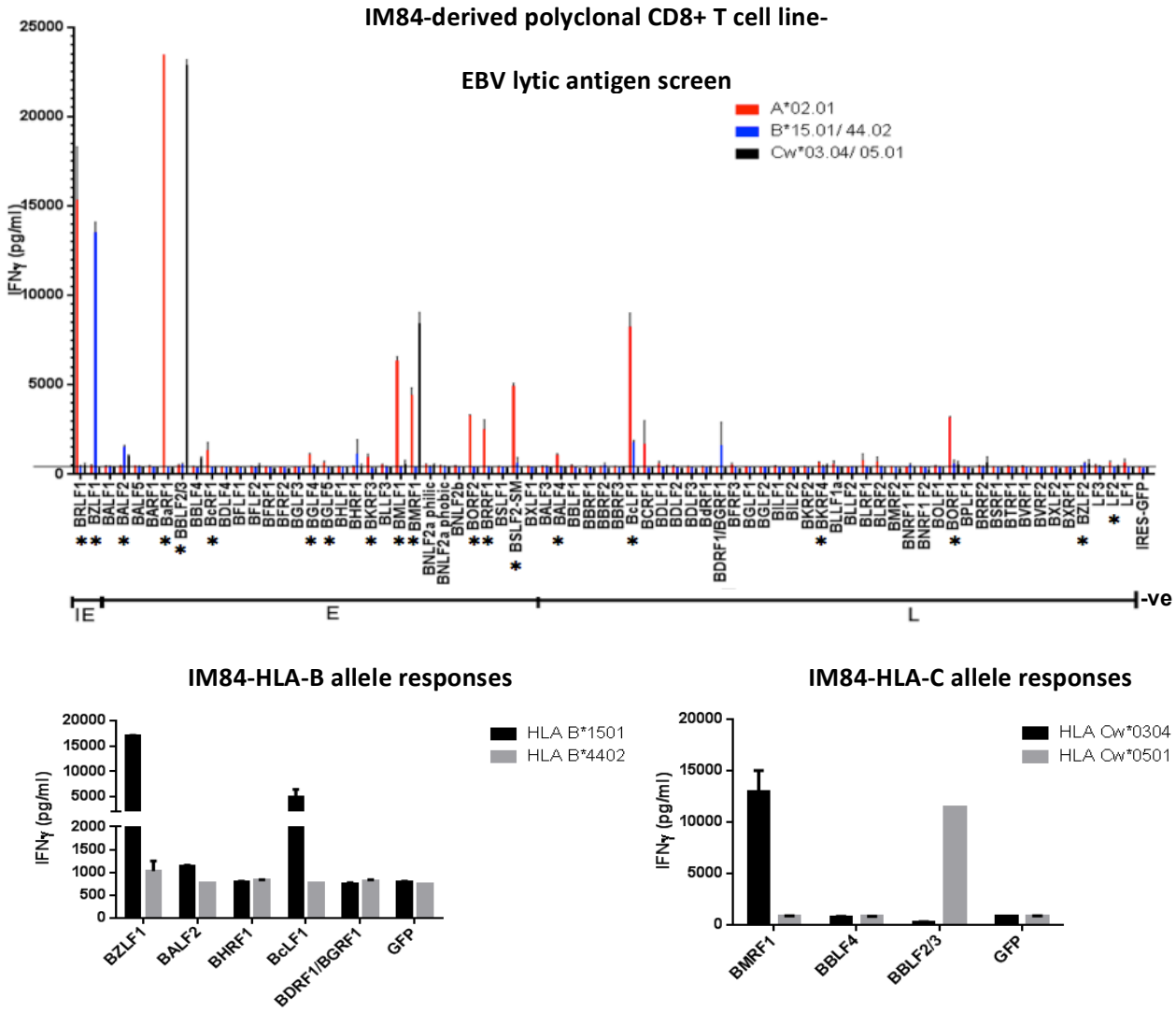


Figure 4.2. Lytic antigen choice within IM84-derived polyclonal CD8+ T cell line
(Top) IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative control (IRES-GFP (empty vector)). (Bottom) Polyclonal line was retested against the non-human cell line, COS-7, for selected EBV lytic antigens and for individual HLA allotypes (B*15.01, B*44.02, C*03.04, C*05.01). HLA-A allotypes were not included as this donor is homozygous for HLA-A*02.01. An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.3.2. Patient IM217 (A*01.01, A*03.01, B*07.02, B*08.01, C*07.01, C*07.02)

A polyclonal CD8+ T cell line from expanded IM217 PBMCs was challenged as previously with the responses shown in figure 4.3. Responses in this polyclonal line were identified to be against 14 individual EBV proteins corresponding to 1 IE protein, 9 E and 4 L proteins. The response in this line of the greatest magnitude was against the E protein BaRF1. Other proteins eliciting clear responses were the IE protein BZLF1, the E proteins BALF2, BBLF2/3, BGLF4 and BORF2, and the L proteins BBRF1, BcLF1 and BVRF2. Out of the 4 responses detected against L proteins only 3 were clear responses, whilst the response to BPLF1 was barely above the threshold line. Of the 9 responses to E proteins, 5 were clear responses whilst the responses to BALF1, BFRF1, BLLF3 and BMRF1 were barely above the threshold.

After separating out the HLA alleles expression in COS-7 cells, a response to BVRF2 was restricted through A*03.01 whilst the response to BBRF1 was restricted through A*01.01. The response to BZLF1 appeared to be restricted through A*03.01 though the response size was low. After separating out the two HLA-B alleles the responses are predominantly restricted through B*07.02 and include the responses to the E proteins BALF2, BGLF4, BBLF2/3, BLLF3, BaRF1, BFRF1 and BORF2 and to the L proteins BcLF1 and BPLF1. The response to BALF2 along with being restricted through B*07.02 also shows a response restricted through B*08.01. The response to BZLF1 is restricted through B*08.01. A response to BALF1 when retested elicited no response. The responses to BBRF1 seen in the first screen using MJS targets show restrictions through HLA-A, B and C alleles. However upon retesting against COS-7 targets, the only response seen is restricted through HLA-A*01.01. No responses were observed to be restricted through HLA-C alleles.

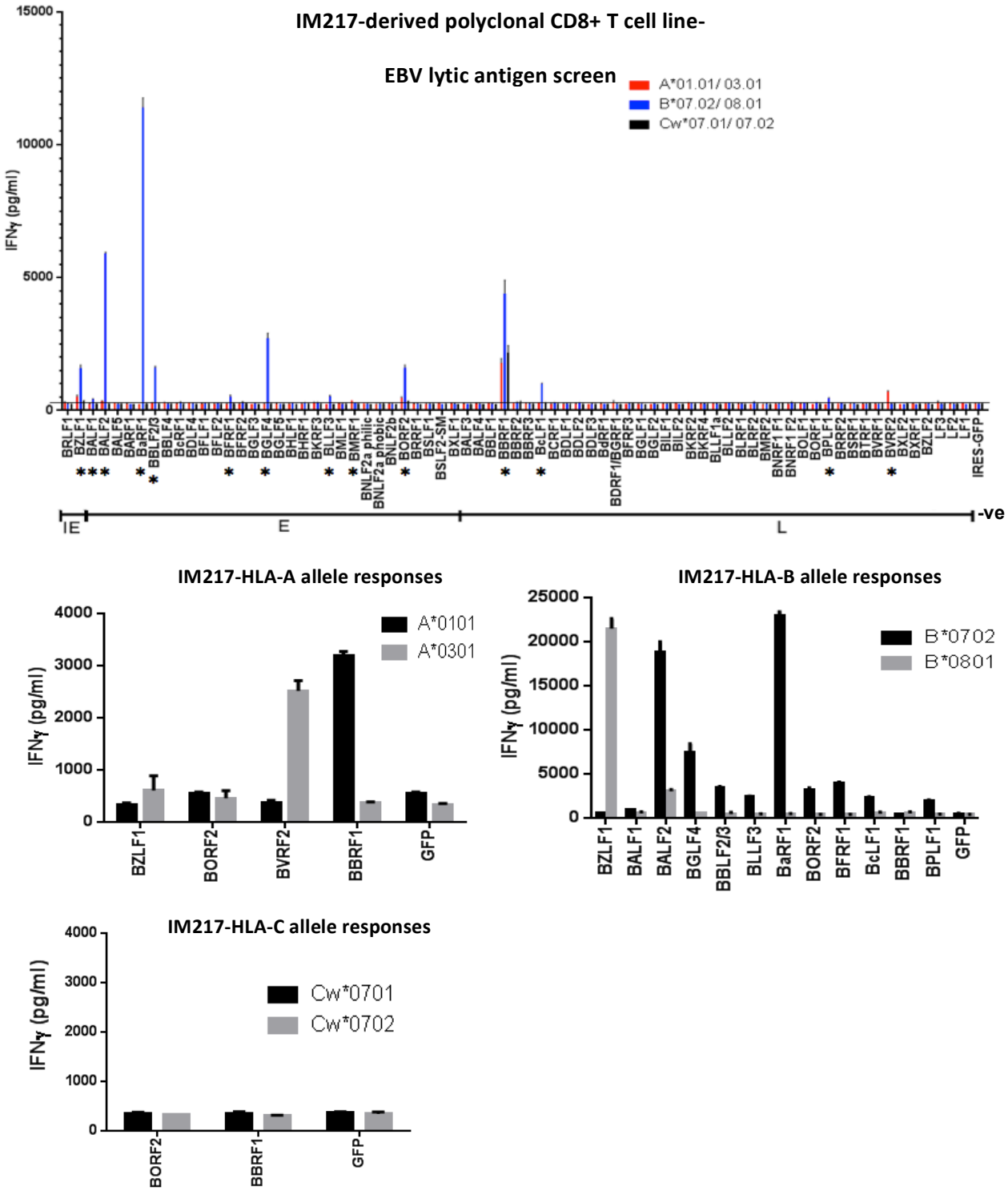


Figure. 4.3. Lytic antigen choice within IM217-derived polyclonal CD8+ T cell line
(Top) IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative control (IRES-GFP (empty vector)). (Bottom) Polyclonal line was retested against the non-human cell line, COS-7, for selected EBV lytic antigens and for individual HLA allotypes (HLA-A*01.01, A*03.01, B*07.02, B*08.01, C*07.01, C*07.02). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.3.3. Patient IM223 (A*02.01, A*24.02, B*08.01, B*40.01, C*03.04, C*07.01)

A polyclonal CD8+ T cell line from expanded IM223 PBMCs was challenged as previously with the responses shown in figure 4.4. Responses in this polyclonal line were identified to be against 24 individual EBV proteins corresponding to 2 IE proteins, 12 E and 10 L proteins. The response in this line of the greatest magnitude was against the E protein BBLF2/3. Other proteins eliciting clear responses were the IE protein BRLF1, the E proteins BALF1, BALF2, BaRF1, BGLF5, BMRF1, BORF2 and BRRF1, and the L proteins BBRF1, BcLF1 and BVRF2. Out of the 10 responses detected against L proteins only 3 were clear responses, whilst the response to BORF1, BPLF1, BSRF1 and BXRF1 were barely above the threshold line. Of the 12 responses to E proteins, 8 were clear responses whilst the responses to BFRF1, BKRF3, SM and BXLF1 were barely above the threshold. The response to BZLF1 was also barely above the threshold line.

After separating out the HLA alleles expression in COS-7 cells, responses to antigens restricted through A*02.01 included the IE protein BRLF1, the E proteins BBLF2/3, BKRF3, BRRF1, BMRF1, BaRF1, and BORF2, and the L proteins BALF4, BcLF1, BBRF3 and fragment 2 of BNRF1. Responses to antigens restricted through A*24.02 included the IE protein BZLF1, the E proteins BALF1, BFRF1 and BORF2 and the L protein BcLF1. After separating out the two HLA-B alleles responses to antigens restricted through B*08.01 included the IE protein BZLF1 and the E protein BMRF1. Responses to antigens restricted through B*40.01 included the E proteins BXLF1, BBLF2/3, BRRF1 and BORF2, and the L proteins BcLF1, BKRF2, BNRF1 and BVRF1. All detected responses to antigens restricted through HLA-C alleles were through C*03.04 and included the E proteins BALF2 and BMRF1 and to the L protein BBRF3. Responses to proteins detected in the initial screen

using MJS targets but not detected in the second screen using COS-7 targets included BSRF1, BPLF1, BXRF1 and BORF1.

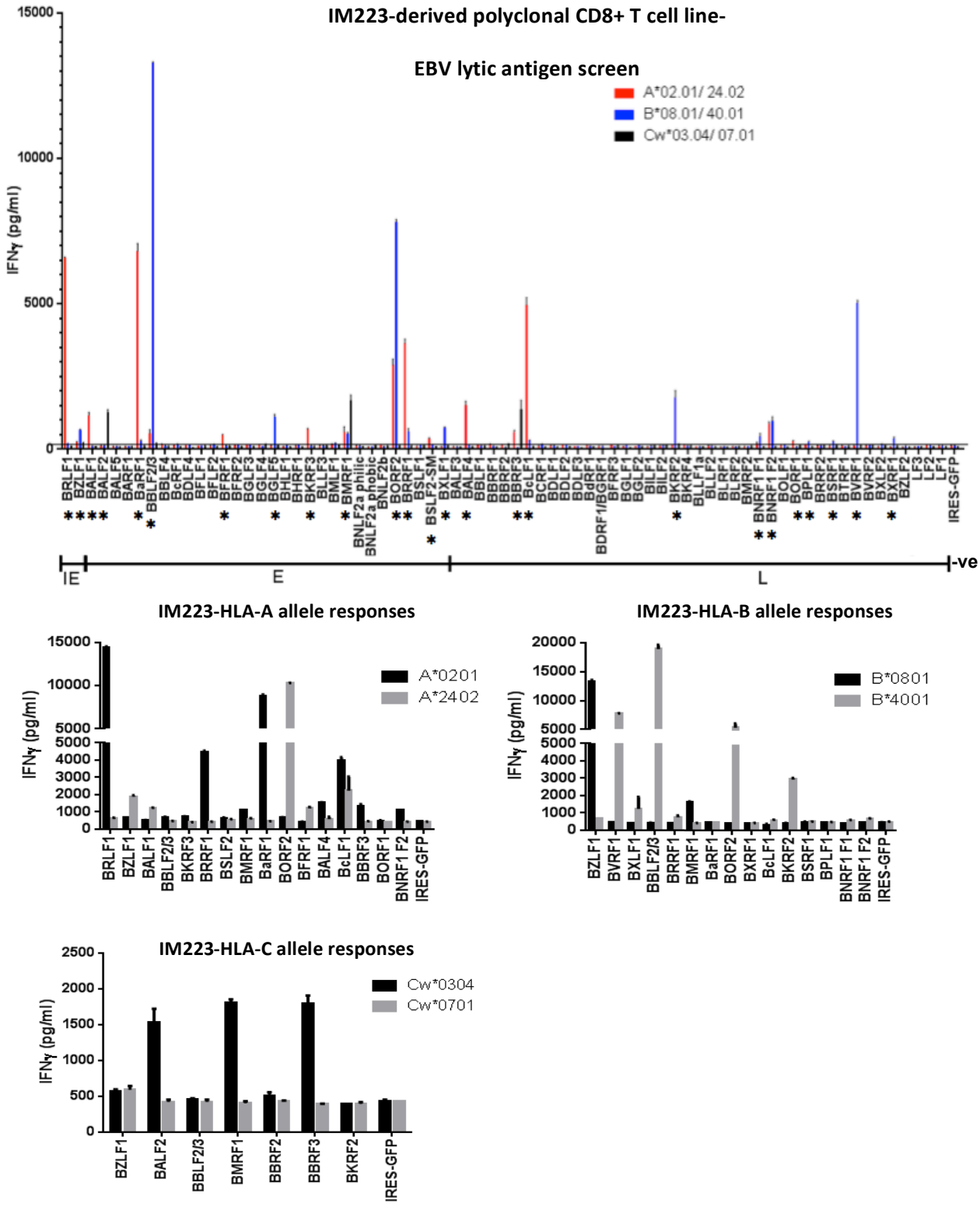


Figure 4.4. Lytic antigen choice within IM223-derived polyclonal CD8+ T cell line
(Top) IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative control (IRES-GFP (empty vector)). (Bottom) Polyclonal line was retested against the non-human cell line, COS-7, for selected EBV lytic antigens and for individual HLA allotypes (HLA-A*02.01, A*24.02, B*08.01, B*40.01, C*03.04, C*07.01). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.3.4. IM239 (A*01.01, A*02.01, B*08.01, B*51.01, C*07.01)

A polyclonal CD8+ T cell line expanded from IM239s PBMCs was challenged was challenged as previously with the responses shown in figure 4.5. Responses in this polyclonal were identified to be against 11 individual EBV proteins corresponding to both IE proteins, 6 E and 3 L proteins. The response in this line of the greatest magnitude was against the L protein BcLF1. Other proteins eliciting clear responses were the IE proteins BRLF1 and BZLF1, the E proteins BaRF1, BMLF1 and BNLF2a, and the L proteins BcLF1 and BNRF1 (F2). Other observed responses were against the E proteins BALF1, BALF2, BXLF1 and the L protein BDLF1. However these responses were barely above the threshold line. The observed response to SM is most likely from the protein BMLF1 and not from BSLF2.

From this screen 8 responses appeared to be restricted through HLA-A alleles, 5 responses through HLA-B alleles and no responses were detected to HLA-C alleles.

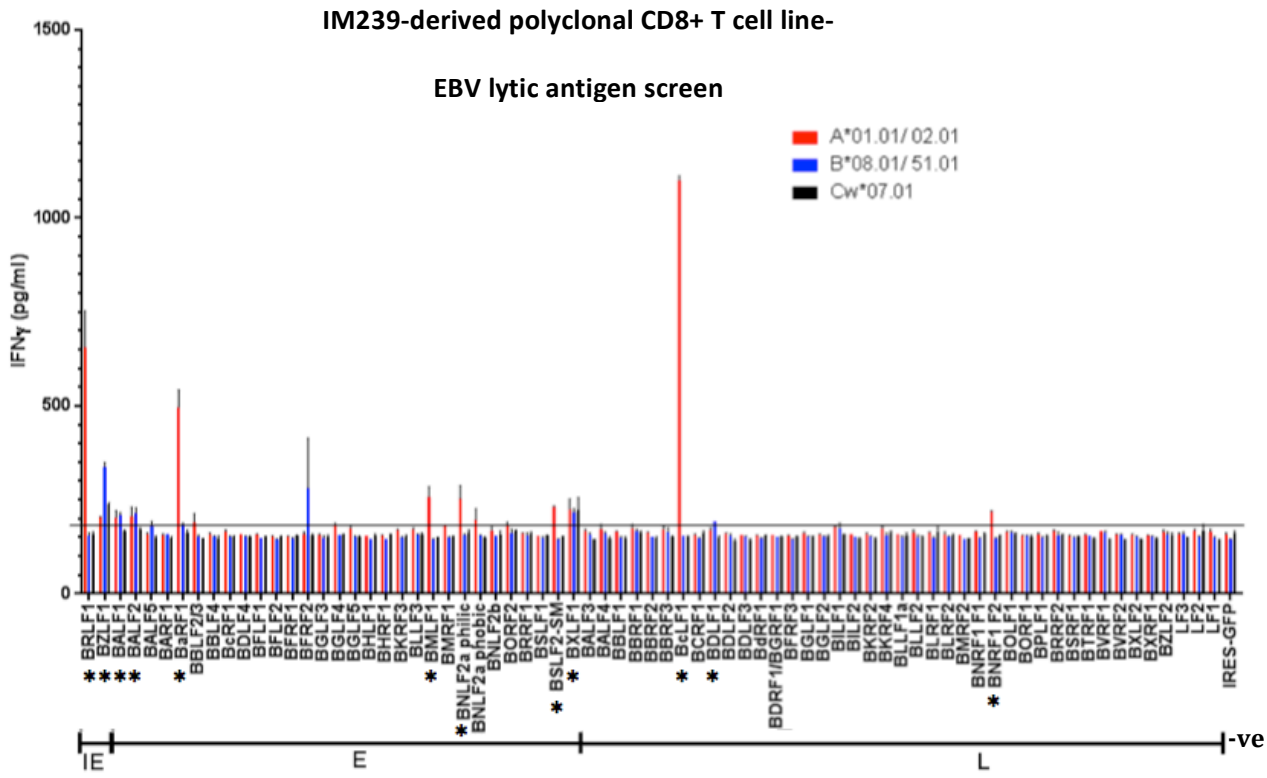


Figure 4.5. Lytic antigen choice within IM239-derived polyclonal CD8+ T cell line
IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative control (IRES-GFP (empty vector)). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.3.5. IM243 (A*01.01, A*23.01, B*08.01, B*44.03, C*04.01, C*07.01)

A polyclonal CD8+ T cell line was expanded from IM243s PBMCs was challenged as previously with the responses shown in figure 4.6. Responses in this polyclonal line were identified to be against 8 individual EBV proteins corresponding to the IE protein BZLF1, 5 E and 2 L proteins. The response in this line of the greatest magnitude was against the E protein BFRF1. Other proteins eliciting clear responses were the IE protein BZLF1, the E proteins BBLF4, BMRF1 and BORF2. The other detected responses to the E protein BALF2 and the L proteins BBRF3 and BORF1 were barely above the threshold line.

After separating out the HLA alleles expression in COS-7 cells, responses to antigens restricted restricted through A*01.01 included the IE protein BZLF1 and the E protein BORF2. Responses to antigens restricted through A*23.01 included the E proteins BBLF4, BFRF1 and BORF2 and the L protein BBRF3. After separating out the two HLA-B alleles responses to antigens restricted through B*08.01 included the IE protein BZLF1 and the E proteins BMRF1 and BORF2. Responses to antigens restricted through B*44.03 included the IE protein BZLF1, and the E proteins BMRF1 and BORF2. After separating out the two HLA-C alleles responses to antigens restricted through C*04.01 included BALF2, BMRF1 and BORF2 and there was one response restricted through C*07.01 which was to the E protein BORF2. The E protein BORF2 was detected across each of the 6 HLA A, B or C alleles whilst BMRF1 was detected across 3 alleles, both HLA-B alleles and HLA-C*04.01.

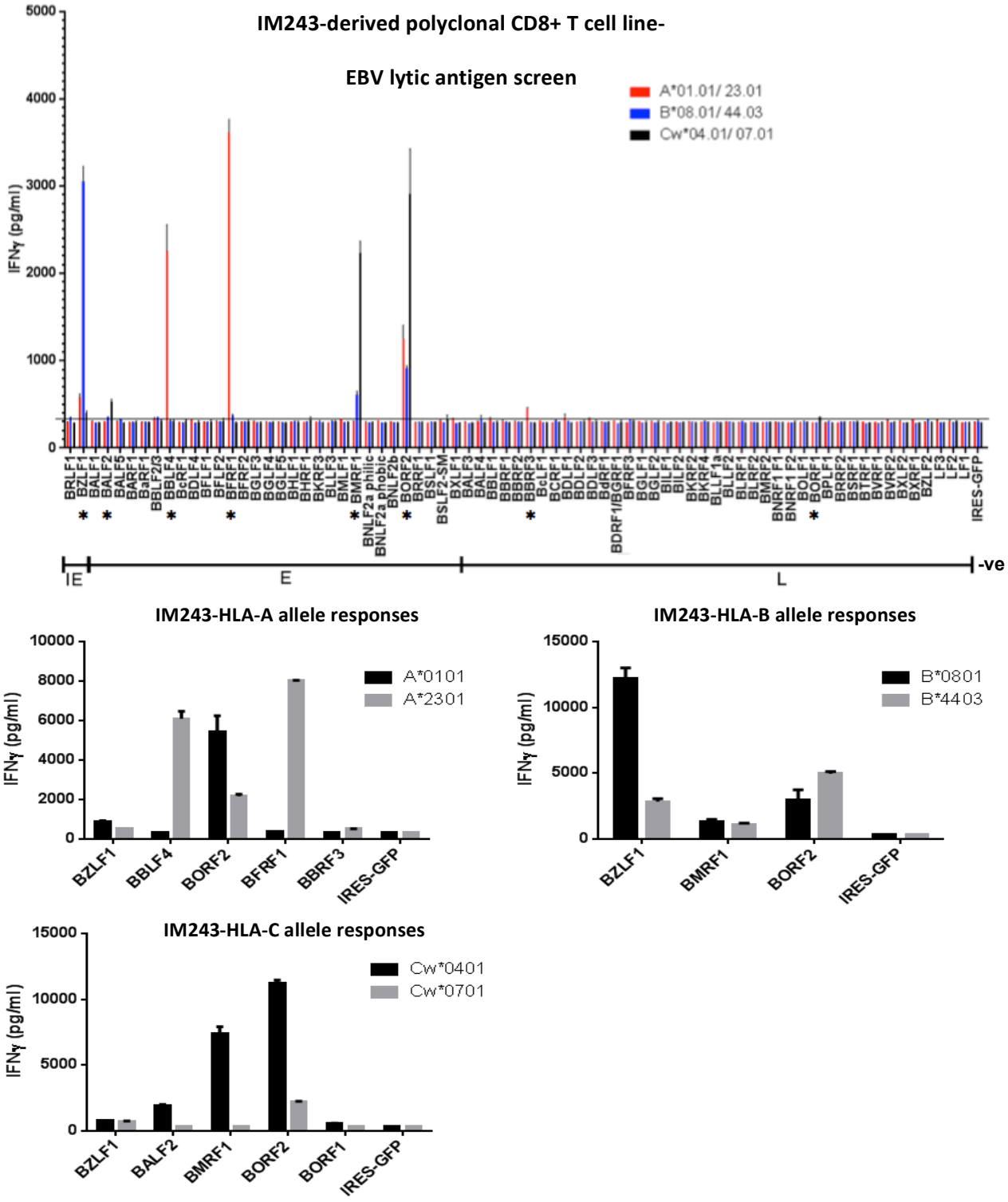


Figure 4.6. Lytic antigen choice within IM243-derived polyclonal CD8+ T cell line
(Top) IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative control (IRES-GFP (empty vector)). (Bottom) Polyclonal line was retested against the non-human cell line, COS-7, for selected EBV lytic antigens and for individual HLA allotypes (A*01.01, A*23.01, B*08.01, B*44.03, C*04.01, C*07.01). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.3.6. IM249 (A*01.01, A*02.01, B*08.01, B*55.01, C*03.03, C*07.01)

A polyclonal CD8+ T cell line was expanded from IM249s PBMCs was challenged as previously with the responses shown in figure 4.7. In the polyclonal line expanded from IM249 and screened against MJS targets, there were responses to 17 individual EBV proteins corresponding to both IE proteins, 9 E and 6 L proteins. The response in this line of the greatest magnitude was against the E protein BaRF1. Other proteins eliciting clear responses were both IE proteins, the E proteins BKRF3, BMLF1 and BORF2, and the L protein BcLF1. The other detected responses to the E proteins BALF1, BALF2, BFRF2, BGLF4, BMLF1, BMRF1 and SM, and the L proteins BALF4, BBRF3, BDLF3, BNRF1 and BVRF2 were barely above the threshold line.

From this screen 12 responses appeared to be restricted through HLA-A alleles, 4 responses through HLA-B alleles and 9 responses restricted through HLA-C alleles.

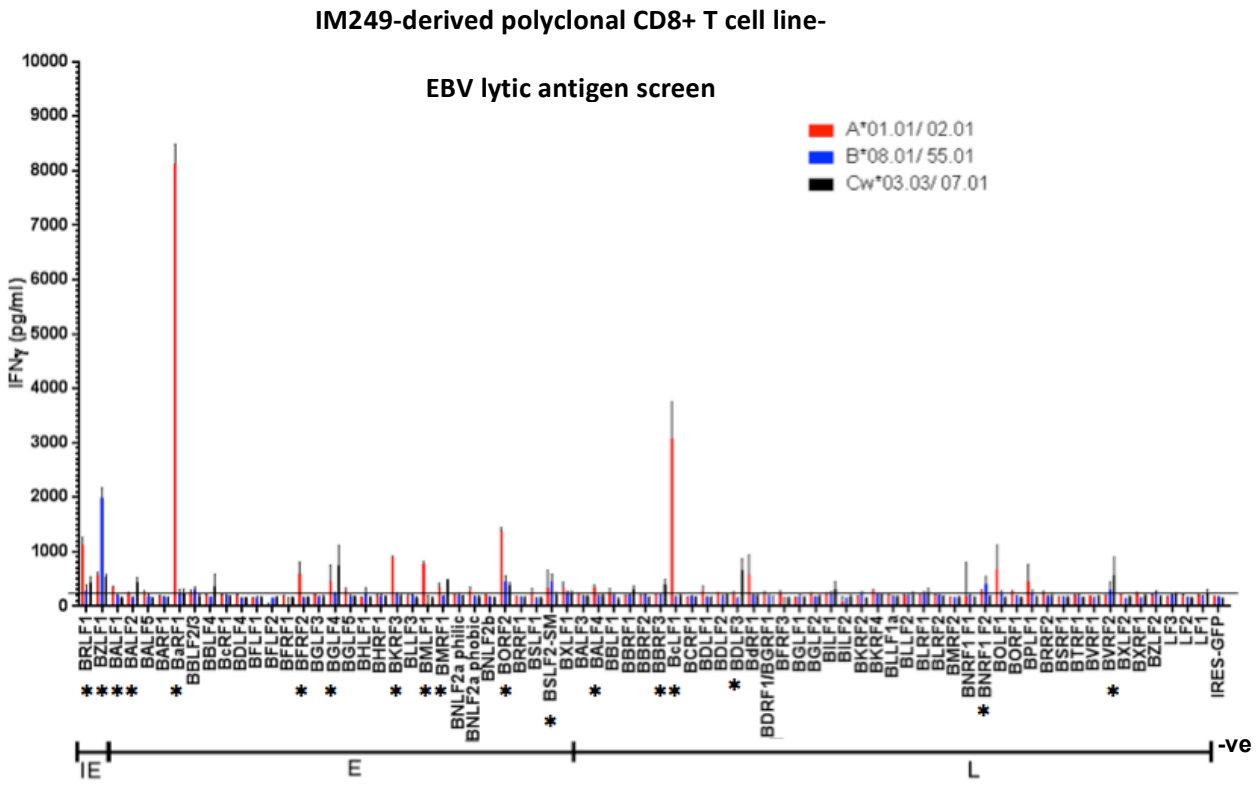


Figure 4.7. Lytic antigen choice within IM249-derived polyclonal CD8+ T cell line
IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative control (IRES-GFP (empty vector)). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.3.7. IM269-AIM (A*03.01, A*11.01, B*15.01, B*35.01, C*03.03, C*04.01)

A polyclonal CD8+ T cell line was expanded from IM269s PBMCs and challenged as previously with the responses shown in figure 4.8. In the polyclonal line expanded from IM269 and screened against MJS targets, there were responses to 12 individual EBV proteins corresponding to both IE proteins, 7 E and 3 L proteins. The response in this line of the greatest magnitude was against the E protein BGLF5. Other proteins eliciting clear responses were the IE protein BZLF1, the E proteins BKRF3 and BMRF1, and the L protein BVRF2. The other detected responses to the IE protein BRLF1, the E proteins BALF2, BBLF2/3, BBLF4 and BORF2, and the L proteins BBRF3 and BcLF1 were all low magnitude responses.

From this screen 3 responses appeared to be restricted through HLA-A alleles, 9 responses through HLA-B alleles and 3 responses restricted through HLA-C alleles.

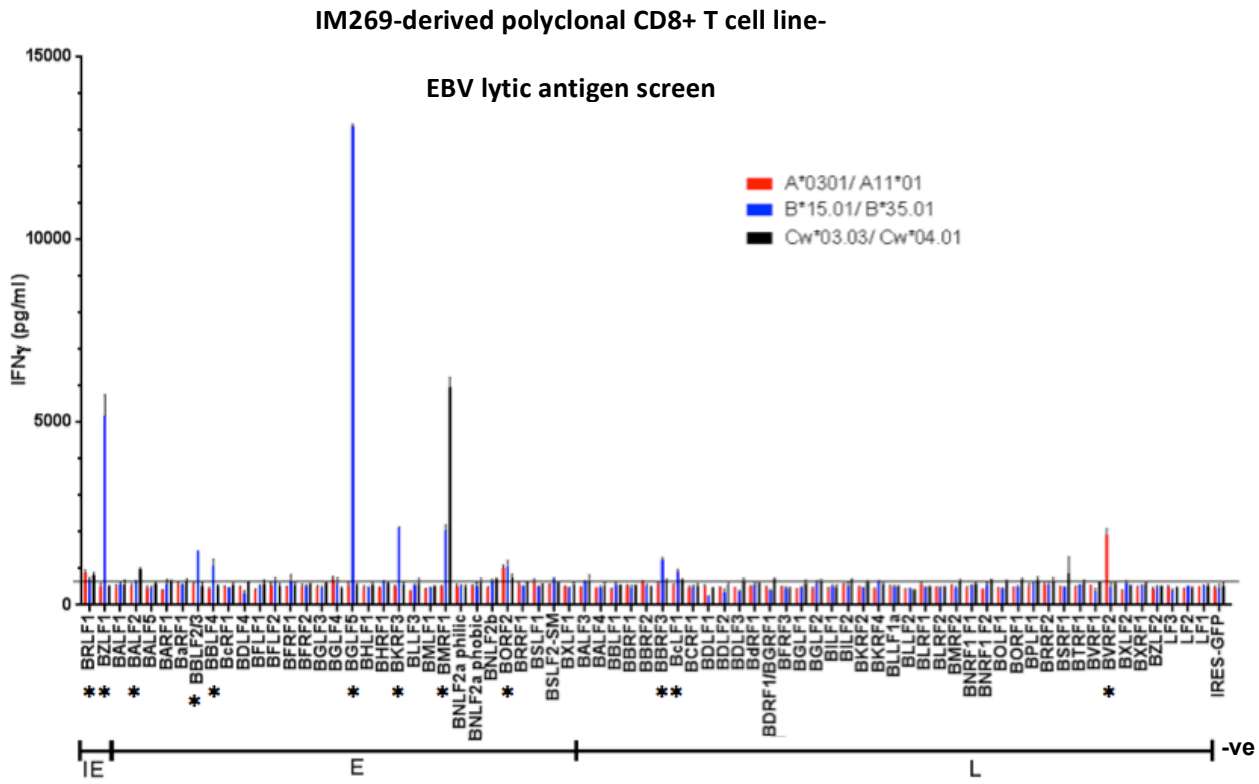


Figure 4.8. Lytic antigen choice within IM269-derived polyclonal CD8+ T cell line
IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative control (IRES-GFP (empty vector)). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.3.8. Summary of CTL responses in IM donors

The above screens shown in figures 4.2-4.8 have been combined to give a summary of the results using polyclonal lines expanded from IM patients (Figure 4.9.). This summary figure shows there is a focusing of responses towards the IE- and E-proteins in both number of responses and in the magnitude of responses. Dominant responses in terms of the size of the response in the expanded polyclonal lines include those against the two IE proteins BRLF1 and BZLF1, the E proteins BaRF1, BBLF2/3, BALF2, BMRF1 and BORF2. Strong responses from L proteins included those to BcLF1 and BVRF1. Dominant responses in terms of the prevalence amongst the individual tested were against the two IE proteins BRLF1 and BZLF1, the E proteins BALF2, BaRF1, BBLF2/3, BMRF1 and BORF2, and the L proteins BBRF3 and BcLF1. The majority of these proteins are from the IE- or E-stages with only BBRF3 and BcLF1 being L-proteins. Of these frequent proteins only BZLF1 and BALF2 were found in all of the 7 donors whilst BORF2 and BcLF1 were found in 6 donors and BRLF1, BaRF1 and BMRF1 in 5 donors. Several of these proteins did induce responses from the polyclonal lines restricted through different HLA types within the same donor and between the different donors.

Across these IM donors the total number of disparate HLA molecules expressed were 6 HLA-A, 8 HLA-B and 6 HLA-C. Common HLA molecules that were expressed in this group of IM patients were HLA A*01.01, B*08.01, C*07.01 (4 donors), A*02.01 (3 donors) and B*15.02, C*03.03, C*03.04 and C*04.01 (2 donors). From these polyclonal lines, detected responses were most frequently restricted through HLA-A*02.01 whilst HLA-B*07.02 and B*40.01 were also common restricted alleles for the responses tested.

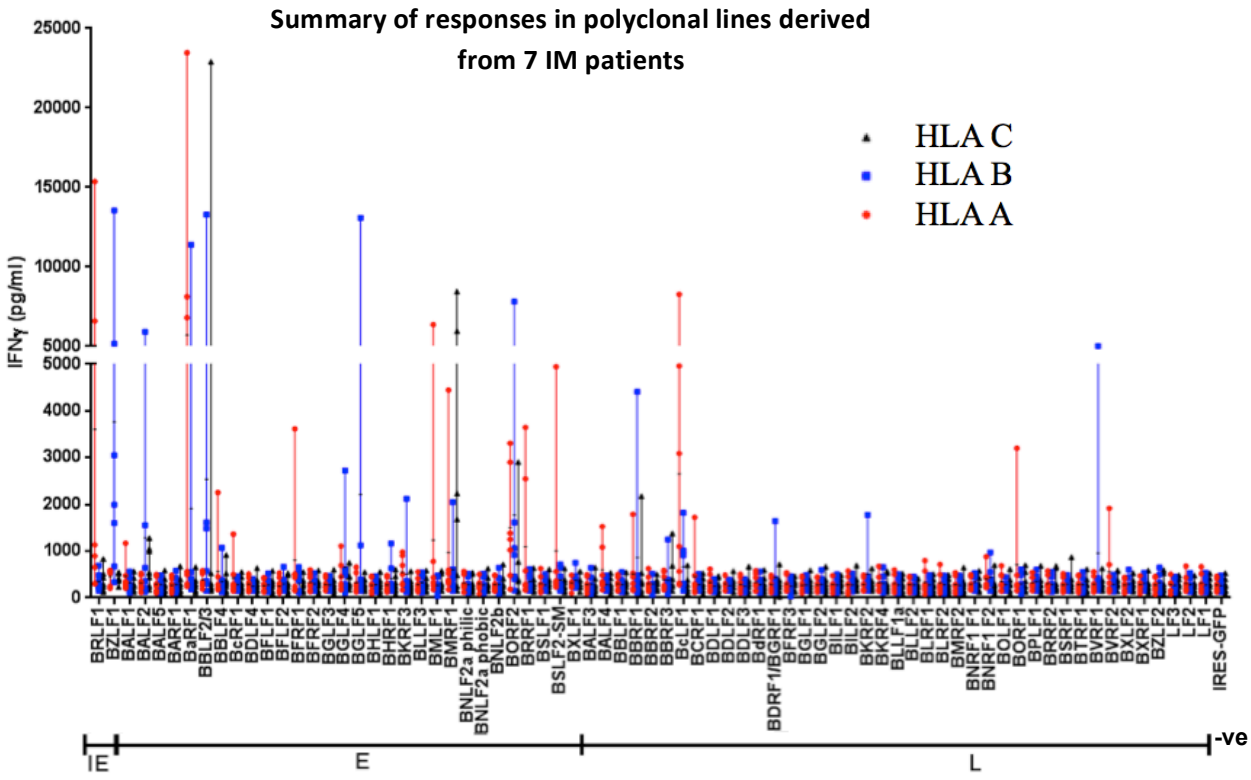


Figure 4.9. Summary of responses from polyclonal CD8+ T cell lines derived from 7 IM patients
IFNγ secretion by polyclonal expanded CTL lines was measured after co-culture with APCs individually expressing each lytic antigen in combination with donor restricted HLA-A (red), B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. An empty vector was used as a negative control (IRES-GFP). Each node represents mean response in one donor.

4.4. Expansion of EBV-specific CTLs from 1 post-IM patient and 6 healthy donors.

During IM EBV-specific CD8+ T cell frequencies have already been expanded within peripheral blood and therefore the above approach of using a non-specific stimulus to bulk up the numbers of cells could be utilised. However to look in donors with persistent disease whose EBV-specific CD8+ T cell pool has contracted a different approach was used and is represented in figure 4.1. This approach addressed in chapter 3 was to use autologous moDCs to cross-present EBV antigens from an EBV (B95.8) infected cell-lysate to stimulate EBV-specific CD8+ T cells within PBMCs that could then be selected and given a general mitogenic stimulus. Upon sorting for expression of the activation marker CD137, typically between 5-10x10^4 cells were collected from a bulk population of around 30x10^6 PBMCs and expanded more than 1000 fold to give a polyclonal population that could be used in the screens described above for lines from IM patients. This procedure was used for 6 healthy donors seropositive for EBV and in a follow-up for the recovered IM269 donor. Several of these donors had previously characterised *ex vivo* responses in an ELISPOT assay using defined peptide epitopes. These responses could help validate responses seen in this study and therefore help validate the procedure itself.

4.4.1. IM269 post-IM (A*03.01, A*11.01, B*15.01, B*35.01, C*03.03, C*04.01)

A CD8+ polyclonal T cell line was generated from the recovered IM patient IM269. This line was generated using the procedure described above in section 4.2. and was challenged against target MJS cells expressing individual EBV genes and donor HLA A, B or C alleles with the responses shown in figure 4.10. Negative controls included the empty vector (IRES-GFP) as well as EBV genes LF1, 2 and 3 that are not present in the B95.8 EBV genome, which was used at the EBV lytic antigen source. Responses in this polyclonal line were identified to be

against 5 individual EBV proteins corresponding to the IE protein BZLF1, 1 E and 2 L proteins. The response in this line of the greatest magnitude was against the L protein BcLF1. Other proteins eliciting clear responses were the IE protein BZLF1, the E protein BALF2 and the L protein BNRF1.

From this screen 3 responses appeared to be restricted through HLA-A alleles, 9 responses through HLA-B alleles and 3 responses restricted through HLA-C alleles.

These results contrast against the responses observed in the polyclonal line expanded from this donors PBMCs at the time of IM symptoms. In that line the largest responses were found against E proteins BGLF5 and BMRF1 and the IE protein BZLF1. Responses to L proteins were small with the response to BcLF1 barely above the threshold line and there being no observed response to BNRF1.

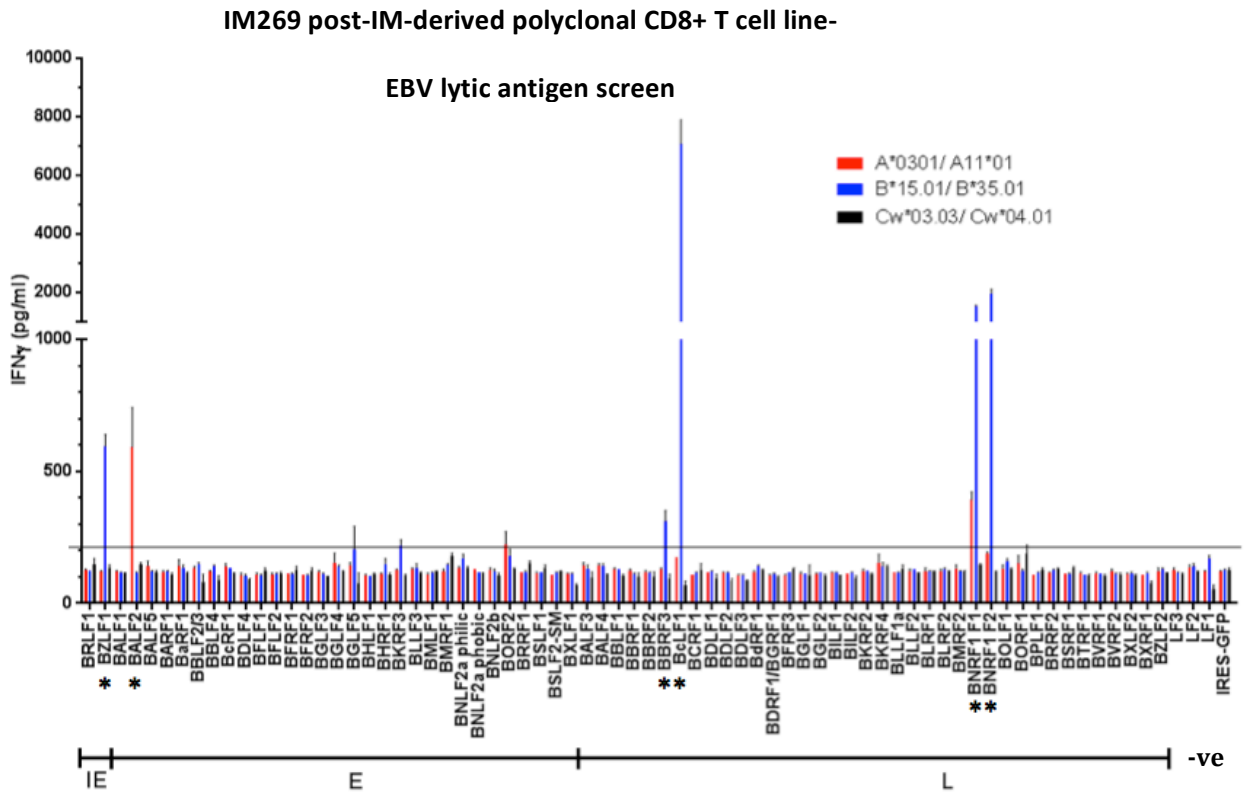


Figure 4.10. Lytic antigen choice within post-IM269-derived polyclonal CD8+ T cell line
IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative control (LF1, 2 and 3 and IRES-GFP (empty vector)). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.4.2. Healthy Donor 1 (A*03.01, A*11.01, B*15.01, B*35.01, C*03.03, C*04.01)

EBV-specific CD8+ T cells were enriched from HD1s PBMCs using the method described above in section 4.2. with the responses shown in figure 4.11. In this polyclonal line responses were identified to be against 10 individual EBV proteins corresponding to the IE protein BZLF1, 4 E and 5 L proteins. The response in this line of the greatest magnitude was against the L protein BcLF1. Other proteins eliciting clear responses were the IE protein BZLF1, the E protein BORF2 and the L proteins BBRF3 and BNRF1. The other detected responses were small and included the E proteins BALF2, BKRF3 and BMRF1 and the L proteins BALF4 and BZLF2.

After separating out the HLA alleles expression in COS-7 cells, responses to antigens restricted through A*03.01 included the L proteins BALF4 and BZLF2. Responses to antigens restricted through A*11.01 included the E protein BORF2 and the L proteins BBRF3, BcLF1, BNRF1 and BZLF2. After separating out the two HLA-B alleles responses to antigens restricted through B*15.01 included the IE protein BZLF1, and the L proteins BcLF1 and BZLF2. Responses to antigens restricted through B*35.01 included the IE protein BZLF1, the E proteins BALF2, BKRF3, BMRF1 and BORF2, and the L proteins BALF4, BBRF3, BcLF1, BNRF1 and BZLF2. After separating out the two HLA-C alleles responses to antigens restricted through C*03.03 included the E protein BMRF1, and the L proteins BBRF3 and BZLF2. Responses to antigens restricted through C*04.01 included the L proteins BBRF3 and BZLF2.

The L protein BZLF2 had detectable responses restricted through all 6 HLA alleles, whilst BBRF3 had detectable responses restricted through 4 alleles (A*11.01, B*35.01, C*03.03 and C*04.01). Other proteins that had detectable responses to two or more HLA alleles included BZLF1 (2), BMRF1 (3), BORF2 (2), BALF4 (2), BcLF1 (3) and BNRF1 (2).

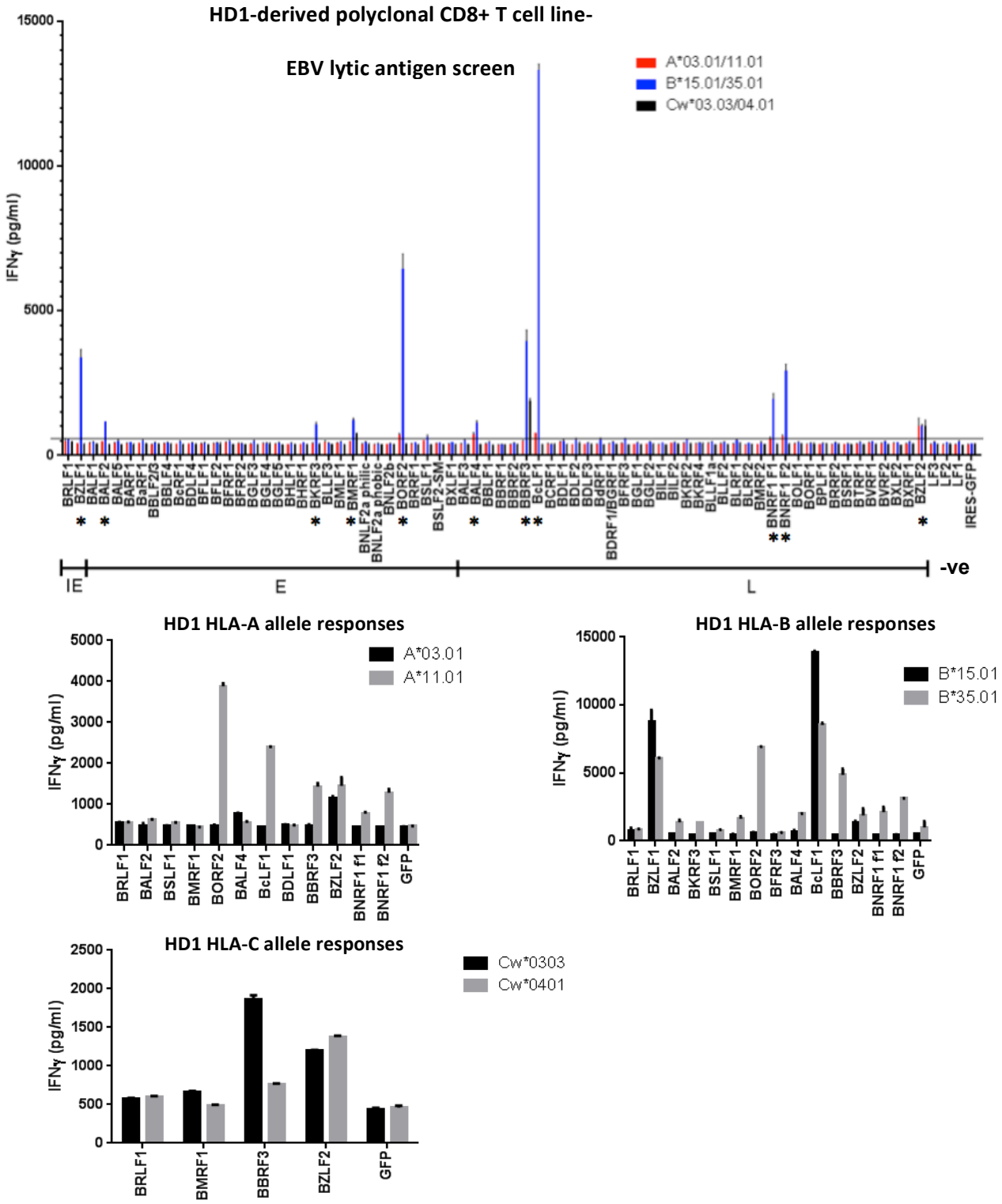


Figure 4.11. Lytic antigen choice within HD1-derived polyclonal CD8+ T cell line
(Top) IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative controls (LF1, 2 and 3 and IRES-GFP (empty vector). (Bottom) Polyclonal line was retested against the non-human cell line, COS-7, for selected EBV lytic antigens and for individual HLA allotypes (A*03.01, A*11.01, B*15.01, B*35.01, C*03.03, C*04.01). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.4.3. Healthy donor 2 (A*01.01, A*11.01, B*07.02, B*35.01, C*04.01, C*07.02)

EBV-specific CD8+ T cells were enriched from HD2s PBMCs using the method described above in section 4.2. with the responses shown in figure 4.12. In this polyclonal line there were responses to 8 individual EBV proteins corresponding to 5 E and 3 L proteins. The response in this line of the greatest magnitude was against the L protein BNRF1. Other proteins eliciting clear responses were the E proteins BORF2, BNLF2b, BALF2, BaRF1, and the L proteins BBRF3 and BcLF1. A small response was also detected against the E protein BRRF1.

After separating out the HLA alleles expression in COS-7 cells, responses to antigens restricted through A*11.01 included the E protein BALF2, and the L proteins BBRF3, and BNRF1. After separating out the two HLA-B alleles responses to antigens restricted through B*07.02 included the L proteins BcLF1 and BZLF2. Responses to antigens restricted through B*35.01 included the IE protein BZLF1, the E proteins BALF2, BKRF3, BMRF1 and BORF2, and the L proteins BALF4, BBRF3, BcLF1, BNRF1 and BZLF2. After separating out the two HLA-C alleles responses to antigens restricted through C*03.03 included the E protein BMRF1, and the L proteins BBRF3 and BZLF2. Responses to antigens restricted through C*04.01 included the L proteins BBRF3 and BZLF2.

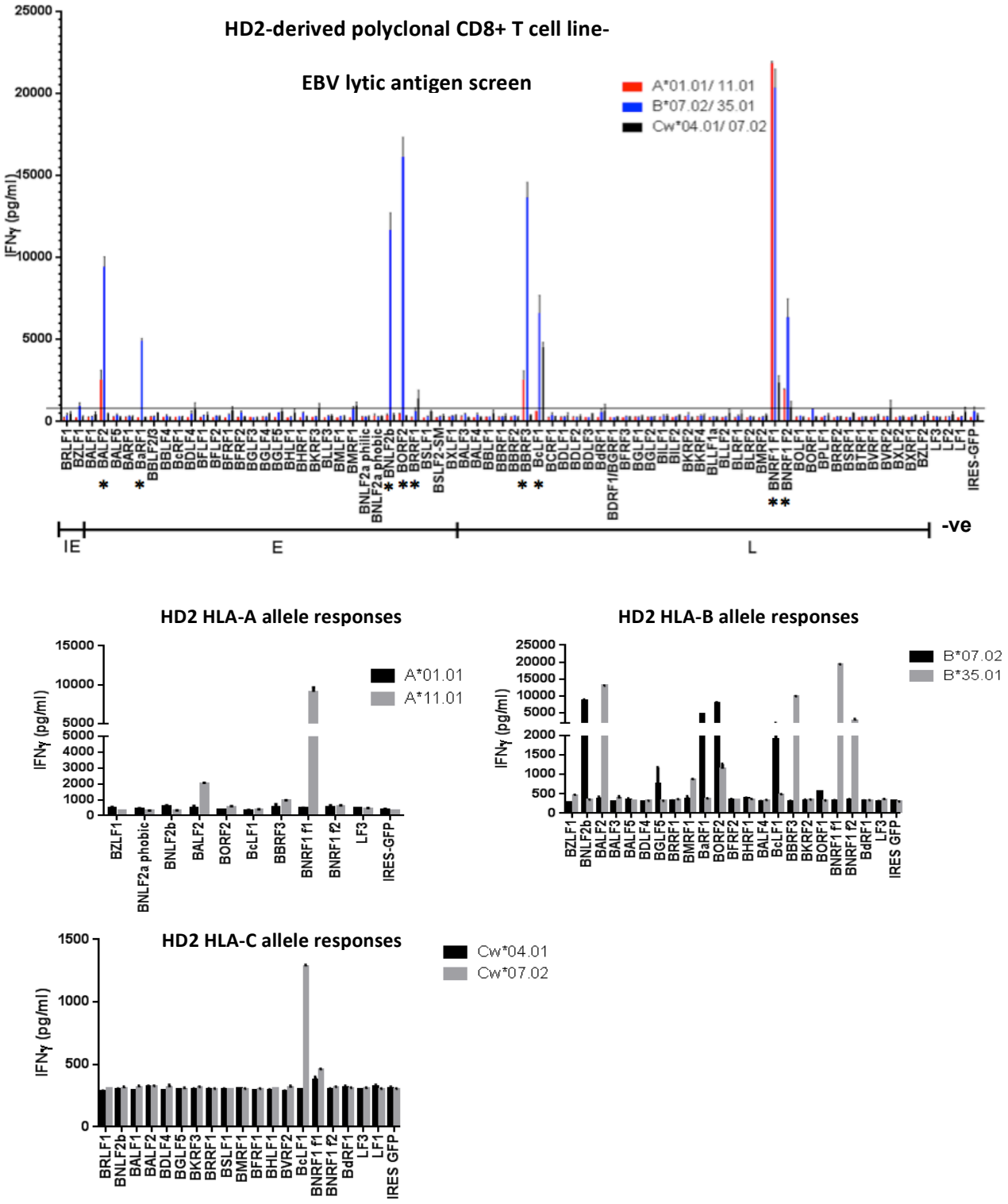


Figure 4.12. Lytic antigen choice within HD2-derived polyclonal CD8+ T cell line
(Top) IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative controls (LF1, 2 and 3 and IRES-GFP (empty vector). (Bottom) Polyclonal line was retested against the non-human cell line, COS-7, for selected EBV lytic antigens and for individual HLA allotypes (A*01.01, A*11.01, B*07.02, B*35.01, C*04.01, C*07.02). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.4.4. Healthy donor 3 (A*02.01, A*24.02, B*39.01, C*06.02, C*07.02)

EBV-specific CD8+ T cells were enriched from HD3s PBMCs using the method described above in section 4.2. with the responses shown in figure 4.13. In this polyclonal line there was only one response and that was to the L protein BcLF1. Other responses were observed on the threshold line to the E proteins BALF1 and BNLF2a and to the L protein BGLF2, but the error bars precluded these from being counted.

After separating out the two HLA-A alleles in COS-7 cells, a large response to BcLF1 was identified to be restricted through A*02.01 and a minor response restricted through A*24.02. No other protein was observed to elicit a response.

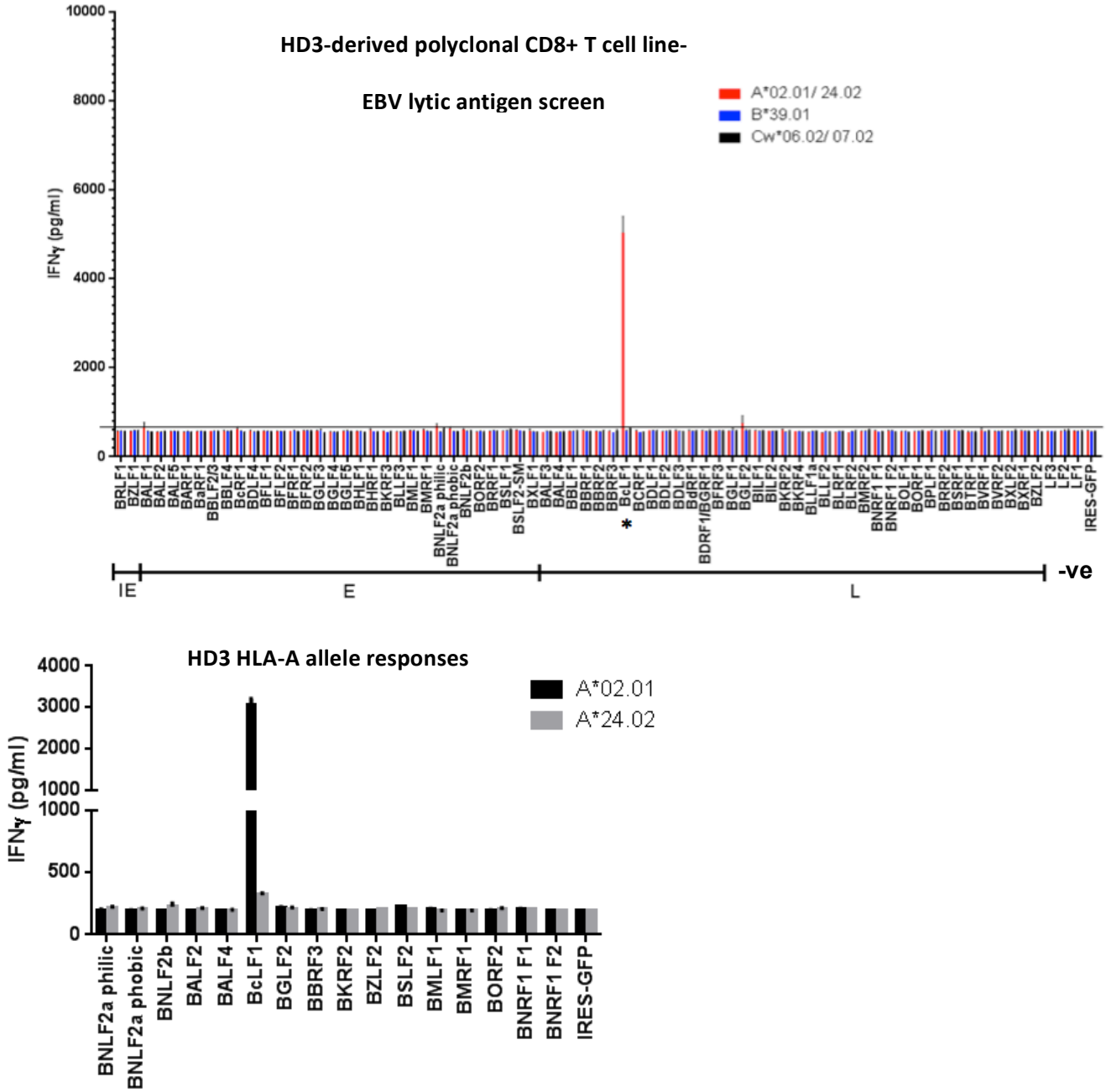


Figure 4.13. Lytic antigen choice within HD3-derived polyclonal CD8+ T cell line
(Top) IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative controls (LF1, 2 and 3 and IRES-GFP (empty vector). (Bottom) Polyclonal line was retested against the non-human cell line, COS-7, for selected EBV lytic antigens and for individual HLA-A allotypes (A*02.01, A*24.02). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.4.5. Healthy donor HD4 (A*01.01, A*02.01, B*39.01, B*40.01, C*03.04, C*12.02)

EBV-specific CD8+ T cells were enriched from HD4s PBMCs using the method described above in section 4.2. with the responses shown in figure 4.14. In this polyclonal line there were responses to 6 individual EBV proteins corresponding to 1 IE protein, 2 E and 3 L proteins. The response in this line of the greatest magnitude was against the L protein BcLF1. Other proteins eliciting clear responses were the IE protein BZLF1, the E protein BALF2, and the L proteins BDLF1 and BKRF2. Small responses were also detected against the E protein BMRF1 and the L protein BDLF2. Other responses on the threshold but were not counted included responses to the L proteins BBRF3 and BDLF2.

No further restriction of the responses into separate individual HLA alleles was carried out. The strongest response in this line was to the L protein BcLF1 that was restricted through an HLA-B allele with a smaller response to this protein also observed restricted through an HLA-A allele. In total there were 3 responses restricted through HLA-A alleles, 4 restricted through HLA-B alleles and 2 responses restricted through HLA-C alleles.

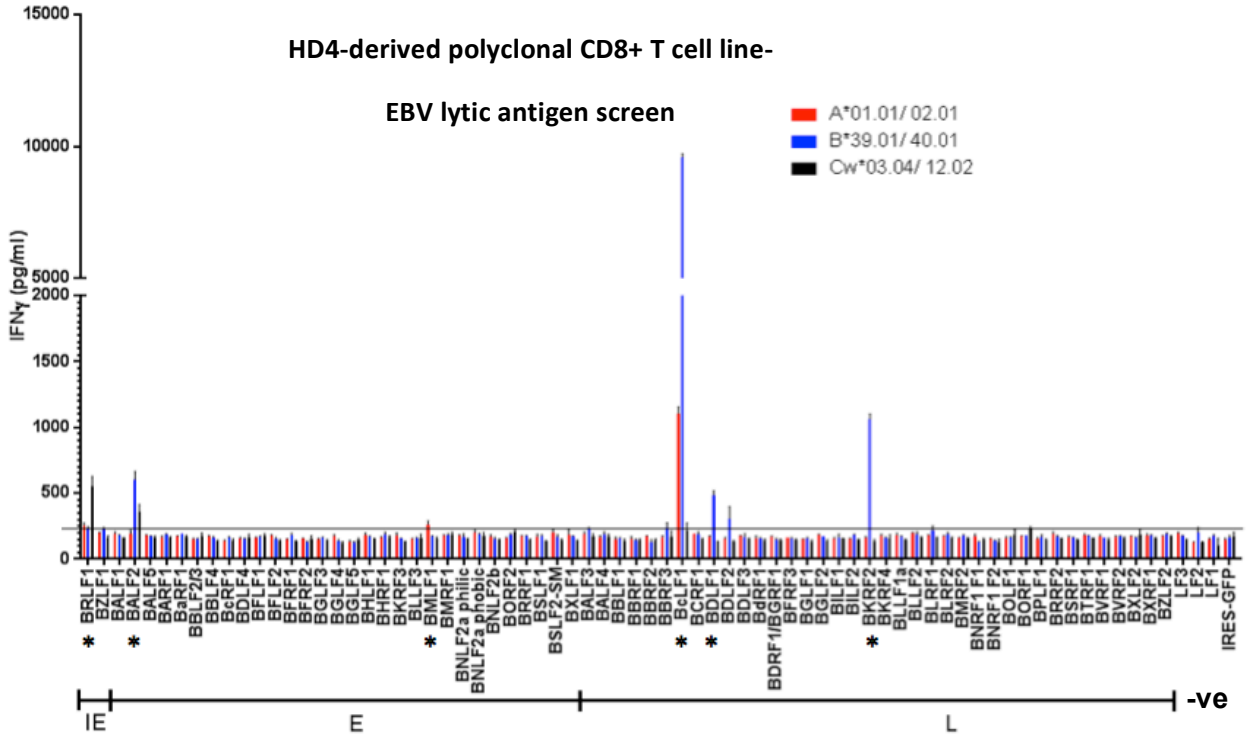


Figure 4.14. Lytic antigen choice within HD4-derived polyclonal CD8+ T cell line
IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative controls (LF1, 2 and 3 and IRES-GFP (empty vector). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.4.6. Healthy donor HD5 (A*02.01, A*11.01, B*35.01, B*44.02, C*04.01, C*05.01)

EBV-specific CD8+ T cells were enriched from HD5s PBMCs using the method described above in section 4.2. with the responses shown in figure 4.15. In this polyclonal line there were responses to 10 individual EBV proteins corresponding to both IE proteins, 4 E and 4 L proteins. The response in this line of the greatest magnitude was against the L protein BcLF1. Other proteins eliciting clear responses were the IE protein BZLF1, E proteins BORF2, BaRF1 and BMRF1 and the L proteins BBRF3 and BDLF1. Small response were also detected against the IE protein BRLF1, the E protein BMLF1 and the L protein BNRF1. The response to BSLF2-SM is presumably elicited from BMLF1.

After separating out the HLA alleles expression in COS-7 cells, responses to antigens restricted through A*02.01 included the IE protein BRLF1, the E proteins BMLF1 and BaRF1and the L protein BcLF1. Responses to antigens restricted through A*11.01 included the IE protein BZLF1, the E protein BORF2 and the L protein BNRF1. After separating out the two HLA-B alleles responses to antigens restricted through B*35.01 included the IE protein BZLF1, the E proteins BMRF1 and BORF2 and the L proteins BBRF3, BcLF1 and BNRF1. Responses to antigens restricted through B*44.01 included the L proteins BDLF1 and BBRF3. After separating out the two HLA-C alleles there was only one response and that was to the E protein BMRF1 restricted through C*04.01. No response was observed to be restricted through C*05.01.

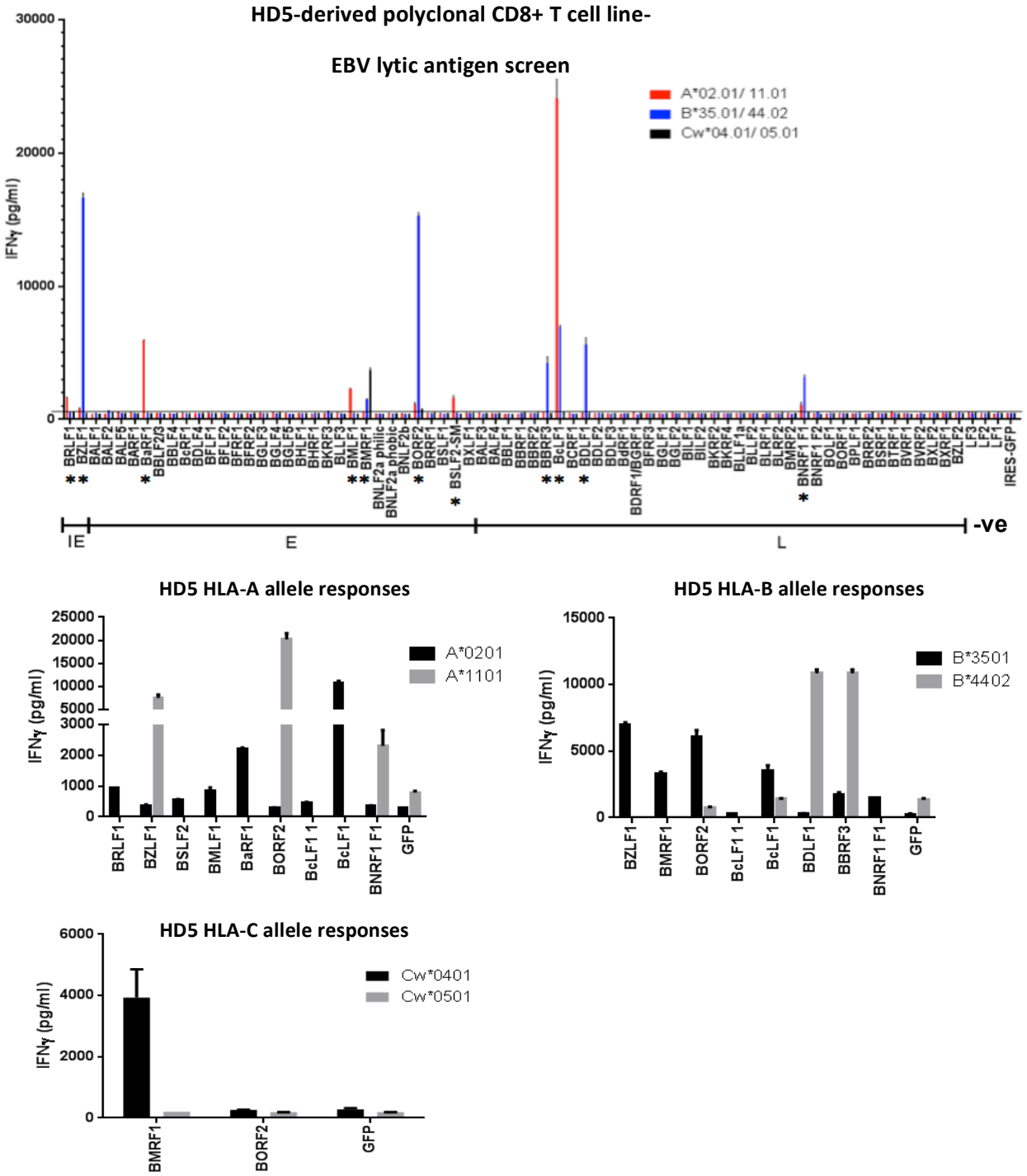


Figure 4.15. Lytic antigen choice within HD5-derived polyclonal CD8+ T cell line
(Top) IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative controls (LF1, 2 and 3 and IRES-GFP (empty vector). (Bottom) Polyclonal line was retested against the non-human cell line, COS-7, for selected EBV lytic antigens and for individual HLA allotypes (A*02.01, A*11.01, B*35.01, B*44.02, C*04.01, C*05.01). An asterisk denotes a positive response to a protein restricted through at least one HLA allele.

4.4.7. Healthy donor HD6 (A*01.01, A*02.01, B*44.02, B*57.03, C*05.01, C*07.01)

EBV-specific CD8+ T cells were enriched from HD6s PBMCs using the method described above in section 4.2. with the responses shown in figure 4.16. In this polyclonal line there were responses to 6 individual EBV proteins corresponding to 1 IE protein, 2 E and 3 L proteins. The response in this line of the greatest magnitude was against the L protein BDLF1. Other proteins eliciting clear responses were the IE protein BZLF1, the E protein BALF2, and the L proteins BcLF1 and BDLF2. A small response was also detected against the E protein BMLF1. Other responses on the threshold but were not counted included responses to the proteins BcRF1, BGLF4, BNLF2a, BBRF1, BDRF1/BGRF1, BILF1 and BZLF2.

No further restriction of the responses into separate individual HLA alleles was carried out. The strongest response in this line was to the L protein BDLF1 that was restricted through an HLA-B allele. In total there were 2 responses restricted through HLA-A alleles, 3 restricted through HLA-B alleles and 1 response restricted through HLA-C alleles.

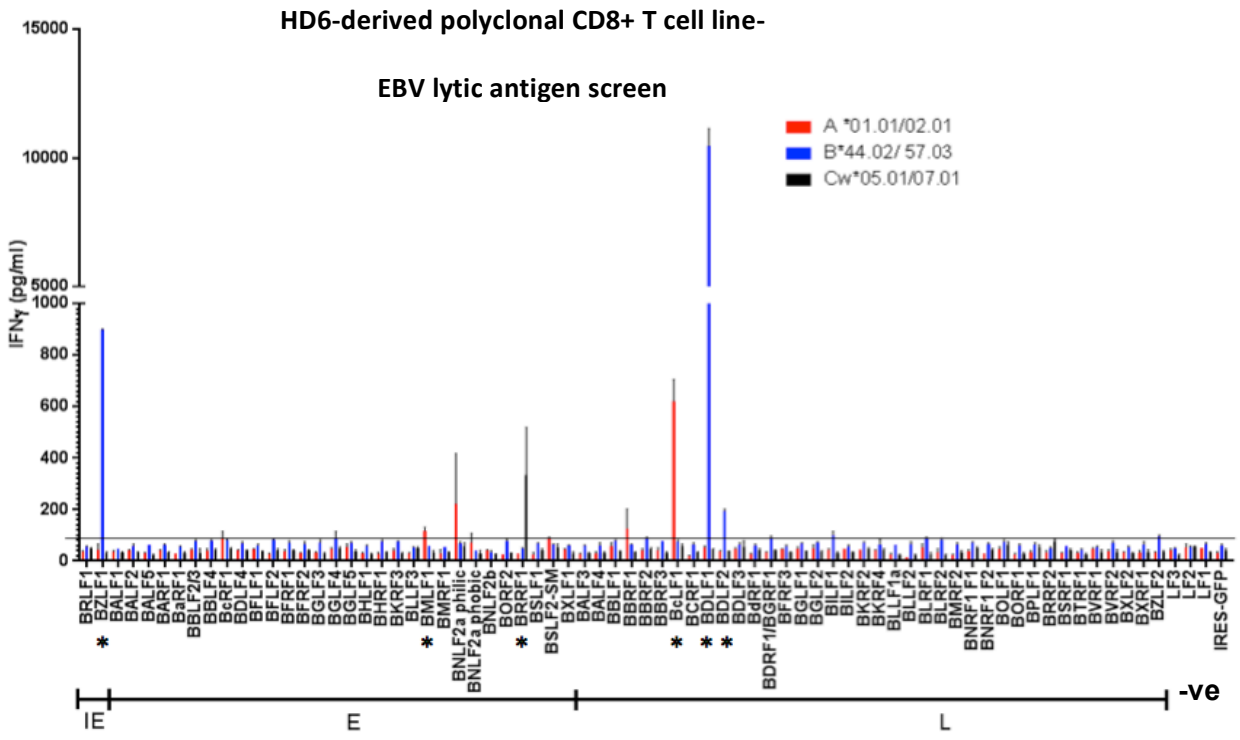


Figure 4.16. Lytic antigen choice within HD6-derived polyclonal CD8+ T cell line
(Top) IFNγ secretion by polyclonal expanded CD8+ T cell lines was measured after co-culture with MJS cells individually expressing each lytic antigen in combination with donor restricted HLA-A (red) or B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Threshold line represents mean of negative controls (LF1, 2 and 3 and IRES-GFP (empty vector).

4.4.8. Summary of EBV-specific responses in healthy virus carriers and post-IM patients.

The above screens shown in figures 4.10-4.16 have been combined to give a summary of the results using polyclonal lines expanded from 6 healthy donors patients and 1 post-IM donor (Figure 4.17.). This approach to amplify EBV-specific CD8+ T cell responses from donors with persistent EBV diseases allowed the identification of a range of responses across each temporal stage of lytic cycle replication. The responses in the lines from 6 healthy donors and 1 post-IM donor are overall less diverse then the responses observed in lines from IM patients and also show more frequent responses to late expressed proteins. However these frequent responses to late proteins are from a restricted set of proteins such as BcLF1 and BNRF1. Dominant responses in terms of the size of the response in the expanded polyclonal lines from healthy donors include those against the IE protein BZLF1, the E proteins BaRF1, BALF2, BNLF2b and BORF2, and the L proteins BBRF3, BcLF1, BDLF1 and BNRF1. Additionally the dominant responses in each donor were typically towards L-proteins with BcLF1 the dominant response in 5 donors and BNRF1 and BDLF1 being dominant responses in 1 donor each. The most frequent response, seen in all 7 healthy donors was against BcLF1. Other frequent responses were found against the proteins BZLF1, BALF2, BBRF3 and BNRF1 occurring in 4 donors and BMRF1, BMLF1 and BORF2 in 3 donors. Proteins that elicited responses in the healthy donors but not in IM donors included: BNLF2b, BDLF1, BDLF2 and BZLF2.

Interestingly in the follow up sample (post-IM) from IM269 there was a very different repertoire of responses. During primary infection IM269 had a wide range of responses, primarily against IE- and E-proteins with the dominant response against BGLF5. However on the follow up bleed, around 4 years after recovery from IM this donor had fewer observed

responses to E-proteins with only BALF2 eliciting a response albeit through a different HLA restriction. Dominant responses were now observed against BcLF1 that elicited only a small response during IM and against BNRF1 that had no observed response during IM.

In regards to HLA restrictions in the healthy donors there were 5 HLA-A, 7 HLA-B and 8 HLA-C unique alleles amongst the 7 donors. The most frequent alleles were HLA-A*02.01, B*35.01 and C*04.01 (4 donors), A*01.01, A*03.01, A*11.01 (3 donors) and B*15.02, B*39.01, B*44.02, C*03.03, C*05.01 and C*07.02 (2 donors). The remaining restricting alleles were just found in 1 donor.

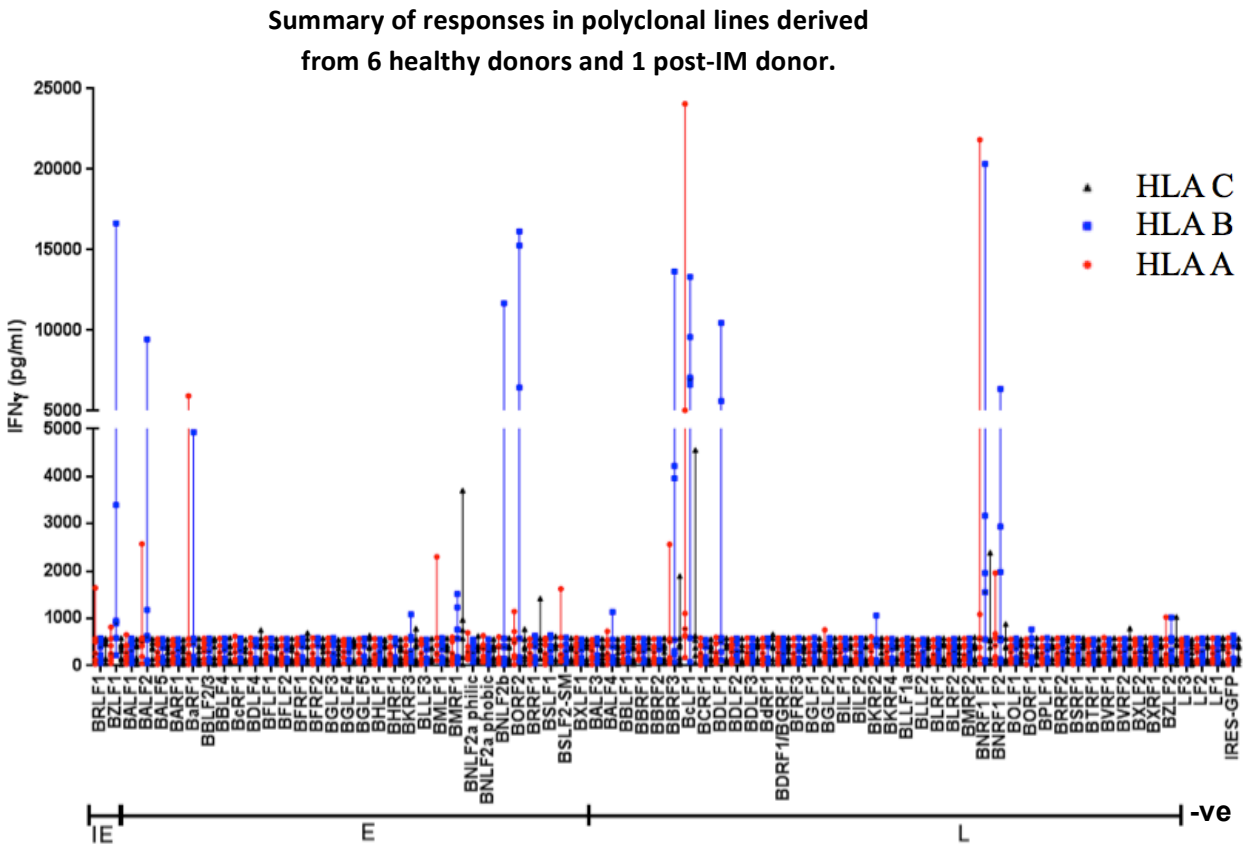


Figure 4.17. Summary of responses from polyclonal CD8+ T cell lines derived from 6 healthy donors and 1 post-IM donor.
IFNγ secretion by polyclonal expanded CTL lines was measured after co-culture with APCs individually expressing each lytic antigen in combination with donor restricted HLA-A (red), B (blue) or C (black) alleles. EBV-lytic ORFs are arrayed along the x axis in nominal order within each lytic phase (IE, E, L) they belong to. Each node represents mean response in one donor.

4.5. Discussion

The observed responses here expand on previous knowledge of immunodominant determinants in EBV infection. In this study the full repertoire of CD8+ T cell responses to EBV-lytic antigens is tested across a range of HLA restrictions compared to less than half this number in previous studies and enabled the identification of novel responses (Pudney et al., 2005, Abbott et al., 2013). This is the first time that responses against the full lytic-antigen repertoire have been tested in IM patients as well as post IM donors and healthy virus carriers. Additionally this approach allows responses to viral antigens to be restricted through a range of HLA class I alleles

Pudney *et al* (2005) showed that IE and E proteins were the most frequent targets of recognition by the CD8+ T cell response during IM (Pudney et al., 2005). In that study the immunodominant antigens were the two IE proteins BZLF1 and BRLF1 and the E proteins BMLF1, BMRF1, BALF2 and BALF5 with all but BMLF1 containing 2-6 epitopes. Responses to L proteins were only identified in 3 out of 11 donors with single epitopes identified each from BALF4, BBRF1 and BILF2. The observations in the present study confirm this pattern of immunodominance but have also shown that the repertoire of CD8+ T cell responses in T cell lines from IM patients is much broader. In that study, one IM donor (IM84) only had a CD8+ T cell response to a single early protein, BMRF1. The responses in this donor were also examined in the present work, confirming a response against BMRF1 but also identifying responses against an additional 18 lytic proteins including several that were targets for recognition in the approach by Pudney *et al* but with no response identified. These proteins included the two IE proteins BZLF1 and BRLF1, the E proteins BALF2, BGLF4 and BMLF1 and the L protein BALF4 with the responses to BRLF1, BZLF1 and BMLF1 in the present study equivalent if not greater to the response seen to BMRF1. This suggests that the

new approach in the present study is not only a broader study but is also more sensitive compared to the previous approach by Pudney *et al*. The two approaches are very different with Pudney *et al* cloning out T cell responses by limiting dilution from IM donors and then using LCL targets expressing individual lytic genes to determine the specificity of the T cell clones. Therefore it is likely that many responses were lost during the limiting dilution cloning or that as autologous LCL targets were used alongside allogeneic PBMCs as feeder cells in the cloning this could preferentially select for some reactivities over others. In the new approach, IM donor PBMCs are first stimulated using allogeneic PBMCs as feeder cells with a non-specific mitogenic stimulus of OKT3 that should expand lines from these donors in an unbiased manner that can then be screened against target cells expressing each lytic gene. Interestingly a number of proteins examined in both studies had no identified responses including BHRF1, BARF1, BLLF2, BCRF1, BILF1 and BLLF1 and perhaps could suggest a hole in the naïve T cell repertoire or that the two approaches weren't sensitive to identify the responses or that a larger cohort of donors is required. Based on the findings by Pudney *et al* and others that IE and E proteins are the most frequent targets of the CD8+ T cell response to EBV most studies have subsequently only used epitopes from these proteins (typically from BZLF1, BRLF1 and BMLF1) as representative targets of the immune response to EBV lytic proteins (Angelini et al., 2013, Landais et al., 2005). However this study has identified responses to many other lytic proteins such as the E protein BALF2, BaRF1 and BBLF2/3 that were frequent and dominant targets suggesting that a broader view of responses rather than a representative view is best.

The present study has identified a much broader picture of the CD8+ T cell response to EBV compared to previous studies with many novel responses identified as dominant targets in many individuals. In particular one protein BaRF1, which is the small subunit of the

ribonucleotide reductase (RR) complex. This protein generated responses in 5 out of 7 IM patient T cell lines and 2 out of 7 healthy donor T cell lines. Interestingly the large subunit (BORF2) of the RR complex was also found in 6 out of 7 IM patient T cell lines and 3 of our 7 healthy donor T cell lines. Other dominant responses in IM were from BBLF2/3, BFRF1, BGLF5 and BcLF1 that encode for the helicase-primase complex, a tegument protein, alkaline exonuclease and the major capsid protein respectively. In one IM patient the dominant response was to BGLF5, which is interesting in the regard that BGLF5 expression can actually lead to the down regulation of several immune components including TLRs and cell surface HLA molecules ad co-stimulatory molecules (van Gent et al., 2011) though its role in immune evasion is thought to be minimal (Quinn et al., 2014). The most prevalent responses were to BZLF1 and BALF2 that were observed in all 7 donors. Other prevalent responses were to BORF2, BcLF1, BRLF1, BaRF1 and BMRF1. These responses were also seen here along with numerous other responses that were not detected in the previous study despite being screened for.

In contrast to the responses seen in the IM patients, the responses observed in the post-IM donor and healthy virus carriers showed a different hierarchy of immunodominance. Recovered IM patients are thought to resemble long-term healthy carriers and the pattern of responses observed in this study does show that the post-IM donor T cell lines is characteristic of the responses seen in the lines from other healthy donors. Additionally IM269 also had an identical HLA restriction as one of the healthy donors (HD1) with both showing a similar repertoire of responses as healthy carriers. In these 7 healthy donors responses were observed across all phases of the lytic cycle with the dominant responses most frequently targeting the L expressed major capsid protein BcLF1. Other dominant responses were found to the IE protein BZLF1, the E proteins BALF2, BaRF1, BNLF2b and BORF2,

and the L proteins BBRF3, BDLF1 and BNRF1. It is possible that the antigen source being used to load the moDCs and stimulate responses is biased towards late expressed lytic proteins and that some early expressed proteins such as BNLF2a, which has been shown to be lost by late lytic cycle, may be missing in our antigen preps (Croft et al., 2009). However the presence of strong responses broadly across the lytic proteome in addition of the presence of responses to BNLF2a in some donors suggests that the antigen source used here contained almost complete coverage of the lytic proteins. In validation of the new approach to proportionately expand up memory CD8+ T cell responses, one of the donors (HD2) used in this study already had known *ex vivo* responses in an ELISPOT assay. These responses were to the peptides RPR, RPG and YPR that are found in the proteins BaRF1, BNLF2b and BNRF1. The responses in the ELISPOT assay gave values of 3, 315 and 27 spot forming cells (SFC) per million PBMCs for the peptides RPR, RPG and YPR. These *ex vivo* response sizes largely correlate with the magnitude of responses seen in this study as the YPR peptide is found in the second fragment of BNRF1 (BNRF1 F2). Another study examining memory CD8+ T cell responses to EBV lytic proteins did identify that frequent though subdominant responses could be identified to some late expressed lytic proteins using overlapping peptides (Abbott et al., 2013). However this study only examined responses to 2 IE, 6 E and 7 L proteins and one of the key findings was that the L expressed protein BNRF1 was also expressed in latency and so was an exception to the observed immunodominance hierarchy. However an observation in both the studies by Pudney *et al* and Abbott *et al* along with the present study is that there was no response to the BLLF1 product gp350, which is interesting as this is the main B cell attachment protein for EBV (Pudney et al., 2005, Abbott et al., 2013).

In a study that investigated the infection of Rhesus macaques with the EBV-related lymphocrytovirus (rhLCV) (Orlova et al., 2011), dominant targets of the CD8+ T cell response included the two IE proteins BZLF1, BRLF1, the E proteins BMRF1, BMLF1, BALF2 and BALF5 and the L proteins BLLF1, BALF4, BNRF1, BVRF2 and BILF2. The authors extended this work to investigate EBV infected healthy virus carriers and identified responses to L proteins BDLF3, BLLF1, BALF4, BNRF1 and BVRF2. Though this study was limited in the number of antigens that were studied, responses to the few L proteins studied were identified. Interestingly many of these proteins also elicited CD8+ T cell responses in EBV, though interestingly some were also absent such as from BALF5, BDLF3, BLLF1 and BILF2. The reason why CD8+ T cell responses to these proteins are absent is unknown and though it could be a technical problem it may also represent a gap in the naïve T cell repertoire (Chen and McCluskey, 2006). These authors also noted that the development of responses to rhLCV L proteins was associated with an increased duration of infection and though responses to these proteins were infrequent in primary infection, they could expand over time. In the present study strong CD8+ T cell responses were detected in healthy donors to L expressed proteins including BcLF1 and BNRF1. BNRF1 as well as being expressed during lytic cycle has also been shown to be expressed in latency, driven off the LMP2 promoter and so this may account for the presence of these responses (Orlova et al., 2011, Abbott et al., 2013). However nothing was previously known about the CD8+ T cell response to BcLF1 and responses to its homolog was not examined in the rhLCV study. Additionally for IM269, the line generated from PBMCs at the time of primary infection showed small or absent responses to BcLF1 and BNRF1 respectively whereas in the post-IM line these two L proteins now elicited the strongest responses, whilst there was no observed expansion of responses to other L proteins. Therefore this could suggest that responses to these L proteins,

unlike other L proteins may expand over time. This could be interesting in determining how this response is being primed or restimulated and how it expands. It could also provide evidence in favour of a model of antigen being cross-presented by dendritic cells during viral persistence.

Other models of immunodominance during virus infection can be used to compare and contrast the data observed here in particular to CMV, HSV-1/2 and VZV. CD8+ T cell responses in healthy virus carriers have been studied using overlapping peptides across the whole proteome of CMV (Sylwester et al., 2005). A significant proportion of the memory CD8+ T cell compartment (10%) was found to be specific for CMV antigens in around a third of individuals. Responses were broadly reactive to antigen from across each phase of the lytic cycle with more than 80% to proteins other than the IE proteins and responses identified to 151 of the 213 (70%) CMV ORFs examined. Cross-presentation by dendritic cells has been shown to be pivotal in generating these responses as direct presentation by CMV-infected DCs or epithelial cells is inhibited by the expression of CMV immune evasion proteins (Busche et al., 2013, Jackson et al., 2011). In comparison to this, the memory CD8+ T cell responses found to EBV in this study targeted a smaller repertoire of proteins though also taken from across each phase of the lytic cycle. However a particular strength of the present study was the ability to study CD8+ T cell responses to primary symptomatic EBV infection in an unbiased manner. This showed that the response in IM patients is relatively broad with around 50% of lytic proteins eliciting a response from the 7 donors examined. In contrast CD8+ T cell responses to primary symptomatic CMV infection have only been tested against peptide pools from two identified immunodominant proteins IE-1 and pp65 (Khan et al., 2007). This study by Sylwester *et al* (2005) benefitted from the high overall CD8+ T cell responses to CMV but for most other viruses T cells are at low abundance in the blood.

Therefore in the study by Jing *et al* (2012) a pre-enrichment step was utilised which was also the basis for this study in EBV (Jing et al., 2012). Jing and colleagues found that the CD8+ T cell response to HSV-1 appears to recognise a broad array of antigens across all kinetic phases and that the antigens were more frequently presented through HLA-A molecules. The authors then took this further and looked for cross-reactivity of these responses against HSV-2 and VZV ORFs (Jing et al., 2016). Interestingly from these studies the authors identified numerous CD8+ T cell responses to antigens that have homologs in EBV and were actually targets in this thesis as well. In the HSV-1 study (Jing et al., 2012) the authors identified UL39 as a candidate vaccine antigen based on its immunodominance across numerous HLA restrictions in different donors. This gene encodes the ribonucleotide reductase (RR) large subunit that in EBV is encoded by BORF2, which represented a frequently targeted antigen in this study, particularly in IM patients. The RR large subunit forms a heterodimer with the RR small subunit encoded by BaRF1 in EBV. Both these proteins were dominant targets of the CD8+ T cell response in this current work but also in the two studies by Jing *et al* (Jing et al., 2012, Jing et al., 2016) for HSV-1 and 2 and for VZV. Additionally a further study has shown that an HLA-A*02.01 restricted epitope from the RR small subunit is cross-reactive across the α-herpesviruses (HSV-1, HSV-2 and VZV) and also for EBV (Chiu et al., 2014). In CMV the homolog of the large RR subunit encoded by BORF2 in EBV is UL45, which elicited both CD4+ and CD8+ T cell responses in around 5% of subjects examined in the study by Sylwester *et al* (2005). However in CMV there is no RR small subunit and the RR large subunit-UL45 lacks many of the catalytic residues and instead appears to be a tegument protein expressed at late stages of infection (Patrone et al., 2003).

Other frequent targets in the studies by Jing *et al* were UL10, UL23 and UL29, which encode for the DNA replication origin-binding helicase, the thymidine kinase and an ssDNA binding

protein. UL10, UL23 and UL29 share homology with the EBV-encoded proteins from BBLF2/3, BXLF1 and BALF2 respectively. In this thesis BBLF2/3 was a dominant target in the IM patients whereas BALF2 was a strong target in both IM patients and in healthy virus carriers. BXLF1 however failed to elicit any responses. These 3 proteins also did not elicit frequent responses in CMV (Sylwester et al., 2005).

Interestingly, away from herpesviruses, studies in other complex viruses have shown dominant responses focused towards antigens expressed early in the replication cycles (Yewdell, 2006). In vaccinia virus, which preferentially infects APCs such as DCs, macrophages and B cells immunodominance studies have shown that there appears to be a bias of responses towards early gene products (Jing et al., 2005, Oseroff et al., 2005). This bias has also been attributed to a block in L antigen presentation and shows that this may be a common strategy deployed by viruses.

HLA restriction is an interesting factor in driving CTL responses and individual allotypes have been associated with increased risk for certain diseases including many autoimmune diseases and has also been associated with graft versus host disease following transplantation (Clark and Chakraverty, 2002). Studies of immunodominance by Brander and colleagues using models of EBV and HIV coinfection identified that responses to HLA-B restricted determinants were the most prevalent and most robust (Bihl et al., 2006, Yewdell, 2006). Further studies have also identified this phenomenon in other viruses including measles (Schellens et al., 2015, Boon et al., 2004). Alternatively for HSV-1 Jing *et al* noted that HLA-A molecules were the most frequent restricting elements (Jing et al., 2012). Responses to HLA-C restricted determinants appears to be much less prevalent compared to HLA-A and –B restricted determinants but this is hardly surprising considering that HLA-C is expressed at lower levels on the cell surface (Snary et al., 1977, Apps et al., 2015). Reasons for this may be

the instability of HLA-C mRNAs or it may be due to the more selective binding of peptide in the ER that prevents its dissociation from TAP (Neisig et al., 1998). Therefore the identification of dominant targets through HLA-C such as with BBLF2/3 through HLA-C*04.01 and BMRF1 through C*03.04 from IM84 was an interesting finding. Other HLA-C restricted responses included those to BALF2, BORF2 and BcLF1. These proteins all elicited dominant responses restricted through other HLA molecules and therefore their presence through HLA-C may indicate an increased abundance of these antigens.

In this thesis HLA-A molecules appeared to be the most prevalent restricting elements for responses in IM patients. However in healthy virus carriers there was a shift towards HLA-B being the most prevalent (data not shown). These observations are difficult to reason as two different approaches were used to generate the polyclonal lines from IM patients and healthy virus carriers. Possible reasons for this difference could be that there is a preference for HLA-B restricting alleles on moDCs compared to B cells or that cross-presentation rather than direct presentation can alter the restricting elements, however no study has looked at this phenomenon.

4.6. Summary

The large screens described in this chapter have led to the identification of many novel CD8+ T cell responses to EBV lytic proteins and has shown that late expressed antigens may be frequent and dominant targets of the immune response. There are caveats with this approach and the differences in polyclonal generation means that it is difficult to rationalise responses seen in IM patients with those in healthy virus carriers. There are also potential issues that the EBV-infected cell lysate contained a higher quantity of L proteins compared to IE and E

proteins. However the initial aim of this project was to determine whether there were any CD8+ T cell responses to L proteins and this has been shown conclusively here. The overall screens in the healthy virus carriers also show that responses were made against IE and E proteins showing that there was still a wide spread of antigens from each phase. The approach to generate polyclonal lines from IM patients was largely unbiased though uncontrollable effects of immunodomination could still occur. The responses in the IM patients still shows that the IE and E proteins are the most frequent targets of the CD8+ T cell response but newly identified dominant targets include BaRF1, BBLF2/3 and BORF2 amongst others. These proteins elicited the largest magnitude responses and were also identified in numerous donors. Several L proteins also elicited responses though these were typically of a lower magnitude compared to responses to IE and E proteins. How epitopes from these proteins are presented despite the immune evasion blocks still needs to be investigated but could offer new insights into how some antigens are processed in primary EBV infection. Additionally in healthy virus carriers, structural proteins were the dominant targets here and in particular BcLF1 and BNRF1. For IM269 a barely detectable response to BcLF1 during IM developed into the immunodominant response 4 years after disease recovery.

In the next chapter an attempt will be made to quantify the *ex vivo* frequency of CD8+ T cells to selected antigens and to identify what might be driving these responses.

CHAPTER 5

Ex vivo qualification of novel CD8+ T cell responses and the ability of these cells to recognise EBV infected target cells.

During lytic EBV replication in B cells, the expressions of several immune evasion proteins serve to inhibit presentation of peptides on the cell surface, minimising recognition by the immune system. Previous studies of the CD8+ T cell response to EBV appeared to show a block in recognition of epitopes from late expressed proteins, though in these studies only a select number of antigens have been looked at (Pudney et al., 2005, Abbott et al., 2013, Quinn et al., 2014). In chapter 4 a more comprehensive screening approach identified numerous novel CD8+ T cell responses to EBV lytic proteins including several responses to L expressed proteins. In particular, one protein BcLF1 was shown to be an immunodominant target in lines from healthy virus carriers but was only a subdominant response during primary EBV disease using lines from IM patients. Following T cell reactivities in one IM donor longitudinally, using T cells lines generated from PBMCs at the time of IM and then following resolution of IM, showed contrasting hierarchies of immunodominance. In the line expanded from PBMCs during IM there were few responses to late proteins, however in the polyclonal line generated after resolution of IM there were dominant responses to the late proteins BcLF1 and to BNRF1. Similar responses to BcLF1 and BNRF1 could also be observed in lines generated from one healthy donor (HD1) who had an identical HLA restriction profile to IM269. In lines from the other healthy donors, BcLF1 was a frequent and dominant target of recognition by CD8+ T cells and was restricted through a variety of HLA class I alleles. It was recently suggested that increasing duration of infection of Rhesus macaques by a related lymphocryptovirus (RhLCV) resulted in the development of CD8+ T

cell responses that were infrequent in primary infection but expanded over time (Orlova et al., 2011). Therefore to investigate whether this expansion could be observed for CD8+ T cell responses to BcLF1 and to examine what driving this response various immunological strategies were employed to define restricting peptide epitopes, identify frequencies of responding cells in IM patients and healthy virus carriers, and to generate T cell clones that could be used in *in vitro* recognition assays.

5.1. Epitope mapping of EBV-gene fragments

Proteins that elicited interesting responses in chapter 4 in terms of magnitude or frequency were deemed good targets to provide follow up analyses. Such proteins included BORF2, BBRF3 and BcLF1 that encode the large ribonucleotide reductase subunit (RR), the glycoprotein gM and the major capsid protein (MCP) respectively. All of these proteins were frequent targets in both IM donors and healthy donors and were dominant targets in at least one individual. However of particular interest was the late protein BcLF1 due to frequent but low magnitude responses in polyclonal lines generated from IM patients but becoming an immunodominant target in the screens on lines generated from healthy donors. Particularly IM269 who showed a barely detectable response to BcLF1 during IM, which became the immunodominant target detected in the lines generated 4 years after resolution of IM.

The approach to epitope map responses from these proteins involved the *in silico* prediction of peptides epitopes from within each protein. Due to the size of the proteins and to cut down on the number of predicted hits, fragments of each of the above proteins were generated and cloned into the pcDNA3.1-V5/HIS expression vector. HLA restricted responses were narrowed down to a single fragment from each protein in all polyclonal lines tested. However

in silico prediction of peptides was unsuccessful in eliciting any responses for the proteins BBRF3 and BORF2 and so responses to these proteins were not pursued further.

The fragments were made by staggering the 3' termination site whilst maintaining the 5' start site with a Kozak sequence (Fig. 5.1. Top). For example, the BcLF1 gene was separated into 4 fragments (Fig. 5.1. Top) and if an epitope (red star) is found within the 5' fragment (F1) a response should be observed for all subsequent fragments, whereas an epitope (blue star) found within the 3' fragment (F4) should only elicit a response to that 3' fragment. These different constructs could then be ectopically expressed in the non-human cell line COS-7 along with individual HLA plasmids. Polyclonal lines that were screened in chapter 4 were then challenged with these targets and responses were measured through the production of IFNγ in an ELISA.

This approach narrowed down regions containing immunogenic epitopes. For BcLF1, epitopes that elicited responses appeared to be in either 5' F1 or 3' F4 fragments of the protein with the central 2kb region appearing not to give any clear responses. Responses to the F1 fragment were observed in polyclonal lines restricted through B*15.01, B*07.02 and B*39.01 whereas responses to F4 were observed in polyclonal lines restricted through A*11.01, B*35.01 and A*02.01. None of the fragments elicited a response for A*24.02 or B*40.01 in the lines tested. For A*24.02 the response observed in HD3 in chapter 4 was very minor and subdominant to the response restricted through A*02.01 so it is possible that this response was lost to the sensitivity of the assay. There was no response restricted through B*40.01 in the line from HD4 and it is possible that this donor only had a B*39.01 restricted epitope.

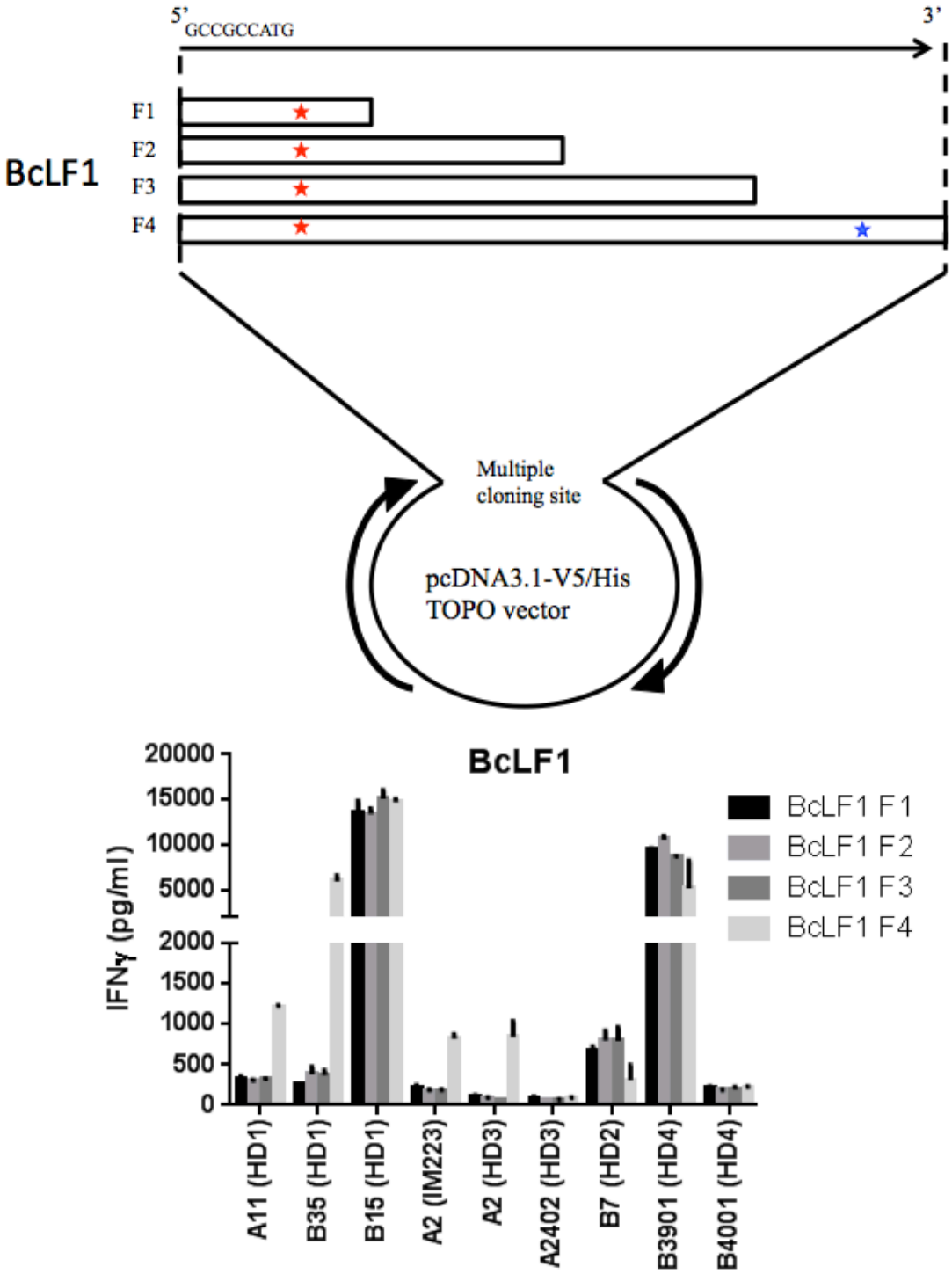


Figure 5.1. Restricting responses to EBV lytic antigen fragments.
(Top) Overlapping PCR fragments of BcLF1 were cloned individually into the pcDNA3.1-V5/HIS TOPO gene expression vector to narrow down the immunogenic epitope. An epitope in fragment 1 (F1-red star) would elicit a response from all 4 fragments. An epitope in fragment 4 (F4-blue star) would elicit a response only from fragment 4. (Bottom) Expression constructs were expressed in COS-7 target cells along with different HLA alleles and used to screen responses from different polyclonal T cell lines previously identified for that response. Supernatant was harvested and amount of IFNγ measured.

By narrowing down the regions of the protein sufficient to generate a response helped to inform the prediction of peptide-epitopes. To predict the epitopes for each restriction a combination of online prediction sites were used; SYFPEITHI and IEDB (see materials and methods), with only the top hits that appeared in both lists used. As previously mentioned, the predicted peptides for BORF2 and BBRF3 were unsuccessful in eliciting a response. For BcLF1, a number of peptides were synthesized for predicted hits restricted through A*02.01, A*11.01, B*07.02, B*08.01, B*15.02 and B*35.01. This was based on the HLA restrictions of other well studied T cell responses that would be examined in parallel in further experiments, the availability of MHC class I tetramers and also on the cost of getting a large number of peptides. From these predictions around 30 peptides were synthesized and were used to load non-lytic LCL targets that could be cultured with polyclonal lines that had observed responses in the previous assay to COS-7 cells ectopically expressing EBV gene fragments and relevant HLA alleles. From these screens only three peptides gave positive responses to epitopes derived from BcLF1 and for the restrictions A*02.01 and B*07.02 (Fig 5.2). Two of these responses for B*07.02 were similar epitopes that only differed by 1 amino acid (valine) at the C-terminus to create 9-mer and 10-mer peptides. Later an additional 11-mer peptide was identified that had an extra valine at the C-terminus and on analysis of T cell avidity appeared to be the target epitope (Fig 5.3).

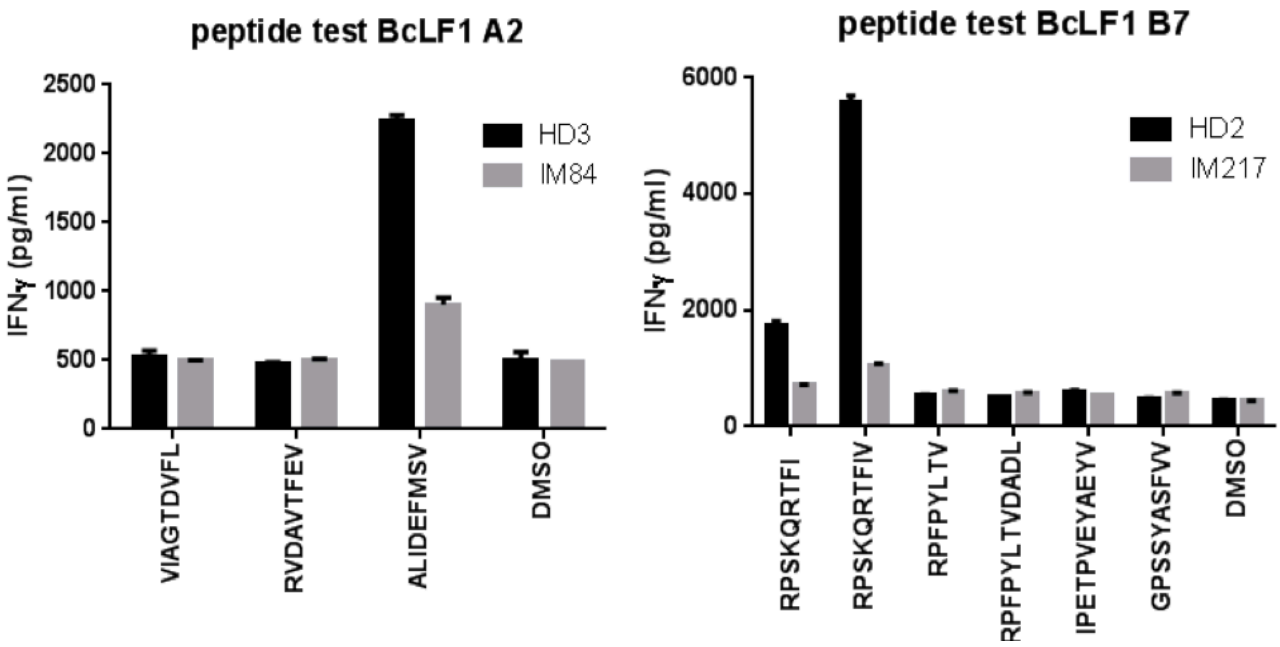


Figure 5.2. Identification of BcLF1 immunogenic peptide-epitopes.
The SYFPEITHI and IEDB prediction software programmes were used to predict epitope-peptides for BcLF1. HLA-A*02.01 and B*07.02 epitopes were identified after screening against polyclonal T cell lines that had previously had these restricted responses in earlier screens. DMSO was used as a negative control. Readout was by IFNγ-capture ELISA.

5.2. Generation of BcLF1 CD8+ T cell clones

CD8+ T cell clones were generated to confirm reactivities against the newly synthesized BcLF1 peptides and so that downstream assays looking at the *ex vivo* response size from the peptides could be compared to assays using these CD8+ T cell clones and looking for recognition of EBV infected cell targets (Fig. 5.3.). To generate these novel BcLF1 reactive CD8+ T cell clones, the previously tested polyclonal lines that had HLA-A*02.01 or B*07.02 reactivities for BcLF1 were stimulated with target COS-7 cells expressing either of these HLA molecules and either with co-expression of the full length BcLF1 gene or sensitised with the synthetic RPS 10mer peptide (Fig. 5.2.) Reactive T cells were then enriched through magnetic bead capture of IFNγ-secreting cells. Both these approaches were successful in generating CD8+ T cell clones reactive for either the B*07.02 epitope RPSKQRTFIV (RPS) 10mer or the A*02.01 epitope ALIDEFMSV (ALI). The approach using the full length BcLF1 gene with the individual HLA molecule could also be applied to other T cell responses without the need for identifying the peptide-epitope.

The A*02.01 restricted CD8+ T cell clone was first tested for reactivity against COS-7 cells expressing the HLA-A*02.01 molecule and pulsed with the newly synthesised BcLF1 peptide ALI as well as another suggested epitope (SMF) that had been shown by another member in our lab to elicit a response in some donors (Fig. 5.3. bottom left). As a positive control the full length BcLF1 gene was co-expressed in the COS-7 cells. There were five T cell clones identified that were specific for BcLF1 and the ALI peptide with clone 4 used in further assays. This clone was subsequently tested for avidity in a peptide titration assay with the functional avidity shown by the dashed line (Fig. 5.3. bottom right).

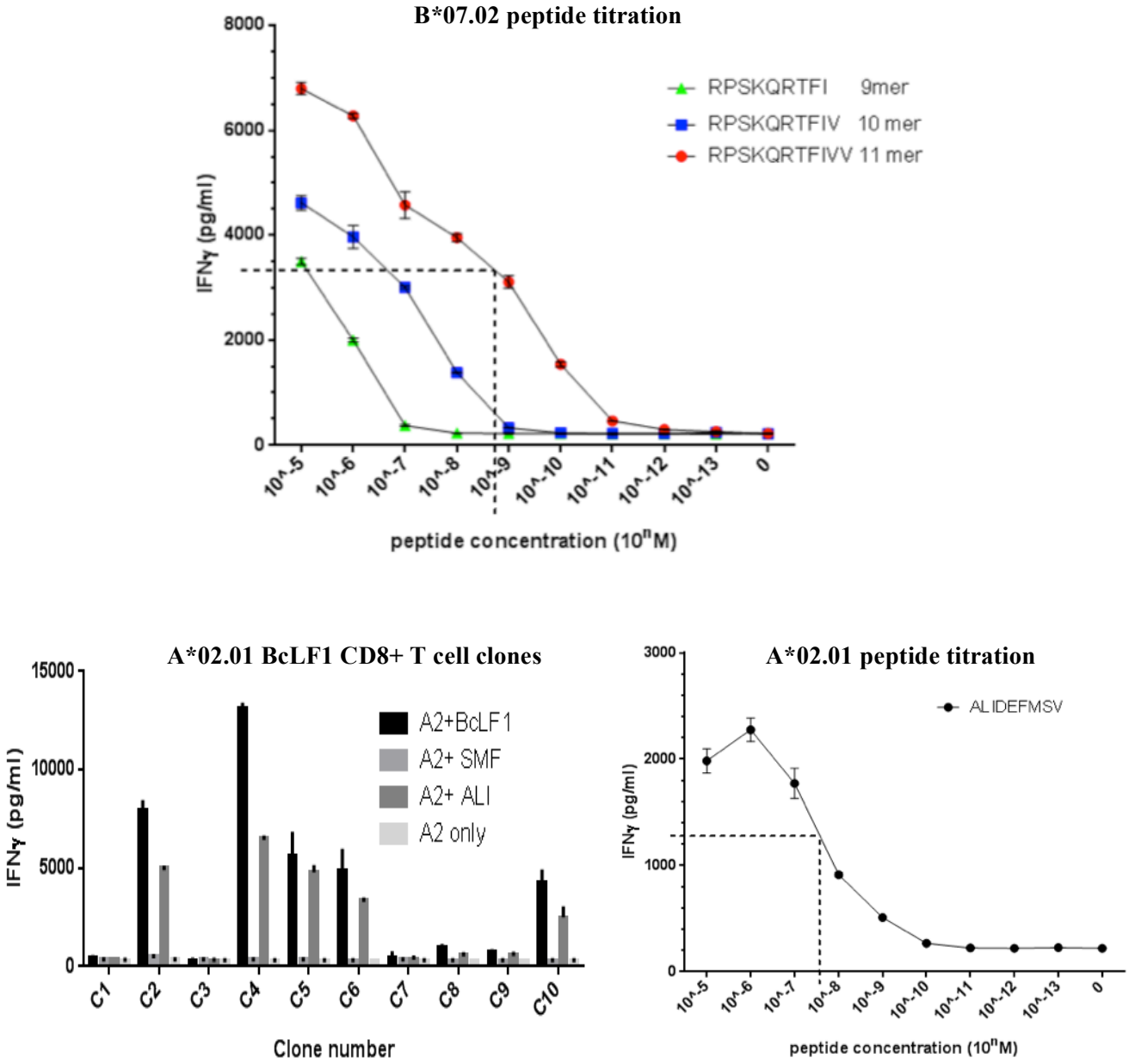


Figure 5.3. Generation of CD8+ T cell clones against BcLF1
(Top) Peptide titration of B*07.02 restricted epitopes from BcLF1 and tested against newly isolated CD8+ T cell clones. Three different lengths of the HLA-B*07.02 restricted peptide RPS (9mer, 10mer and 11mer) were titrated out against the responding T cell clone. (Bottom left) BcLF1 responding CD8+ T cell clones against tested against COS-7 targets ectopically expressing HLA-A*02.01 and BcLF1, HLA-A*02.01 alone or loaded with synthetic peptides of either SMF or ALI, and HLA-A*02.01 alone with no added peptide. Readout for each assay was by IFNγ-capture ELISA. (Bottom right) Clone 4 was then tested for functional avidity towards the A*02.01 restricted ALI 9mer peptide.

The B*07.02 restricted RPS 10mer CD8+ T cell clone was also tested for avidity against RPS 9mer, RPS 10mer and RPS 11mer peptides with the 10mer and 11mer peptides containing an additional 1 and 2 valine residues respectively (Fig. 5.3 Top). This CD8+ T cell clone had the highest avidity for the RPS 11mer peptide (red line) and therefore this peptide was used in all further assays.

5.3. Frequency of CD8+ T cell responses to BcLF1

Responses to BcLF1 in the screens in chapter 4 showed that in lines generated from IM patients these responses were present but typically subdominant to IE and E-proteins. However in lines from healthy donors, responses to BcLF1 typically had the greatest magnitude. Additionally in the donor (IM269) with both IM and follow up screens a negligible BcLF1 response in the line established from IM PBMCs increased to become the dominant target in the line developed using PBMCs from 4years later. Therefore to test *ex vivo* frequencies to this protein in PBMCs from IM donors and healthy donors, MHC-I tetramers were generated using the above ALI and RPS peptides.

The A*02.01 monomers were sourced from the protein expression facility at the University of Birmingham and contained a UV-cleavable peptide that could be substituted for other A*02.01 restricted peptides. This monomer was used for the ALI peptide and for the FLD peptide found within the late protein BALF4. A separate A*02.01 monomer containing the GLC peptide from the early expressed protein BMLF1 was also tetramerised and used to detect responses within donor PBMCs. For the B*07.02 monomers, B*07.02 and β2M protein were sourced from the protein expression facility and monomers were generated by Dr

Andrew Hislop containing the RPS (11mer) peptide. Another B*07.02 monomer containing the peptide RPR found within the early expressed BaRF1 was also used.

To analyse *ex vivo* frequencies of T cell responses to lytic proteins a number of IM donor PBMCs and healthy donor PBMCs were used as well as 2 donors who were seronegative. The PBMCs were stained for expression of CD3, CD8 and for the tetramers containing relevant HLA-A*02.01 or B*07.02 peptides (Fig 5.4). Frequencies of tetramer positive cells were then measured as a percentage of that donors CD8+ population. As a negative control, EBV seronegative donors were also tested for responses to each of the different HLA-A*02.01 and B*07.02 epitopes. Access to EBV seronegative donors was difficult and only two A*02.01 and one B*07.02 were found from the available donors. The A*02.01 seronegative donors had no or only low frequencies of T cells specific for each epitope though one of these donors was also B*07.02 positive and did appear to show some reactivity with the B*07.02 BcLF1 epitope RPS.

Measuring the frequency of EBV lytic epitope-specific CD8+ T cell responses in IM donor PBMCs showed stronger responses as compared to the healthy donors and this was true for all tested epitopes. The highest frequency of tetramer positive CD8+ T cells for both A*02.01 and B*07.02 restrictions were towards the E-proteins (BMLF1 or BaRF1). These responses were significantly lower in healthy donor PBMCs compared to IM donor PBMCs, however responses to ALI showed no significant difference in frequency between these two groups. Interestingly whilst the frequency of ALI-specific T cells was relatively low in the IM patient and the healthy donor with less than 0.5% of the CD8+ T cell compartment specific, the frequency of RPS-specific T cells had a wider spread of frequencies in the donors tested reaching more than 15% of the CD8+ T cell compartment in one IM patient. Within the donors tested here there were also three IM donors and five healthy donors that had been used

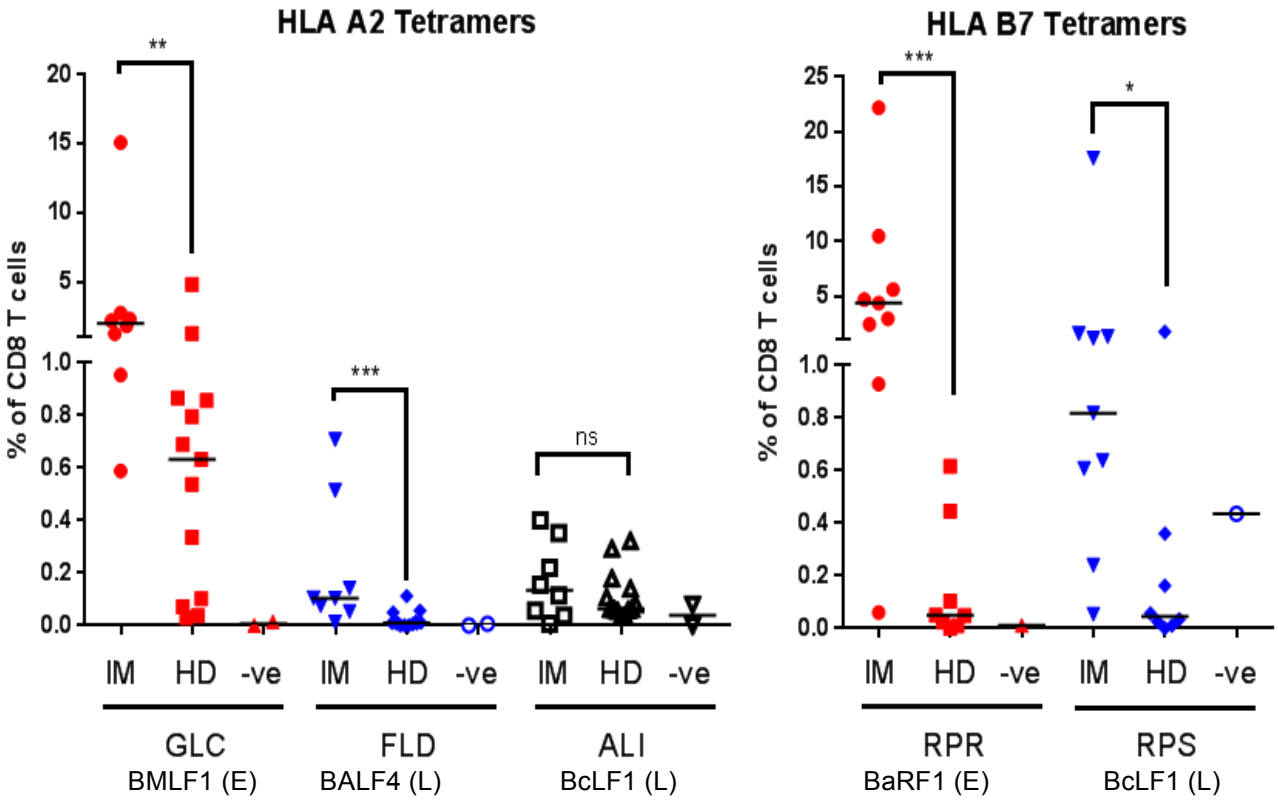


Figure 5.4. Frequency of EBV-specific CTLs in IM donors and in healthy donors
HLA-A*02.01 and B*07.02 tetramers were used to probe the *ex vivo* frequency of EBV-specific CD8+ T cells in the blood of IM patients and healthy virus carriers. The HLA-A*02.01 monomer contained a UV cleavable peptide that could be substituted for other A*02.01 restricted peptides under UV light. HLA-A*02.01 restricted peptides includes GLC (BMLF1), FLD (BALF4) and ALI (BcLF1). The HLA*B*07.02 restricted peptides includes RPR (BaRF1) and RPS-11mer (BcLF1). Mann-Whitney U test was used to test for significance.

	A*02.01			B*07.02	
	GLC	**FLD**	**ALI**	**RPR**	**RPS**
IM217	-	-	-	4.373	0.819
IM239	0.781	0.051	0.351	-	-
IM249	1.854	0.514	0.155	-	-
HD2	-	-	-	0.104	0.360
HD3	0.866	0.011	0.034	-	-
HD4	0.337	0.006	0.036	-	-
HD5	0.795	0.050	0.061	-	-
HD6	4.832	0.008	0.082	-	-

Table 5.1. Ex vivo frequencies of EBV lytic antigen-specific CD8+ T cells in previously screened IM patients and healthy donors.
Previously screened IM patients and healthy donors from chapter 4 were examined for the *ex vivo* frequency of CD8+ T cells to the HLA-A*02.01 epitopes GLC, FLA and ALI derived from BMLF1, BALF4 and BcLF1 respectively or to the HLA-B*07.02 epitopes RPR and RPS derived from BaRF1 and BcLF1 respectfully. Frequencies are measured in comparison to the entire CD8+ T cell compartment.

in the previous screening approach in chapter 4 and these are shown in table 5.1. The two donors, IM217 and HD2 with the B*07.02 allele did show proportional frequencies of CD8+ T cells to RPR (BaRF1) and RPS (BcLF1) compared to the responses in the screens shown in chapter 4. However in the donors with the A*02.01 allele the responses in chapter 4 appeared to under represent the frequency of the CD8+ T cells to GLC (BMLF1) whereas the responses to BcLF1 appeared to be over represented compared to the *ex vivo* frequencies seen with the tetramer to ALI. The lack of any noticeable decrease in the frequency of responses to ALI in healthy donor PBMCs compared to IM donor PBMCs led us to examine frequencies of responses in donors with IM and in matched follow up samples from the same donor at least 1 year post-IM symptoms (Fig 5.5). As IM resolves the EBV-specific T cell pool contracts to leave a population of memory T cells at a low frequency. This is what is seen here with the responses to GLC, FLD, RPR and RPS. However in 3 of 4 donors the frequency of ALI-specific CD8+ T cells actually increased substantially and in the 1 donor where the response decreased the frequency was substantially higher than the others during IM and remained higher post-IM. Though the frequency of ALI-specific CD8+ T cells appears to increase post-IM the absolute numbers of CD8+ T cells were not measured in this study but can be 3-5 fold higher during IM than in healthy individuals. Therefore the donors IM224 and IM226 most likely saw a contraction in number of ALI-specific CD8+ T cells whilst the donors IM239 and IM279 saw the absolute numbers maintained.

In comparison to the observed frequencies of ALI-specific CD8+ T cells, the RPS-specific CD8+ T cells were present at considerably higher frequencies during IM and contracted post-IM to leave a memory pool of comparable size to that of the ALI T cells. The graphs in Fig 5.5 are colour coded according to donor with one donor, IM279 tested for all five epitopes (Top-green line; bottom-pink line). Indeed the frequency of RPS-specific CD8+ T cells

increased post-IM in IM279, albeit minutely, and was the only B*07.02 restricted donor where this was observed. However due to the limited number of donors that could be accessed, the significance of these results regarding the kinetics of responses between primary IM and recovered IM could not be tested. However the pattern of these results do suggest that the A*02.01 and B*07.02 epitopes may be handled differently in terms of their processing and presentation.

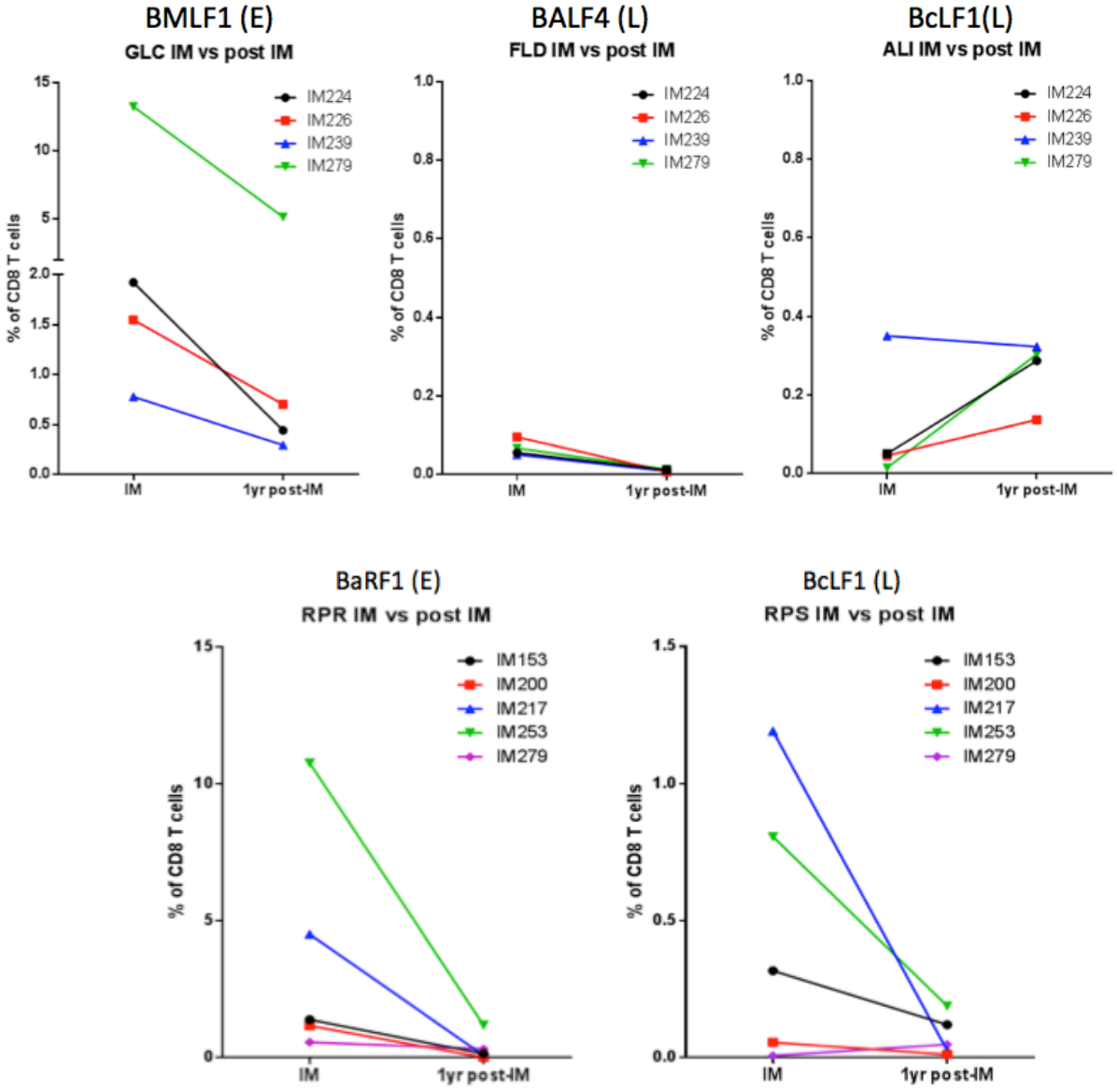


Figure 5.5. Frequency of EBV-specific CTLs during- and post-IM
PBMCs from IM patients originally collected at the time of IM symptoms with a follow up sample after one year were thawed and stained with LIVE/DEAD stain, CD3 and CD8 antibodies and MHC class I tetramers containing the HLA A*02.01 peptides GLC, FLD or ALI; or the B*07.02 peptides RPR or RPS.

5.4. Recognition of lytic and non-lytic LCLs by lytic-antigen CD8+ T cell clones.

Previous studies have examined the efficiency by which representative CD8+ T cell clones to IE, E and L proteins recognise lytically infected B cells and have shown that the efficiency of recognition drops with progress through the lytic cycle (Pudney et al., 2005, Abbott et al., 2013, Quinn et al., 2014). This decreasing efficiency is thought to be due to the influence of EBV-encoded immune evasion proteins that act progressively through the lytic cycle to decrease the pool of epitopes that can be presented by the infected cell (Quinn et al., 2014). This hypothesis was therefore used to suggest why CD8+ T cell responses to late expressed lytic proteins were so infrequent and subdominant to responses to IE and E proteins. However in this study, strong responses were shown to some L proteins, such as BcLF1, in lines from healthy virus carriers. Additionally the *ex vivo* analysis of CD8+ T cell responses to BcLF1 showed that frequency of T cells to this protein were typically superior to those against the well-studied and 'representative' FLD epitope from the L protein BALF4. Therefore the two CD8+ T cell clones specific for the A*02.01 epitope ALI and the B*07.02 epitope RPS from BcLF1 were tested in parallel with other HLA-A*02.01 or B*07.02 restricted CD8+ T cell clones specific for epitopes from representative early and late expressed proteins. Established lymphoblastoid cell lines (LCLs) from a number of donors were used as targets. These LCLs had either been established using the WT B95.8 virus and were therefore semi-permissive to lytic replication, or were established using the BZLF1-KO strain and were therefore incapable of entering lytic cycle. WT and BZLF1-KO LCLs from the same donor were used in the majority of cases, however where a matched pair was not available the WT LCL was used alone.

Previous studies (Abbott et al., 2013) have shown that CD8+ T cell responses can be found against the L lytic protein BNRF1 in both WT and BZLF1-KO LCLs and is therefore also

expressed in latency though typically at lower levels. This was also observed in this study (data not shown). As positive controls BZLF1-KO LCLs were sensitised with the T cells cognate epitope peptide and these positive controls were used as the maximum amount of IFNγ that the T cell can release. Responses from T cells to the other targets was measured relative to this. As negative controls T cell clones were challenged with an HLA mismatched WT LCL or in the absence of target (effector only).

Figure 5.6 shows results of 3 assays testing E and L-specific CD8+ T cell clones in parallel for recognition of HLA matched WT LCLs. Using HLA-A*02.01 restricted CD8+ T cell clones against WT and BZLF1-KO targets (Fig. 5.6 top) there was little recognition of donor 1 BZLF-1 KO LCL targets over that of the negative controls of the effector only and mismatched LCL target. Similar to previous studies, there was good recognition of WT LCL targets by the GLC-specific CD8+ T cell clone whilst there was little recognition by the FLD-specific CD8+ T cell clones (Pudney et al., 2005, Quinn et al., 2014). However the ALI-specific CD8+ T cell clones did not resemble the other late T cell clone and did show good recognition of donor 1 WT LCL target. These results were similar for each of the donor LCLs tested using the A*02.01 restricted T cell clones. Similarly for the B*07.02 restricted T cell clones, there was good recognition of most WT LCL targets by the RPQ-specific CD8+ T cell clone targeting the early expressed protein BMRF1. Additionally against 2 of the 4 donor WT LCL targets there was also good recognition by the RPS-specific CD8+ T cell clones. However surprisingly, the other CD8+ T cell clone specific for the TPS epitope from the late expressed BFRF3 protein also showed good recognition of WT LCL targets from each HLA matched donor and these were typically the strongest response against most donor WT LCLs. Therefore in these assays, spontaneously lytic LCLs were capable of presenting peptides from

some late expressed proteins and could be detected by CD8+ T cell clones specific for these epitopes.

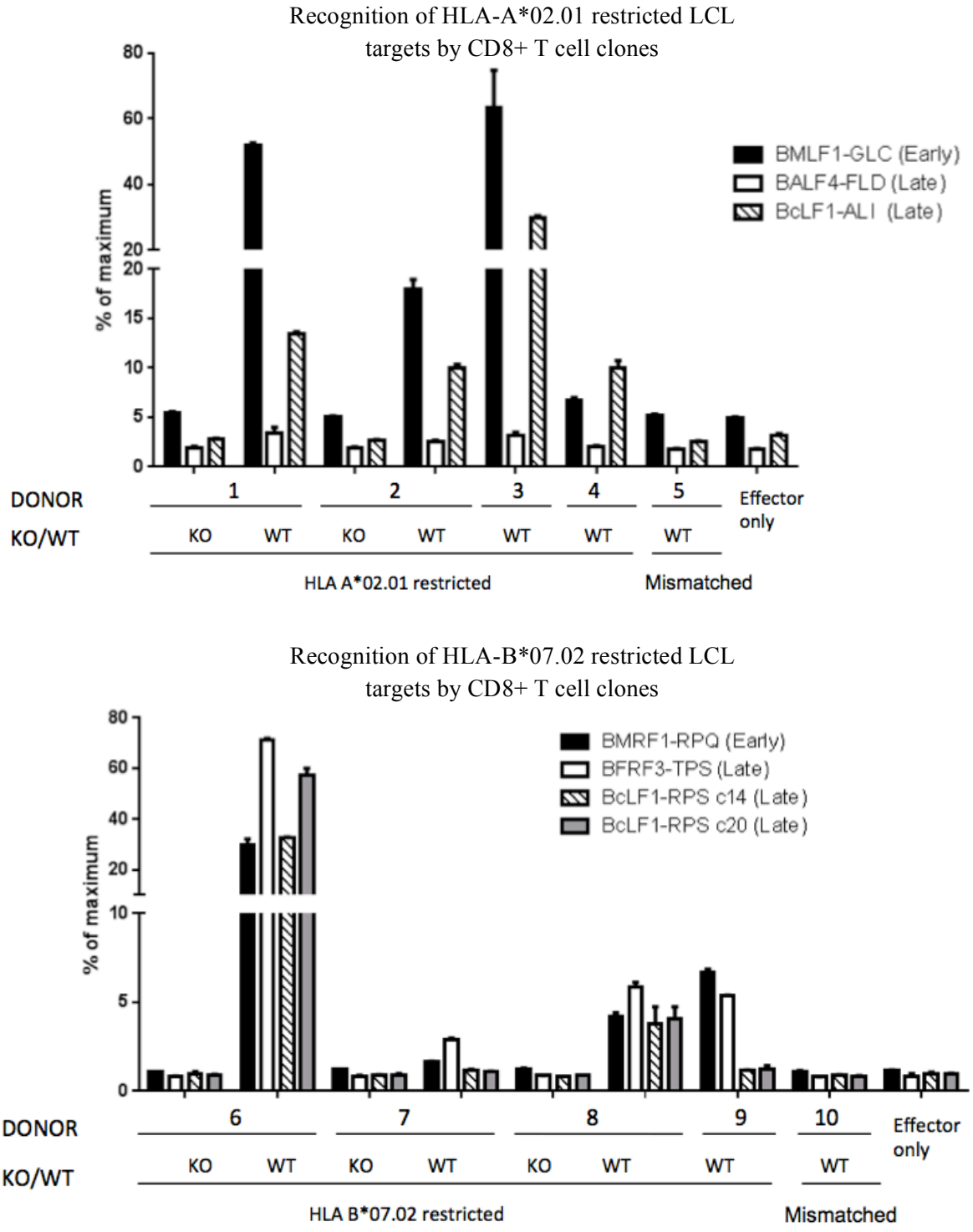


Figure 5.6. Recognition of WT and BZLF1-KO LCLs by lytic-antigen CD8+ T cell clones
Paired Donor HLA A*02.01 (Top) or B*07.02 (Bottom) restricted BZLF1 K/O LCLs and WT LCLs and HLA mismatched LCLs were used as targets for recognition by HLA A*02.01 or B*07.02 restricted T cell clones specific for EBV lytic antigens from various phases. Secretion of IFNγ was used as a readout in an IFNγ-capture ELISA. Responses are measured as percentages of positive peptide pulsed controls. HLA mismatched LCLs and T cell clones cultured without targets (effector only) were used as a negative control for background responses. Results representative of 3 separate assays.

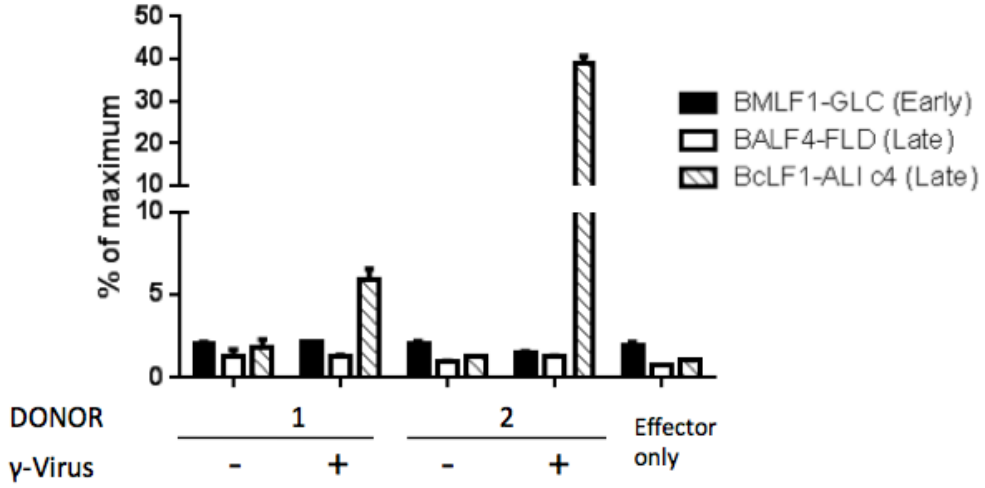
5.5. Recognition of virus-antigen loaded non-lytic LCLs by T cell clones.

The EBV particle itself has been suggested as a source of antigen either upon infection of a B cell or endocytosed by an already infected B cell (Adhikary et al., 2007). EBV relies on the endocytic pathway to infect B cells and undergoes uncoating to allow the release of the virus genome into the cell (Hutt-Fletcher, 2007). This process may allow for the remaining proteins to be processed and presented by the B cell and though these may be small amounts, potentially significant amounts of epitope-peptide may be generated from abundant virion proteins such as BcLF1 (Johannsen et al., 2004). Such antigens include many structural and surface glycoproteins of the virion.

In this study, gradient-purified EBV particles, sourced from Dr Shannon-Lowe, were irradiated to prevent replication and were used as a source of antigen for processing by BZLF1-KO LCL targets. These antigen-loaded targets were then use to challenge different T cell clones, including two clones specific for epitopes from the early proteins BMLF1 (GLC) and BMRF1 (RPQ) which are not virion components and require replication for their expression. In parallel, CD8+ T cell clones specific for structural proteins BALF4 (FLD), BcLF1 (ALI and RPS) and BFRF3 (TPS) were also challenged with these virus-loaded targets. As positive controls BZLF1-KO LCLs were sensitised with the T cells cognate epitope peptide and these positive controls were used as the maximum amount of IFN□ that the T cell can release. Responses from T cells to the other targets was measured relative to this. As negative controls T cell clones were challenged with BZLF1-KO LCLs were not loaded with purified virus particles or in the absence of target (effector only). Responses could be observed against all virus-loaded LCL targets using the T cell clones specific for the TPS, ALI and RPS epitopes from the structural proteins BFRF3 and BcLF1 respectively. However no response was observed against these virus-loaded targets using T cell clones

specific for the GLC and RPQ epitopes from the E proteins BMLF1 and BMRF1 or for T cell clones specific for the FLD epitope from the L protein BALF4.

Recognition of virus-loaded LCL targets by
HLA-A*02.01 CD8+ T cell clones



Recognition of virus-loaded LCL targets by
HLA-B*07.02 CD8+ T cell clones

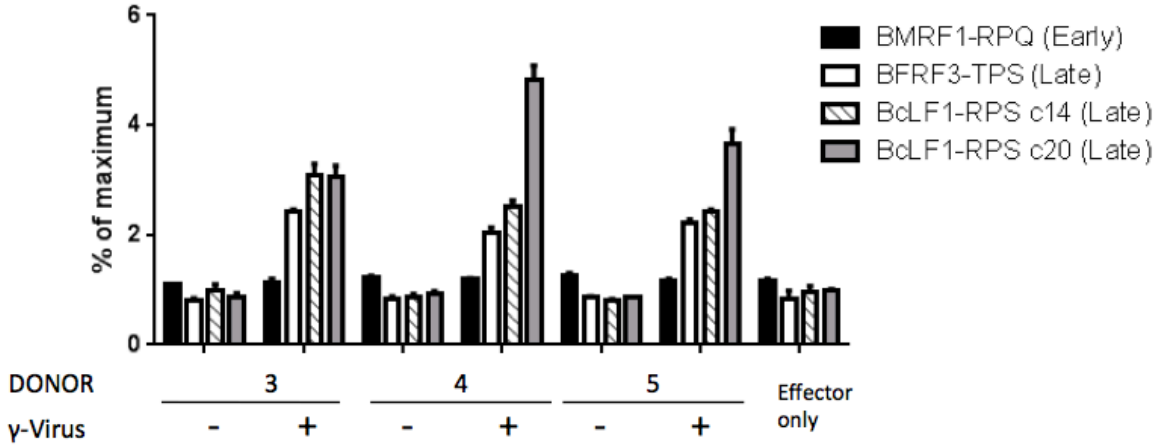


Figure 5.7. Recognition of virus-antigen loaded non-lytic LCLs by T cell clones.
Donor HLA A*02.01 or B*07.02 restricted BZLF1 K/O LCLs were pulsed with inactivated, purified EBV particles (γ-virus) at an MOI of 100. A range of HLA A*02.01 or B*07.02 restricted T cell clones to different EBV lytic antigens from various phases were challenged with the loaded HLA matched BZLF1 K/O LCLs. Secretion of IFNγ was used as a readout in an IFNγ-capture ELISA. Responses are measured as percentages of positive peptide pulsed controls. T cell clones cultured without targets (effector only) were used as a negative control for background responses. Results representative of 2 separate assays.

5.6. Discussion

Understanding the kinetics of T cell responses to specific antigens as well as recognition of target cells can provide clues as to how those antigens are processed and presented during an infection. From studies of primary EBV infection in patients with IM there is an observed hierarchy of responses focussed against the IE and some E antigens with rare, subdominant responses seen against L antigens. This hierarchy matches the falling efficiency of epitope presentation from successively expressed antigens primarily as a result of a number of EBV encoded immune evasion proteins (Pudney et al., 2005, Croft et al., 2009, Quinn et al., 2014). These evasion proteins reviewed in Ressing *et al* (Ressing et al., 2015), affect how newly synthesized peptides are transported into the ER and loaded onto MHC class I molecules as well as affecting the number of HLA A and B molecules on the cell surface. Interestingly the immune evasion protein BILF1 leads to the down regulation of HLA-A and –B molecules but has been shown to have little effect on HLA-C molecules, possibly to evade NK cell recognition (Griffin et al., 2013).

In contrast to the reported immunodominance hierarchy of CD8+ T cell responses in IM, CD4+ T cell responses have been reported to be broadly reactive to proteins across the EBV lytic proteome (Long et al., 2013). This broad CD4+ T cell repertoire has been proposed to be stimulated by antigens being reprocessed and presented by dendritic cells or LCLs to T cells and it could potentially be by such a pathway that the novel CD8+ T cell responses to L expressed proteins seen in this study are being restimulated. This could be similar to what is observed during CMV infection where cross-presentation by dendritic cells is thought to be a crucial driver of the host CD8+ T cell response due to the presence of immune evasion proteins that prevent direct presentation in lytically infected cells (Busche et al., 2013, Snyder et al., 2010, Torti et al., 2011). Alternatively some of these CD4+ T cell responses could be

driven by *de novo* infection of B cells. EBV infection of B cells begins with adsorption of the virus to the B cell membrane and is then followed by viral endocytosis and fusion of the virus envelope with the endosomal or cell membrane. This allows the release of the nucleocapsid into the cytoplasm and the transport of the EBV genome to the cell nucleus (Hutt-Fletcher, 2007, Mautner and Bornkamm, 2012, Feederle et al., 2006). Therefore EBV glycoproteins and tegument proteins may access the endosomal compartment for degradation and loading onto MHC class II molecules whereas the capsid proteins may be degraded by cytoplasmic proteases (Blum et al., 2013).

In this chapter HLA-A*02.01 and HLA-B*07.02 restricted epitopes from the major capsid protein encoded by BcLF1 were identified using online prediction software. Attempts were made to map epitopes to other proteins that were of interest from chapter 4 however the few peptides that scored highest on the prediction algorithms and were tested here did not elicit any responses. This is a limitation with attempting to predict epitopes and why previous studies have tended to test large numbers of peptides. For example one study by Oseroff *et al* (2005) used more than 6000 *in silico* predicted peptides spanning 258 VV antigens and only 48 peptides from 35 VV antigens were identified as immunogenic (Oseroff et al., 2005). Therefore a more suitable approach could be to take the proteins that were of particular interest in chapter 4 and to generate overlapping peptides that span these proteins. This approach would still only focus on a subset of proteins and would be expensive. Other strategies detailed in the introduction could also be attempted to identify the immunogenic epitope.

Tetramers were subsequently generated that contained either the HLA-A*02.01 or B*07.02 epitopes and were used to measure the frequency of reactive CD8+ T cells in relation to responses to other EBV lytic proteins in IM patients, post-IM patients and healthy virus

carriers. Whilst these results largely confirmed previous studies (Hislop et al., 2007b) the frequency of HLA-A*02.01 restricted ALI-specific CD8+ T cells showed different kinetics and increased following resolution of IM though were still at a lower frequency compared to the GLC-specific CD8+ T cells. Interestingly the B*07.02 restricted RPS-specific CD8+ T cell frequency followed the previously observed kinetics and decreased post-IM. Additionally, many of the donors that were screened for responses in chapter 4 were also screened for *ex vivo* responses to the A*02.01 epitopes GLC, FLD, ALI, or the B*07.02 epitopes RPR and RPS in figure 5.4. and with individual frequencies shown in table 5.1. For all the donors examined using the A*02.01 epitopes, the frequency of reactive CD8+ T cells to GLC and ALI were disproportionate to the response sizes observed in chapter 4. In the *ex vivo* screens, GLC had the highest frequency of reactive CD8+ T cells whereas the frequency of ALI-specific T cells was lower. However in chapter 4 the HLA-A responses to BcLF1 were always larger than the responses to BMLF1. This could suggest that the ALI epitope is not the dominant epitope in these lines as this effect was observed both in the lines from the IM patients and in the lines from the healthy donors. In contrast to this, the *ex vivo* frequency of RPS-specific CD8+ T cells did appear to match the responses observed in HD2 and in IM217. However the B*07.02 restricted responses observed in chapter 4 were typically subdominant to other HLA-B restricted responses such as from B*15.01, B*35.01 and B*40.01 and so the newly observed inflation of CD8+ T cell responses to BcLF1 may still hold true for these other HLA-B allotypes. Unfortunately despite efforts to map epitopes to these responses none of the predicted peptides that were tested elicited a response. Therefore this observation with the ALI-specific T cell response may just be a unique feature of the HLA-A*02.01 restriction. It has been shown that some EBV epitopes can access the ER in a TAP-independent manner due to the high hydrophobicity of these peptides (Lautscham et al.,

2003). This has been shown for HLA-A*02.01 epitopes such as GLC from BMLF1 and may be a feature of other A*02.01 peptides.

To test what might be driving the increase in frequency of ALI-specific responses following primary infection, CD8+ T cell clones specific for the ALI and RPS epitopes were generated and were tested alongside other established EBV lytic CD8+ T cell clones for recognition of different WT, BZLF1-KO and antigen-loaded LCL targets. None of the CD8+ T cell clones asides from those specific for epitopes from BNRF1 were able to recognise the non-lytic LCLs and therefore it would appear that these other proteins are expressed solely during lytic replication. Previous studies have shown that CD8+ T cell clones specific for IE and some E proteins recognise HLA matched lytic LCL far better than CD8+ T cell clones specific for L proteins, though recognition can be recovered through the selective knockdown of immune evasion protein expression (Quinn et al., 2014). This previous study used GLC (BMLF1) and FLD (BALF4)-specific CD8+ T cell clones, which were also used in this study and showed similarly that the GLC-specific T cell clones recognised the lytic LCL targets efficiently whilst the FLD-specific T cell clones did not. However in this study, while there was poor recognition of the LCL targets by the T cell clone specific for the epitope from the other L protein BALF4, there was good recognition of lytic LCLs by CD8+ T cell clones specific for the L-expressed proteins BcLF1 (ALI and RPS) and BFRF3 (TPS). This is interesting as both L proteins recognised were capsid proteins whilst the other L protein BALF4 is a glycoprotein (gB). This could represent the different cellular compartments that these proteins are found in and the availability of antigen to be processed and presented (Henson et al., 2009, Neuhierl et al., 2002). Alternatively the MCP (BcLF1) has been shown to be one of the most abundant proteins in the EBV virion along with the MTP (Major tegument protein, BNRF1) (Johannsen et al., 2004). Along with the repertoire of responses seen in chapter 4, these responses could

be driven by cross-presentation though this has only previously been suggested for CD4+ T cell responses to these antigens (Mautner and Bornkamm, 2012). Attempts were made to investigate the role of cross-presentation through T cell recognition of a matched non-lytic LCL after antigen transfer with a mismatched lytic LCL (Adhikary et al., 2007). However these experiments were unsuccessful in our hands possibly due to a low proportion of cells undergoing lytic cycle and there being insufficient antigen released. Alternatively by loading non-lytic LCL targets with a high MOI of irradiated, gradient-purified EBV particles, good recognition of target cells could be achieved by ALI-specific T cells but also to a lesser degree by TPS and RPS-specific T cells. This shows that EBV virion antigens can be taken up by LCLs and cross-presented to virion-specific T cells. Additionally activated B cells that express co-stimulatory molecules are capable of priming CD8+ T cell responses through this pathway (Castiglioni et al., 2005, Schildknecht et al., 2007). This observation of cross-presentation is probably mediated through attachment and endocytosis of the purified EBV particle and therefore solely using purified BcLF1 protein would unlikely mediate recognition by virion-specific CD8+ T cells. Another experiment would be to check whether virion-specific CD8+ T cells such as those towards BcLF1 (ALI and RPS) or BFRF3 (TPS) would be able to recognise *de novo* infection of B cells as this could present a therapeutic method to prevent or limit the spread of EBV infection in B cells and would also use a physiological level of virus.

Aside from the therapeutic potential of these new virion-specific CD8+ T cells, identifying the determinant epitopes of the responses seen in chapter 4 could not only strengthen the hypothesis that cross-presentation is potentially driving some responses to L-expressed proteins but could also further help model the cellular response to EBV. In this chapter two immunogenic peptides were identified from BcLF1 despite numerous other restricting

responses. Identifying these other BcLF1 peptide determinants and performing similar experiments seen in this chapter could further strengthen the idea that cross-presentation has an important role in the development of T cell responses to EBV. Similar studies with peptide determinants from the other L proteins and in particular dominant targets such as the glycoprotein BBRF3 (gM) and the capsid protein BDLF1 could help address if compartment localisation could be a factor in generating these responses and could help create a model of how T cell responses to L expressed proteins are formed.

5.7. Summary

In this chapter, HLA-A*02.01 restricted CD8+ T cell responses to the late expressed protein BcLF1 have been shown to increase in frequency following IM using MHC class I tetramers. CD8+ T cell clones generated from this epitope were capable of recognising HLA matched lytic LCL targets along with purified virus loaded HLA matched non-lytic LCL targets. These responses are in contrast to other late-expressed proteins along with previous observations that late-expressed proteins are poor targets of the CD8+ T cell response. However the observations in this thesis that abundant late proteins such as BcLF1 can be recognised efficiently by CD8+ T cell clones suggests that some CD8+ T cell responses to EBV proteins could be driven through cross-presentation. These observations are in line with the broad repertoire of CD4+ T cell responses across the lytic proteome and the immunodominance of CD4+ T cell responses to virion proteins that together are thought to be driven by cross-presentation (Long et al., 2013, Adhikary et al., 2006, Mautner and Bornkamm, 2012).

Chapter 6

Final Discussion

During these studies a new protocol was developed for stimulating EBV-specific CD8+ T cell responses within healthy virus carriers using autologous monocyte-derived dendritic cells. Many different DC subsets have been investigated, predominantly in mice, for their ability to cross-present different forms of antigens (Shortman et al., 1997, Joffre et al., 2012). In this study *in vitro* generated moDCs along with blood PDCs were tested for their abilities to cross-present virus infected cell lysates to autologous CD8+ T cells from healthy virus carriers. Both subsets were capable of inducing a strong expansion of virus specific CD8+ T cells within IL-2 driven polyclonal lines. However DC subsets like PDCs and myeloid DCs occur at low frequencies within the blood and are therefore difficult to enrich to an appropriate purity and to get sufficient numbers to perform the experiments. Additionally DCs can be difficult to handle in culture as even gentle manipulation may result in their maturation (Pierre et al., 1997, Salskov-Iversen et al., 2005). Therefore *in vitro* generated moDCs were chosen as they can be differentiated from monocytes, which occur at frequencies of around 10% of PBMCs and can be enriched to a high purity. The phenotype of these differentiated cells could be scrutinised carefully using a panel of cell surface markers for HLA class I and II molecules, co-stimulatory molecules and adhesion molecules. Maturation could be shown after culture with either inflammatory cytokines or TLR agonists. Both conditions were capable of inducing a mature phenotype, however TLR agonists were chosen for all further studies. This was due to studies that have shown that DC maturation cocktails containing TLR agonists rather than inflammatory cytokines are capable of driving potent Th1 responses that can aid CTL expansion (Langenkamp et al., 2000, Jensen and Gad, 2010).

In this study an EBV antigen source was generated from the HEK293-p2089 cell line that contains the EBV bacmid p2089 and by trans expression of BZLF1 could drive lytic gene expression (Delecluse et al., 1998). The cells were harvested 48-hours after transfection of BZLF1 with a high proportion of cells expressing a late expressed glycoprotein (gp350) and therefore indicating that the cells had transitioned through the lytic cycle. However due to the lack of availability of antibodies to all lytic proteins the presence of every lytic protein could not be determined. Alternative antigen sources such as virus antigens from lysates of cells ectopically expressing each EBV lytic gene could be used though this would likely be very time consuming. Alternatively a method by Milosevic and colleagues used an approach where they expressed fragments of the EBV genome in bacterial cells and fed these cultures to non-lytic LCLs for stimulation of EBV-specific CD4+ T cell responses (Milosevic et al., 2006). This approach could potentially be applied to stimulating CD8+ T cell responses by using DCs as the APC though the expression of gene fragments in bacteria would miss post-translational modifications of whole proteins (Yewdell, 2006) and could potentially induce high background responses of activated CD8+ T cells from the use of bacterial cells (van Schaik and Abbas, 2007).

The approach used in this study to stimulate EBV-specific CD8+ T cell responses was similar to one used to examine CD8+ T cell responses to HSV-1 (Jing et al., 2012). In that study the authors generated immature moDCs and loaded them with apoptotic HSV-1 infected HeLa cells. No additional maturation stimulus was supplied, presumably relying on the presence of DAMPs and PAMPs within the antigen prep as apoptotic cells have been shown to be particularly immunogenic for cross-presentation by dendritic cells (Blachere et al., 2005). These antigen-loaded moDCs preps were then co-cultured with donor CD8+ T cells for 20 hours and then enriched by FACS for the expression of CD137. However in the present work

studying EBV responses, CD137 expression was not detected on GLC-specific CD8+ T cells until 48 hours post-stimulation with antigen-loaded moDCs. The proportion of GLC-specific CD8+ T cells expressing CD137 then increased 72 hours post-stimulation with antigen-loaded moDCs. This delay in activation could be the result of the route of antigen delivery with delivery of soluble antigen showing delayed kinetics of cross-presentation (Schnurr et al., 2005). Therefore optimising this protocol for different systems is important and whilst the authors of the HSV-1 study demonstrated that they could enrich for HSV-1 specific CD8+ T cell populations after 20 hours using a potentially more immunogenic antigen source, a longer co-culture time was required in this system with EBV.

This study also takes advantage of the availability of MHC class I tetramers within our lab that meant certain T cell populations could be easily quantified in polyclonal cultures. Initial studies of testing antigen processing and presentation by dendritic cells looked for recognition and effector function by CD8+ T cell clones. However we were unable to detect clear recognition of the antigen-loaded DCs by specific CD8+ T cell clones. MHC class I tetramers were then tested and were crucial in showing that co-culture with antigen-loaded moDCs led to the stimulation and expansion of specific antiviral CD8+ T cells. However the reasons for why we were unable to detect recognition using T cell clones was unclear but a possibility is that the CD8+ T cell clones were not incubated with the antigen-loaded moDCs for a sufficient amount of time or that the IFNγ-capture ELISA was not sensitive enough.

A particular strength of this work was the generation of the complete cDNA library of EBV lytic proteins that can be used to screen responses in polyclonal lines generated from either IM patients or healthy virus carriers. From this work, the primary CD8+ T cell response to EBV has been shown to be more diverse than previously thought due to the ability of being able to screen for responses to every EBV lytic protein. This study has identified new

dominant responses particularly to the E proteins BaRF1 and BBLF2/3 as well as others. Several novel responses to late expressed proteins were also identified, though in the IM patients, these responses were typically much smaller and reconciles well with the previously observed pattern of immunodominance for IM patients with IE and E proteins the most frequently targeted (Pudney et al., 2005). However in the T cell lines derived from healthy donors there was observed dominant responses to some select late expressed proteins such as BcLF1, BDLF1 and BNRF1 and so it was postulated that these responses may develop over time as was suggested by studies of Rhesus lymphocryptovirus infection (Orlova et al., 2011).

In chapter 5 MHC class I tetramers were used that enabled the quantification of *ex vivo* responses to the newly identified A*02.01 and B*07.02 epitopes from BcLF1 (ALI and RPS) in parallel with previously well-studied epitopes from BMLF1 (GLC), BALF4 (FLD) and BaRF1 (RPR). Examining *ex vivo* responses to the BMLF1 encoded GLC-epitope were relatively strong in healthy donors while weaker responses were found to the GLC-epitope in DC stimulated lines from these donors. By contrast while *ex vivo* responses to the BcLF1 encoded ALI-epitope were present, surprisingly strong A*02.01 restricted responses to BcLF1 expressing cells were detected in lines from these donors. Why this disparity occurs is not immediately clear but may relate to their being T cell specificities to epitopes other than ALI derived from BcLF1 presented to cognate T cells by A*02.01. To resolve this, using libraries of overlapping peptides to map epitopes, including those we were unable to predict for B15 and B35, may be required. However it was shown in 3 out of 4 IM donors that the frequency of ALI-specific CD8+ T cells increased in frequency one year after disease resolution suggesting that responses may evolve differently compared to responses to the other L protein BALF4 (FLD). Responses to BcLF1 in chapter 4 were also observed to be restricted through a wide range of HLA-A, B and C molecules and it would be interesting to examine the *ex vivo*

frequency of responses to these epitopes to confirm whether responses to BcLF1 do expand over time. It would also be interesting to identify the immunogenic epitopes for all of the responses identified in chapter 4 and use them in combination with MHC multimers to examine the frequency of responses in EBV seropositive donors. A combinatorial method of using a large number of MHC multimers labelled with different fluorochromes has been developed to examine different T cell populations in parallel and could be used here to further examine the immunodominance hierarchy in healthy donors in an unbiased manner (Andersen et al., 2012). Additionally, identifying epitopes to a wide range of proteins could also discover new cross-reactive epitopes that can be broadly recognised across the herpesvirus family as has been shown to one epitope from the early expressed BaRF1 protein (Chiu et al., 2014).

During an acute primary virus infection there is a large expansion of virus-specific CD8+ T cells as is seen in patients with IM (Hislop et al., 2007). Following resolution of primary infection the majority of expanded cells die by apoptosis and the remaining small number of virus-specific CD8+ T cells enter the long-lived memory compartment. However this contraction of the primary response may not be equal for each epitope as responses to some EBV lytic and latent antigens appear to be disproportionately culled following resolution of IM (Hislop et al., 2002a). Therefore the pattern of immunodominance observed during IM may not reflect that seen in persistence. Indeed responses to the latent protein EBNA3 show different kinetics to those seen against lytic proteins and actually increase in frequency following resolution of IM (Hislop et al., 2002). Similarly this is seen for CD4+ T cell responses to another latent protein EBNA1, which are absent during primary infection and may arise several months after (Long et al., 2013). In the present study novel responses to the L protein BcLF1 were shown to increase over time following resolution of IM. Other proteins that were shown to be subdominant components of the CD8+ T cell response in IM patients

such as the L proteins BDLF1 and BNRF1 were shown in some healthy virus carriers to be dominant components. The tegument protein BNRF1 does show some expression during latency as was observed in this work and in others (Abbott et al., 2013). However CD8+ T cell responses to the minor capsid protein BDLF1 was not studied further and could potentially in some individuals show a similar evolution as is seen for the responses to the major capsid protein BcLF1. CD8+ T cell clones generated from BcLF1 and BDLF1 were shown not to recognise non-lytic LCLs whereas they could recognise lytic LCL targets (Fig. 5.6. and data not shown). This suggests that these L proteins are not expressed during latency, unlike BNRF1, and potentially could be being reprocessed through receptor-mediated uptake by and presentation by semi-permissive LCLs. This pathway was suggested by Adhikary *et* al (2007) for driving CD4+ T cell responses to EBV structural proteins such as BcLF1 and BDLF1 amongst others in LCL-stimulated T cell lines (Adhikary et al., 2006, Adhikary et al., 2007). Therefore CD8+ T cell responses to late lytic structural proteins could be being driven or at least maintained by low-grade chronic antigen stimulation through this pathway. Additionally there is a role for dendritic cells scavenging free virus particles or infected cells and stimulating responses through cross-presentation.

The observed pattern of immunodominance in IM patients does suggest that the antiviral CD8+ T cell response is driven by antigen processed and presented by infected B cells during primary infection of EBV (Pudney et al., 2005). CD8+ T cells are typically primed in secondary lymphoid organs by dendritic cells that provide additional signals of co-stimulation and inflammatory cytokines. However activated B cells have also been shown to be capable of priming CD8+ T cell responses and as EBV infection can drive the activation of B cells this may be the mechanism for how the initial CD8+ T cell response is elicited (Castiglioni et al., 2005, Calender et al., 1987). IM patients are typically only diagnosed once they display

symptoms which can be several weeks after initial infection and therefore it isn't known whether the primary response is being primed by DCs and then the later observed expansion of CD8+ T cells is being driven by lytically infected B cells. During viral persistence there appears to be a shift in this immunodominance hierarchy with a number of late-expressed structural proteins providing the dominant targets as shown in this study. This may therefore represent an alternative route of antigen processing and presentation such as cross-presentation by dendritic cells. The route of antigen presentation could be resolved by examining responses in patients with X-linked lymphoproliferative disease (XLP) who have become infected with EBV. EBV infection in these patients results in an exaggerated IM-like disease that is often fatal and is a result of the inability of T cells to recognise B cells (Hislop et al., 2010). Therefore examining responses to these late lytic structural proteins in XLP patients could be used to determine whether these responses are being driven by B cells or by DCs as only DCs will be competent to stimulate EBV-specific T cell responses.

This proteome wide approach using donor HLA molecules employed in this study did lead to the identification of at least one HLA-A*01.01 restricted epitope that was then used to isolate a CD8+ T cell clone. HLA-A*01.01 expression is a risk factor for developing EBV-associated Hodgkin lymphoma (HL) which is also coincidental with the lack of any identified HLA-A*01.01 epitope from an EBV protein (Huang et al., 2012). The A*01.01 epitope identified here had previously been identified by mass spectroscopy analysis of EBV-transformed LCLs though no T cell response has previously been ascribed to this epitope (Kinch et al., 2016). This A*01.01 T cell clone was specific for an epitope derived from the late expressed protein BBRF1 and had poor functional avidity in a peptide titration assay and was not studied further. Another putative A*01.01 response was observed for the early expressed protein BORF2 and though this response was not studied further here, it is interesting that this protein

also had an A*01.01 epitope assigned to it through mass spectroscopy analysis (Harndahl et al., 2011) and is accessible on the IEDB website (see materials and methods). It would be interesting to examine the frequency and function of these specificities in healthy donors and Hodgkin lymphoma patients to determine if there are differences between these two groups correlating with the development of disease.

In this thesis a novel method has been developed to stimulate and expand EBV-specific CD8+ T cells from the blood of healthy virus carriers and to screen these T cell lines for reactivity against all EBV lytic proteins and across each of the donors HLA class I alleles. This is also the first time that a proteome wide approach has been applied to a complex virus such as EBV to examine CD8+ T cell responses during primary infection. This approach has developed our understanding of the cellular immune response to EBV and has identified numerous novel responses from all phases of the EBV lytic cycle, whilst largely supporting previous observations of an immunodominance hierarchy during primary infection. The approach also identified novel responses to a subset of EBV late expressed proteins that do not conform to previous observations and may provide evidence for a role of virus-antigen reprocessing by virus infected B cells and stimulation of T cell responses to virion components. However the next step would be to examine whether CD8+ T cell clones specific for EBV virion proteins are capable of recognizing *de novo* infected B cells using more physiological levels of virus. If this was possible it could represent a novel therapeutic target for limiting the spread of EBV virions to neighbouring cells within the body by targeting the infected B cell before viral replication can occur.

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