EBV immune evasion genes modulating $CD8^+$ T cell recognition during lytic cycle replication

By

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Abstract

During lytic cycle replication EBV expresses at least three genes; BNLF2a, BILF1 and BGLF5, which individually act to inhibit efficient processing and presentation of $CD8^+$ T cell epitopes. This thesis sets out to assess the relative contribution of these potential immune-modulating proteins to the evasion from $CD8^+$ T cells at different stages of EBV lytic cycle. Lentiviral vectors for shRNAs were used to silence expression of these individual viral genes in EBV-transformed B-cells, which were then probed with $CD8^+$ T cell effector clones of specificities for epitopes derived from the three phases of the EBV lytic cycle; allowing us to determine the contribution each immune evasion gene makes towards the inhibition of antigen presentation during lytic cycle.

Cells replicating viruses lacking BNLF2a were more efficiently recognised by $CD8^+$ T cells specific for immediate early and early expressed antigens relative to those lacking BGLF5 and BILF1. Conversely, cells lacking the expression of BILF1 were better recognised by $CD8^+$ T cells specific for early and late lytic antigens. These data suggest that whilst the role BNLF2a plays in interfering with antigen presentation diminishes as lytic cycle progresses (IE>E>>L), BILF1 plays a more active role with the progression of lytic cycle (IE<E<<L).

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Publications arising from this thesis

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

Misstear K, Chanas SA, Rezaee SA, Colman R, Quinn LL, Long HM, Goodyear O, Lord JM, Hislop AD, Blackbourn DJ. (2012). Suppression of antigen-specific T cell responses by the Kaposi's sarcoma-associated herpesvirus viral OX2 protein and its cellular orthologue, CD200. J Virol. 86(11):6246-57

Abbott RJM, Quinn LL, Leese AM, Scholes H, Pachnio A and Rickinson AB. (2013). CD8+ T cell responses to lytic Epstein-Barr virus infection: late antigen specificities as subdominant components of the total response. J Immunol. (Accepted)

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List of Abbreviations

ABC	ATP-binding cassette
AP-1	Activator protein-1
APCs	Antigen presenting cells
ATP	Adenosine triphosphate
BCR	B cell receptor
BHV	Bovine herpersvirus
CAEBV	Chronic active EBV
CR	Central repeat
Cr	Cr-sodium-chromate (Cr)
DCs	Dendritic cells
Dox	Doxycycline
DRiPs	Degradation of defective ribosomal products
E	Early
EBNA	EBV nuclear antigen
EBV	Epstein Barr Virus
EHV	Equine herpesvirus
ELISA	Enzyme linked immunosorbant assay
ENKTL	EBV-associated NK/T cell lymphoma
ER	Endoplasmic reticulum
ERRAP	ER-associated aminopeptidase

Abbreviations

FasL	Fas-ligand
FCS	Foetal calf serum
FS	Forward scatter
Gly-Ala	Glycine-alanine
HC	Heavy chain
HCMV	Human cytomegalovirus
HHV	Herpesvirus
HLA	Human leukocyte antigens
HPLC	High performance liquid chromatography
HSV	Herpes simplex virus
HuS	Human serum
IE	Immediate early
IFN	Interferon
IM	Infection mononucleosis
KSHV	Kaposi’s sarcoma
L	Late
LCL	Lymohoblastoid cell lines
LMP	Latent membrane proteins
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complexes
miRs	MicroRNAs
NBD	Nucleotide binding domain

Abbreviations

NFkB	Nuclear factor-kB
NK	Natural killer
NLRs	NOD-like receptors
NOD	Nucleotide oligomerisation domain
OriP	Origin of viral replication
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PHA	Phytohaemagglutinin
PLC	Peptide loading complex
PRR	Pattern-recognition receptors
PRV	Pseudorabies virus
PTLD	Post transplant lymphoproliferative disorder
qRT-PCR	Quantitative real-time polymerase chain reaction
RACE	Rapid amplification of the cDNA ends
RIG-I	Retinoic acid-inducible
RLRs	RIG-I- like receptors
shBGLF5-LCLs	B95.8 LCLs transduced with lentivirus expressing shRNA-BGLF5
shBILF1-LCLs	B95.8 LCLs transduced with lentivirus expressing shRNA-BILF1
shBNLF2a-LCLs	B95.8 LCLs transduced with lentivirus expressing shRNA-BNLF2a
shRNA	Small hairpin RNAs
siRNA	Small interfering RNA
TAP	Transporter associated with antigen processing

Abbreviations

TCR	T cell receptors
TLR	Toll-like receptors
TNFR	Tumour necrosis factor receptor
TOP	Thimet oligopeptidase
TPA	12-O-tetradecanoylphorbol-13-acetate
TPPII	Tripeptidyl peptidae II
TRAM1	Translocating chain associated membrane protein 1
Ub	Ubiquitin
wt-2089	2089 EBV strain
ZREs	Z-responsive elements
ΔBZLF1	BZLF1 deleted recombinant EBV strain
ΔBGLF5	BGLF5 deleted recombinant EBV strain
ΔBILF1	BILF1 deleted recombinant EBV strain
ΔBNLF2a	BNLF2a deleted recombinant EBV strain
β2m	Beta-2-microglobin

CHAPTER 1

INTRODUCTION

1.1. **The host response to viral infection**

Viruses are obligate intracellular infectious agents that hijack the cellular machinery of their hosts in order translate viral proteins and replicate their genome. As a consequence of this, they are exposed to a plethora of host immune controls; a pressure under which they need to survive. The course of infection differs greatly between viruses; some viruses infect a host, rapidly replicate and then transmit, so that the pressure to survive long term in the harsh immune environment is less important. Other viruses, such as those in the herpesviridae family, establish a persistent infection which is often asymptomatic, although occasional reactivation can occur. The requirement of a functional immune system is essential in order to control viral infections and maintain a healthy host. However, since viruses have been co-evolving with their hosts for many years, some have adopted strategies to modulate the host immune responses. This interplay between the immune response of the host and immune modulation by the virus is essential for the maintenance of a lifelong viral infection of a healthy host.

There are two arms to the human immune system, both of which play important roles in responding to viral infection. These are the innate and adaptive immune system and will be discussed in more detail below.

1.2. **The innate immune response to viral infection**

In the first stages of viral infection, it is the innate immune system which is most important in order to detect incoming viruses, and is necessary to activate the adaptive immune response. The innate immune response is activated via detection of viral components through pattern-recognition receptors (PRRs) (Akira *et al.*, 2006). PRRs detect components including DNA, single and double stranded RNA and viral proteins. There are three classes of PRRs which are known to detect these components in innate immune cells, toll-like receptors (TLR), retinoic acid-inducible (RIG-I)- like receptors (RLRs) and nucleotide oligomerisation domain (NOD)-like receptors (NLRs). Following the detection of viral components, these receptors activate intracellular signalling cascades, resulting in the secretion of type I interferons (IFN), proinflammatory cytokines and chemokines. Type I IFNs active intracellular signalling pathways involved in inducing the apoptosis of infected cells, and priming uninfected cells for resistance to incoming viral infection. Proinflammatory cytokines and chemokines are important for inducing inflammation and recruiting other immune cells to the site of infection (reviewed by (Takeuchi and Akira 2009). These responses are important for activating the adaptive arm of the immune system in the host.

1.3. **The adaptive response to viral infection**

The adaptive immune system protects the host from viral infection through more specific responses and leads to the generation of immunological memory. It is comprised of two arms; the humoral, which is responsible for the production of antibody by B cells; and cell-mediated immunity in which T cells play a central role. Classically, B cells detect and bind their specific antigens, which tend to be located in the extracellular environment, via their cell surface B cell receptor (BCR). This engagement of BCR with antigen can lead to the formation of plasma cells which then secrete specific antibodies. By binding to epitope on antigens, antibodies can confer viral immunity in three ways: neutralise viral infection by binding to them; opsonise viral particles, leading to their elimination; activation of the complement system; and opsonisation of infected cells to mediate antibody dependent cell mediated cytotoxicity.

Cell mediated immunity involves T cells, which are able to recognise specific peptide epitopes via their T cell receptors (TCR). These peptide epitopes are derived from the degradation of intracellular proteins, which may have originated from the extracellular or intracellular environment. The products of protein degradation are then presented on the cell surface by major histocompatibility complexes (MHC), also known as human leukocyte antigens (HLA), in humans. Upon recognition of these MHC:peptide complexes, T cells which have already experienced their antigen then elicit an effector function such as cytotoxicity of the target cell, whereas naïve T cells, that have not previously experienced their antigen, are primed to become effector cells.

T cells are further subdivided into two categories, depending upon their surface marker expression; those which express CD4 recognise peptides presented by MHC class II molecules, whereas those expressing CD8 recognise peptides presented by MHC class I molecules. This thesis is focussed on the interplay between EBV infection and $CD8^+$ T cell responses. There follows, therefore, a review of the molecular and cellular components of $CD8^+$ T cell responses, and of the relevant aspects of EBV biology.

1.4. **The MHC class I antigen processing pathway**

MHC class I molecules are found on nearly every cell of the body. They are heterodimers composed of an MHC heavy chain (HC) associated with a beta-2-microglobulin (β2m) and peptide. The extracellular region of the HC folds into three domains, with β2m forming the fourth. Two of the HC extracellular domains form a peptide binding groove, which bind specific peptide epitopes that are around 8-11 amino acids in length (Falk and Rotzschke 1993). The heavy chain region of MHC class I molecules, encoded by the HLA-A, -B and –C genes on chromosome 6, are highly polymorphic, in that heterozygous individuals can express up to 6 different alleles. Since it is the sequence of the peptide binding groove which dictates the peptides that bind, this allows for a high diversity of epitopes which can be presented on MHC class I molecules. In addition, the peptide binding groove interacts strongly with only a few amino acid side chains of peptides, therefore further increasing the repertoire of peptides that can be presented.

Assembled MHC class I molecules are loaded with peptide in the lumen of the endoplasmic reticulum (ER) and then exported to the cell surface via the Golgi apparatus. Once at the cell surface, MHC:peptide complexes are accessible for probing by circulating $CD8^+$ T cells (Fig 1.1).

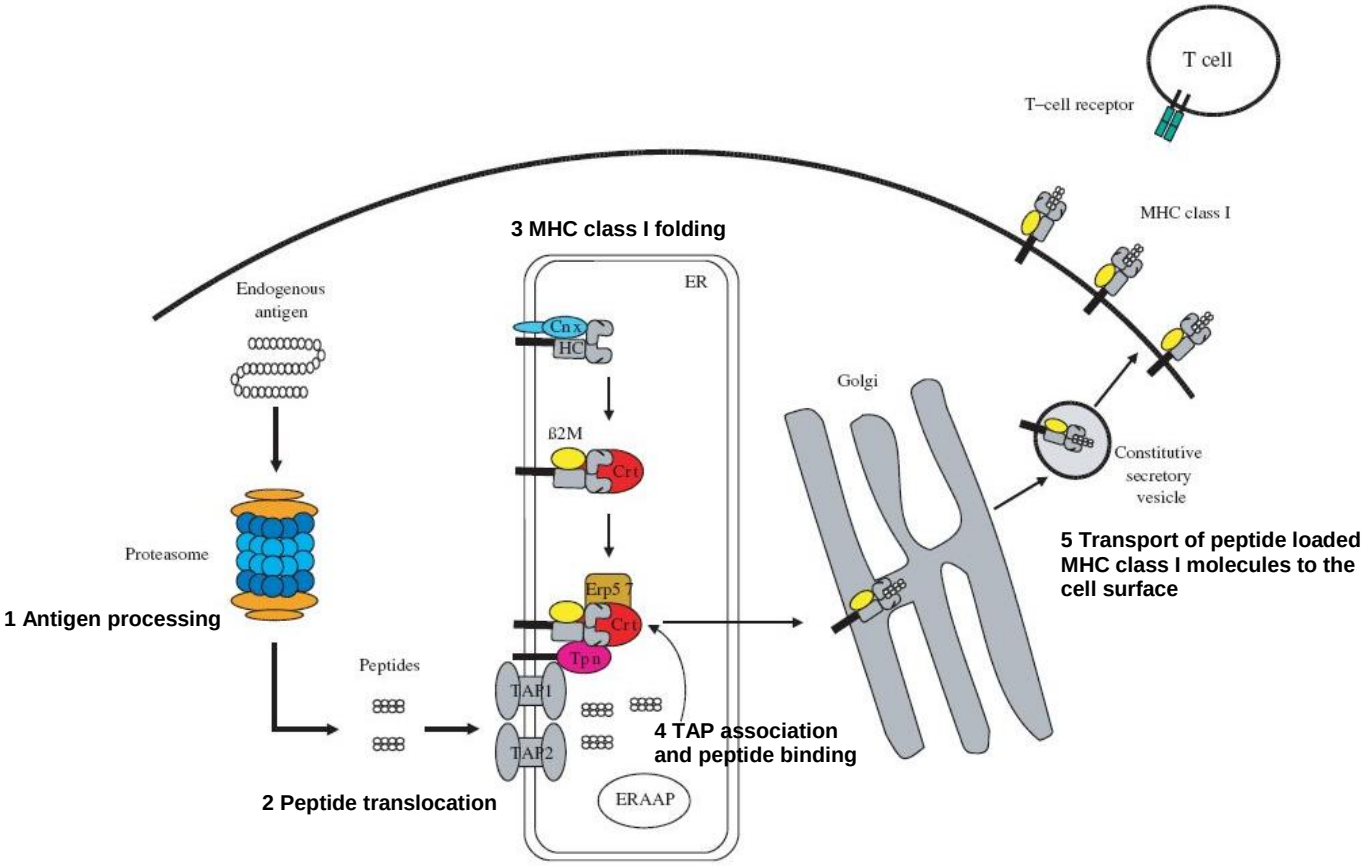


Figure 1.1 MHC class I antigen processing pathway. MHC class I antigen presentation pathway.

(Adapted from (Danchin *et al.*, 2004)). (1) Proteins are degraded in the cytosol by proteasomes into short peptides. (2) Peptides are then translocated into the ER lumen by TAP. (3) Newly synthesised MHC class I HCs and β_2m fold and assemble in the ER lumen with the aid of calnexin, calreticulin and ERp57. (4) MHC class I molecules associate with TAP and tapasin facilitates peptide binding. (5) Peptide loaded MHC class I molecules dissociate from TAP and are transported through the secretory pathway to the plasma membrane where they are displayed at the cell surface to circulating $CD8^+$ T cells.

1.4.1. Degradation of proteins and generation of peptide epitopes

Peptide epitopes in complex with MHC class I molecules originate from degraded intracellular proteins. The source of these peptides has been an area of conflicting opinions for many years. One school of thought, proposed by Rock *et al.* is that the major source of peptides is derived from the degradation of pre-existing functional proteins. This involves the ubiquitination of proteins, by ubiquitin (Ub) ligase enzymes, most commonly at lysine residues. Once one Ub has been tagged, this then signals other ligases to attach additional Ub molecules at the lysine residues within the previous Ub. The presence of at least four Ub molecules marks the protein for proteasomal degradation (discussed below).

In 1996 it was proposed by Yewdell *et al.* that the majority of peptides are not derived from pre-existing protein degradation, but instead come from the rapid degradation of defective ribosomal products (DRiPs) (Yewdell *et al.,* 1996). DRiPs are thought to occur when; mRNA translation is terminated early; there is a ribosomal frame shift during translation or; from the degradation of newly translated misfolded proteins. It since has been found that around 30% of mRNA translation products are rapidly degraded DRiPs and that the presence of peptides corresponds to the level of mRNA rather than the steady state level of full-length protein (Schubert *et al.,* 2000; Khan *et al.,* 2001; Qian *et al.,* 2005). DRiPs are now widely believed to contribute a larger pool of peptide than the degradation of stable protein. A number of studies have been carried out that show that DRiPs likely are the major source of peptides presented on MHC class I. For example Mackay *et al* showed that upon inducing the expression of a viral protein encoded by Epstein Barr Virus (EBV), there is a rapid increase in peptide display and thus T cell recognition, before there is steady state of protein expression (Mackay *et al.,* 2009). In contrast, when de novo expression of viral protein was suppressed,

there was a rapid decline in peptide display and T cell recognition, despite the presence of mature protein (Mackay *et al.*, 2009). In agreement with his finding, a more recent study has shown, using a different model, that the production of peptides occurs well before there are detectable levels of the corresponding protein, and moreover, the insertion of a premature stop codon in this mRNA still yielded high levels of antigenic peptide, despite the lack of protein production (Apcher *et al.*, 2011). These findings support the idea that it is DRiPs which serve as the major source of peptides available for MHC class I loading. In terms of responses to viral epitopes this is perhaps more advantageous since viral protein translation occurs rapidly, before the accumulation of stable protein, and since there is a high error rate in the translation process, it is likely an ideal source for rapid peptide supply for presentation to $CD8^+$ T cells and subsequent recognition of infected cells.

These proteins, whether functional or defective, are then degraded by the cellular proteasome (Goldberg *et al.*, 2002). In humans, the 26S proteasome is more commonly used. This is composed of a hollow 20S core and capped at each end by a 19S complex. The core is comprised of four symmetrical rings, which are themselves made up of α- and β-subunits; these rings form a hollow cylinder with ends that can be opened to allow entry of substrates. It is the 19S subunits which recognise Ub tagged proteins and unfold them to allow access to the core. The β subunits within the inner two rings of the core contain peptidase sites, which act to cleave proteins at specific sites, creating a pool of diverse peptides (Kloetzel 2004). Cells are also capable of producing immunoproteasomes, in which there is the replacement of the constitutive subunits for the IFN-γ induced homologous β-catalytic subunits (LMP-2, -7 and -10) (reviewed, (Yewdell 2005)). Immunoproteasomes carry different proteolytic sites and as a result generate a different diversity of peptides, thereby increasing the repertoire of peptides presented on MHC class I molecules. Immunoproteasomes are expressed constitutively in

immune tissues, however their production can be increased upon exposure to IFN-γ, which is released upon detection of viral infection. It widely believed that the immunoproteasome does not completely replace the proteasome pool, and that it is the expression of both within the same cell that provides a greater repertoire of peptides for MHC class I presentation.

Following proteasomal degradation of proteins, peptide products are released into the cytosol where they may be subject to further trimming by cytoplasmic peptidases. Products which are greater than 4-6 residues in length are trimmed predominantly by two peptidases, tripeptidyl peptidae II (TPPII) and thimet oligopeptidase (TOP). TPPII cleaves three residues at a time from the N-terminus of substrates and appears to prefer peptides that are more than 15 residues in length (Reits *et al.,* 2004), which will account for a small amount of the proteasomal degradation product (York *et al.,* 2006). It has been found that silencing the expression of TPPII does not decrease the supply of peptides to the MHC class I pathway, due to only a small proportion of peptide products being more than 15 residues long (York *et al.,* 2006). TOP is considered to play the major role in the trimming of proteasomal products and it degrades peptides which are 9-15 residues in length (Kessler *et al.,* 2011), accounting for a larger fraction of the proteasomal output (Saric *et al.,* 2004). Indeed, silencing the expression of TOP results in increased presentation of peptides, while its over expression decreases peptide presentation (York *et al.,* 2003). This over expression of TOP limits the amount of peptides available for presentation by destroying them, thus limiting the supply of peptides to the ER. It should be noted that other peptidases have been identified such as an IFN-γ leucine aminopeptidase, that could trim an extended version of SIINFEKL, and was identified in the cytosol of HeLa cells (Beninga *et al.,* 1998). However, these have been studied less extensively. Following trimming, peptides are then transported into the ER where they can be loaded onto MHC class I molecules.

1.4.2. Peptide transport into the ER

Peptides are predominantly translocated into the ER via the transporter associated with antigen processing (TAP). TAP belongs to the adenosine triphosphate (ATP)-binding cassette (ABC) superfamily and is a heterodimer which spans the ER membrane. It is made up of TAP1 and TAP2 subunits, which each have an N-terminal hydrophobic transmembrane domain, containing an ER retention signal, and a C-terminal nucleotide binding domain (NBD) (Abele and Tampe 1999).

Peptide translocation across TAP begins with the binding of peptides to the cytoplasmic loops of TAP1 and TAP2, in an ATP-independent manner. These loops are located close to a hydrophobic pore, made up of the transmembrane domains of TAP1 and TAP2, through which peptides are translocated. Following peptide binding, ATP binds to and is hydrolysed by the NBD of TAP1 and TAP2, providing the energy required to transport peptides from the cytosol to the lumen of the ER (van Endert *et al.,* 1994). Interestingly, TAP2 mediated ATP hydrolysis is essential whereas TAP1 mediated hydrolysis has been found to be non- essential (Karttunen *et al.,* 2001). The length of peptides that TAP can transport ranges from 7 to 20 amino acids, although those which are 8-13 amino acids long are preferentially transported (Momburg *et al.,* 1994).

Although proteasome degradation and TAP transportation is considered the conventional route for peptide processing, other TAP-independent pathways may also contribute. Using TAP deficient T2 cells, it was noted that some EBV derived peptides are presented in the absence of TAP, indicating an alternative processing route (Lautscham *et al.,* 2001). In TAP deficient individuals MHC class I peptide loaded molecules are still displayed, albeit to a

much lower extent. It has been found that some peptides, derived from signal sequences, are presented on TAP deficient T2 cells, and are also found on normal, TAP expressing cells (Henderson *et al.*, 1992; Wei and Cresswell 1992), which would suggest that these alternative pathways may also be active in cells expressing TAP. More recently it was identified that in the absence of TAP, HLA-E molecules no longer present leader sequences and instead present a novel repertoire of peptides which are much like those peptides presented by HLA-A2 molecules (Oliveira *et al.*, 2010; Lampen *et al.*, 2013). However, cells which expressed TAP were not able to present this novel pool, indicating that perhaps there is competition between the two pathways, where the alternative pathway is only present in the absence of TAP. Although the evidence points towards an alternative antigen processing pathway, the mechanism has yet to be identified. It has been proposed that there may be novel transport mechanisms via access to the vesicular pathways, which allow peptides into the ER, or that the ER may not be required and peptides may gain access on the secretory route, where they may displace poorly bound peptides and finally there is the suggestion that autophagy may play a role. Indeed, recently it was shown that an epitope derived from the HCMV encoded protein, pUL138 required autophagy for its presentation, via a TAP-independent class I processing pathway (Tey and Khanna 2012). The exact mechanisms involved here are yet to be elucidated and it is yet to be established how much of a role these alternative pathways play when TAP is present.

1.4.3. Assembly of MHC class I:peptide complexes and trafficking to the cell surface

Following translocation into the lumen of the ER, peptides are loaded onto nascent MHC class I molecules. Prior to MHC class I loading, peptides often require further trimming by the ER-associated aminopeptidase (ERAAP) enzyme (Serwold *et al.,* 2002). Interestingly the expression of ERAAP is increased in the presence of IFN-γ.

The loading of peptides onto MHC class I molecules begins with the synthesis of the HC and β2m subunits, which then enter the ER, via signal sequences. In the ER the HC rapidly associates with the ER-resident chaperone molecule calnexin (Germain and Margulies 1993). In the presence of β2m, HC subunits fold appropriately and calnexin dissociates. The loading of these MHC class I molecules relies on the peptide loading complex (PLC), which centres around TAP. Firstly, TAP recruits the HC-β2m dimer complex via the adaptor tapasin. This transient interaction is stabilised by the thiol disulphide oxidoreductase protein, ERp57 and the ER chaperone calreticulin, allowing tapasin to serve as a bridge between TAP and the MHC class I molecule (Hulpke *et al.,* 2012). In addition, tapasin functions to edit the peptides which are to be loaded, thereby ensuring optimal peptide binding (Williams *et al.,* 2002). It has recently been discovered that two tapasin molecules are present in the PLC, one bound to each TAP subunit, this is thought to maximise effective loading (Hulpke *et al.,* 2012). The blocking of tapasin interaction with TAP diminishes the level of MHC class I presentation, indicating an important role for tapasin in the PLC and antigen processing pathway (Hulpke *et al.,* 2012). Furthermore, the cell line .220, which lacks the expression of tapasin, shows defective MHC class I association with TAP, peptide loading and decreased MHC class I surface levels (Sadasivan *et al.,* 1996).

Once high affinity peptides have bound to the grove of MHC class I molecules the MHC:peptide complex dissociates from the PLC and is transported to the cell surface via the Golgi network. Some studies suggest that the MHC class I molecules may cycle out to the Golgi and be transported back for peptide loading, perhaps as a means of quality control (Garstka *et al.,* 2007). Subsequent transport to the cell surface requires the presence of cargo receptors, such as Bap31 which facilitates the trafficking (Paquet *et al.,* 2004), since MHC class I molecules lack trafficking signals in the cytoplasmic tail.

Upon arrival at the cell surface, stable MHC class I peptide loaded complexes present the endogenously generated peptides to the TCR of $CD8^+$ T cells.

1.4.4. Activation of naive $CD8^+$ T cells

During infection of the host, naive $CD8^+$ T cells become primed when they encounter their MHC class I presented antigen in the secondary lymphoid organs, such as the lymph node and, perhaps more importantly the lymph node periphery (Hickman *et al.*, 2008). Antigenic peptides are presented by antigen presenting cells (APCs) such as B cells, macrophages and dendritic cells (DCs). Although macrophages and B cells are efficient APCs, $CD8^+$ T cells appear to prefer DC presentation of their cognate antigen. DCs can themselves be infected, resulting in antigen presentation to $CD8^+$ T cells, or they may present the antigen via cross presentation by sampling antigens form the extracellular environment and processing and presenting these on MHC class I molecules, as described above. The antigen specific contact between naïve $CD8^+$ T cells and other APCs, at specific anatomical sites, results in $CD8^+$ T cell activation and expansion.

The way in which naive T cells are able to home to lymphoid tissue is via the use of chemokine receptors and integrins. Chemokines such as CCL17, which are produced by DCs, have been shown to attract naive $CD8^+$ T cells expressing the chemokine receptor CCR4 during priming (Semmling *et al.*, 2010). There is also an implied role of the chemokine receptor pair XCL1-XCR1. This receptor pair has recently been demonstrated to be important for the activation of $CD8^+$ T cells. XCR1 expression is restricted to lymphoid tissue resident DCs and the XCL1 ligand, which is a chemoattractant for the DCs, is abundantly expressed by activated $CD8^+$ T cells. The absence of XCL1 results in poor priming and expansion of $CD8^+$ T cells thus, this interaction is required for maximal priming and expansion of CD8+ T cells (Parish and Kaech 2009). Therefore there is perhaps interplay between chemokines of

$CD8^+$ T cells and DCs in order to achieve maximal recruitment of naïve $CD8^+$ T cells and antigen presenting DCs, during priming.

Following priming, naïve CD8+ T cells are induced to proliferate and differentiate into both effector and memory cells (Gerlach *et al.,* 2010), although there is controversy surrounding the fate of naive T cells, and exactly what dictates this fate. The short lived effector cells, which account for the majority of $CD8^+$ T cells in primary infection, mostly diminish once infection is cleared. However, a small proportion will remain and establish a memory pool (Parish and Kaech 2009). These memory cells can proliferate and expand rapidly in response to antigen re-encounter, which allows the immune system to respond to successive infections much faster and more efficiently.

The fate of the effector cell is very different from that of the naive. Following the recognition of their cognate antigen, activated T cells upregulate cytokine receptors which allow them to enter the peripheral tissues and home to the site of infection. Here, effector $CD8^+$ T cells will encounter and, via the TCR, will engage with cognate antigen presented by MHC class I molecules. This can result in cytolysis of infected cells and the release of cytokines, such as IFN-γ, which essentially establishes an anti-viral state in neighbouring cells by inducing the upregulation of MHC class I molecules and more. $CD8^+$ T cells also release the anti-inflammatory cytokine IL-10, as a means of regulating the severity of tissue damage (Trandem *et al.,* 2011).

1.4.5. $CD8^+$ T cell killing of infected cells

Activated $CD8^+$ T cells are able to lyse infected cells in one of two ways; the granule exocytosis pathway or; via the upregulation of Fas-ligand (FasL). Both stimulate the caspase cascade, leading to apoptosis and cell death (Shresta *et al.,* 1998). In order to minimise any destruction of neighbouring cells, $CD8^+$ T cells establish an immunological synapse, upon interaction of their TCR with peptide loaded MHC class I molecules. This synapse encloses the TCR:MHC class I complex, so that the release of cytotoxic proteins is concentrated on the target cell only, thereby minimising bystander killing and maximising cytotoxic effects on infected cells. The granule exocytosis pathway relies upon the release of cytotoxic proteins which are stored in secretory lysosome-like granules within $CD8^+$ T cells. Two types of cytotoxic proteins are stored in these granules; perforin and granzymes. Perforin exists in a monomeric form in the granules, rendering it inactive. Upon release into the immunological synapse, perforin polymerises to form transmembrane pores in membrane of the target cell. Granzymes are serine proteinases which induce the caspase dependant apoptotic pathway and it is the combined effect of perforin and granzyme which results in lysis of target cells (reviewed (Harty *et al.,* 2000)).

Although the granule exocytosis pathway is thought to account for the majority of target cell death, $CD8^+$ T cells also have a second method of killing, via expression of FasL. Upon activation, $CD8^+$ T cells upregulate FasL, which ligates to Fas on target cells and activates the caspase apoptotic pathway, resulting in target cell death.

1.4.6. Immunodominance

Although virally infected cells generate a vast number of viral peptides, only a small fraction of these actually induce a $CD8^+$ T cell response (Yewdell and Bennink 1999). This results in a hierarchy of responses known as immunodominance. A number of factors contribute to immunodominance, such as the affinity of the peptides bound to MHC class I molecules, the number of complexes at the cell surface, suppression of some responses by more dominant populations and the interference with the antigen processing pathway by viruses. This viral interference may preferentially inhibit the presentation of a subset of potential peptide antigens.

1.5. **Viral immune evasion**

As a consequence of the immune pressure exerted on viruses during infection, it is unsurprising that many have co-evolved with their hosts in order to dampen down the immune responses mounted. The mechanisms used by different viruses vary, and they target multiple immune-response pathways. However, the MHC class I antigen presentation pathway is a particularly common target, with several stages targeted by different immune modulating viral proteins.

1.5.1. Interfering with peptide production

Many viruses have evolved to limit the generation of viral peptides by limiting protein expression and degradation, thus decreasing the availability of peptides for presentation to $CD8^+$ T cells.

One such viral protein, which is able to minimise its own proteasomal degradation and production of peptide is the EBV nuclear antigen I (EBNA1) protein (Blake *et al.,* 1997; Levitskaya *et al.,* 1997; Yin *et al.,* 2003). EBNA1 encodes an extensive glycine-alanine (Gly-Ala) repeat region which was first shown to minimise EBNA1 peptide presentation by preventing proteasomal degradation (Blake *et al.,* 1997; Levitskaya *et al.,* 1997). This was originally thought to be the reason that EBNA1 was able to prevent its own presentation to T cells, although this effect of the gly-ala repeat on proteasomal degradation is now thought to be an artefact of overexpression. More recently it was shown that the role of the gly-ala region is to inhibit the initiation of EBNA1 mRNA translation (Apcher *et al.,* 2009; Apcher *et al.,*

2010). EBNA1 mRNA is purine rich, as a result of the gly-ala region, which results in a lack of secondary structure, thereby inhibiting translation and reducing antigen presentation, thus avoiding DRiP formation (Yin *et al.*, 2003; Tellam *et al.*, 2008). Moreover, the expression level of EBNA1 has been found to be relatively low (personal communication, Dr Rose Tierney). Such a small level of transcript which is poorly translated probably results in low levels of DRiP formation and small amounts of peptide available for presentation. It is possible that the effect of the Gly-Ala repeat region may come in to play to protect residual DRiPs from proteasomal degradation.

The γ-herpesvirus, Kaposi's sarcoma (KSHV) has also evolved a similar mechanism in order to evade peptide presentation of its viral genome maintenance protein LANA1. LANA1, a homologue of EBNA1, has been shown to decrease its peptide presentation via the central repeat (CR) region. This CR region differs from that of EBNA1 as it is composed of repeats rich in glutamine, glutamate and aspartate. This can be further divided into three subdomains: CR1, CR1 and CR3. Although the CR2 and CR3 regions have been shown to retard protein synthesis (Kwun *et al.*, 2007). The CR1 subdomain is now known to be primarily responsible for the prevention of peptide presentation by MHC class I, by a process which has yet to be identified, although it is known to be prior to translocation into the ER. This process is not dependent upon the inhibition of translation or on proteasomal processing (Kwun *et al.*, 2011), instead it has been suggested that this region may act to uncouple the proteasomal processing from the ER translocation machinery. Therefore, although LANA1 protein is a homologue of EBNA1, the mechanisms the two employ in order to inhibit their own peptide presentation appear to differ.

1.5.2. Inhibiting TAP-mediated peptide transport into the ER

There are numerous examples known of viruses targeting the TAP transporter as a means of interfering with antigen processing and presentation. This is likely as a consequence of the vast majority of viral peptides requiring translocation by TAP, and since the sole function of TAP is in antigen processing, TAP inhibition will perhaps have little effect on the rest of the cell.

The first discovered viral product seen to interfere with TAP was the herpes simplex virus (HSV) cytosolic protein ICP47. ICP47 was initially shown to block the transport of MHC class I molecules (York *et al.,* 1994). This protein binds to the peptide binding site of TAP with a 10-fold higher affinity than the highest affinity peptides (Tomazin *et al.,* 1996), thus acting as a competitive inhibitor of peptide binding (Ahn *et al.,* 1996; Ahn *et al.,* 1996; Tomazin *et al.,* 1996). ICP47 binding to TAP it is thought to destabilise the structure of TAP (Lacaille and Androlewicz 1998), thereby altering the conformation and inhibiting peptide binding and ATP hydrolysis. As a result there is a decrease in peptide loaded MHC class I molecules at the surface of infected cells. Where normal peptides stabilise TAP and stimulate ATP hydrolysis, ICP47 does not, and is not translocated across the membrane, remaining associated with TAP (Lacaille and Androlewicz 1998).

More recently, distinct molecular mechanisms of viral targeting of TAP was demonstrated by the UL49.5 protein encoded by the varicelloviruses; bovine herpersvirus-1 (BHV-1), pseudorabies virus (PRV) and equine herpesvirus 1 (EHV-1) and 4 (EHV-4). These three related UL49.5 genes all interfere with TAP function, leading to reduced expression of stable MHC class I complexes at the cell surface of virus infected cells, which can be restored following deletion of the UL49.5 gene (Koppers-Lalic *et al.,* 2005; Koppers-Lalic *et al.,*

2008). The way in which UL49.5 inhibits the function of TAP differs between these viruses. BHV-1 UL49.5 is able to target TAP for proteasomal degradation, a function which requires the C-terminal tail. BHV-1 UL49.5 is also able to block the conformational changes of TAP that are required for peptide transport, which interestingly requires only the core complex of TAP, and this function requires the ER luminal domain of UL49.5 (Koppers-Lalic *et al.,* 2005; Verweij *et al.,* 2008; Verweij *et al.,* 2011; Verweij *et al.,* 2011). Both PRV and EHV-1 are thought to induce conformational changes which prevent the translocation of peptides across TAP (Koppers-Lalic *et al.,* 2008). In addition they are able to interfere with the energy supply that TAP requires for peptide translocation in different ways; PRV is able to block the conformational changes which follow ATP binding while EHV-1 blocks the binding of ATP to TAP (Koppers-Lalic *et al.,* 2008)).

Yet another molecular mechanism for interfering with TAP is displayed by the US6 gene product of Human cytomegalovirus (HCMV). This gene encodes an ER-localised integral membrane protein, which is able to interfere with the function of TAP (Hengel *et al.,* 1997). US6 interacts with TAP and inhibits peptide translocation, preventing MHC class I assembly by binding to the ER lumen exposed loops of TAP at the ER luminal face (Ahn *et al.,* 1997; Hengel *et al.,* 1997). US6 binding to TAP induces a conformational change in TAP, which renders the NBD of TAP1 unable to bind ATP, thereby depriving TAP of the energy source required for peptide translocation, without affecting peptide binding (Hewitt *et al.,* 2001). Since the ER luminal domain of US6 is required for ER retention and is sufficient to inhibit ATP binding, despite the NBDs being localised on the opposite side of the ER membrane, this is thought to be an indirect conformational effect exerted upon TAP1 NBD (Ahn *et al.,* 1997; Hewitt *et al.,* 2001). Like the above mentioned TAP inhibitors, this results in inhibition of

peptide transport by TAP and thus lower levels of cell surface MHC class I, thereby preventing $CD8^+$ T cell recognition of infected cells.

The most recent TAP inhibitor to be identified is the membrane protein CPXV12 encoded by cowpox virus, although the exact mechanism of action has yet to be identified (Wilkinson and Lehner 2009).

The remarkably diverse molecular mechanisms employed by several different persistent viruses to target TAP is testimony to the key role of this transporter complex in regulating MHC class I antigen presentation to $CD8^+$ immune effector cells.

1.5.3. Retention of MHC class I molecules in the ER

Some viruses have also evolved to interfere with the export and trafficking of MHC class I molecules from the ER to the plasma membrane. The first example of such a mechanism was provided by the adenovirus gene product E3/19K (E19). Although subsequently shown to have dual immune evasion functions, E19 was originally shown to bind tightly to MHC class I $\alpha1\alpha2$-domains in the ER and prevent their transport to the plasma membrane, thereby reducing cell surface levels of MHC class I (Andersson *et al.,* 1985). E19 is able to prevent MHC class I departure from the ER via two distinct mechanisms. Firstly, the cytosolic tail of E19 contains an ER retrieval motif, resulting in its transport from the Golgi apparatus back to the ER (Gabathuler and Kvist 1990). However, E19 mutants lacking this retrieval motif are still able to inhibit MHC class I transport from the ER, which is now known to be due to E19 binding to TAP and preventing the tapasin-mediated binding of MHC class I molecules to TAP, thereby acting as a tapasin inhibitor (Bennett *et al.,* 1999). This dual function of E19 is thought to be a means of overcoming the lack of tight binding of E19 to some MHC class I molecules, therefore allowing it to interfere with the egress of those MHC class I molecules with which E19 shows weak association.

A second paradigmatic example of virus mediated retention of MHC class I complexes in the ER is the HCMV US3 gene product. US3 encodes an ER-resident type I integral membrane protein which also promotes the retention of MHC class I peptide loaded molecules in the ER (Ahn *et al.,* 1996). The association of US3 with MHC class I molecules is transient, after which it is degraded, thus there needs to be a constant supply of US3 (Gruhler *et al.,* 2000). The exact mechanism by which this US3 functions is not fully understood. It is not thought to be via a retrieval signal since there is no known ER retrieval motif, in addition both the

transmembrane and luminal domain of US3 are required for to retain MHC class I molecules in the ER, although this may require an intermediate ER resident protein (Lee *et al.*, 2000).

1.5.4. Diverting class I molecules to lysosomes

Rather than preventing MHC class I complex trafficking by retaining them in the ER, some viral proteins have been shown to prevent transport by actively diverting the complexes to lysosomes for degradation. A prime example is the U21 protein of human herpesvirus 7 (HHV7). U21 is an integral membrane protein which binds tightly to MHC class I molecules shortly after synthesis and rather than preventing their transport, targets them for lysosomal degradation (Hudson *et al.*, 2001). Although the exact mechanism is yet unknown, it has been shown that there is no known lysosomal sorting sequence in the cytosolic tail of U21, and the luminal domain of this protein is known to be responsible for both associating with MHC class I molecules and the rerouting of these molecules to lysosomes, however, it does not localise to lysosomes along with MHC class I molecules. It is thought that there may be a cellular protein requited to mediate U21 redirecting of MHC class I molecules to the lysosomal compartment (Glosson *et al.*, 2010).

A second example of this immune evasion strategy is provided by the gp48 protein of murine cytomegalovirus (MCMV). gp48 also binds to MHC class I molecules via the luminal/transmembrane domain of the protein. These complexes are transported out of the ER and then redirected to the for lysosomal degraded (Reusch *et al.*, 1999).

1.5.5. Redirection of MHC class I molecules to the cytosol

Under normal circumstances, the assembly of MHC class I molecules undergoes a quality control step in which misfolded MHC class I molecules are exported out of the ER to the cytosol, where they are subsequently degraded by the proteasome. This is another step in the MHC class I antigen processing pathway of which viruses have evolved to take advantage.

HCMV encodes two such proteins, US2 and US11. These are ER resident integral membrane glycoproteins which induce degradation of newly synthesised MHC class I molecules. In cells expressing US11 it has been demonstrated that newly synthesised HCs are rapidly destroyed, displaying a much decreased half-life (Wiertz *et al.,* 1996). Furthermore, this destruction can be blocked using proteasome inhibitors and those class I molecules which are not destroyed are found predominantly in the cytosol, which would suggest that US11 induces the retrograde transport of HC molecules to the cytosol, where they are deglycosylated and subsequently degraded by the proteasome (Wiertz *et al.,* 1996). The transient interaction of US11 with MHC class I is thought to be what retains US11 in the ER, as it has no known ER retention signal. US2 also causes a translocation of class I molecules into the cytosol and subsequent proteasomal degradation, although this occurs more slowly than that seen for US11 (Wiertz *et al.,* 1996), indicating that US2 is able to target HCs that are in complex with β2m, sometime after their insertion into the ER. A major difference between US11 and US2 is that US2 is itself translocated into the cytosol, along with MHC class I molecules (Wiertz *et al.,* 1996). Following translocation, HCs are deglycosylated and degraded, in the same way as is seen in the presence of US11. This translocation process has been shown to involve the ER translocon Sec61 (Wiertz *et al.,* 1996), the translocon through which HC are initially inserted into the ER. Other cytosolic proteins are thought to be required, such as calnexin and

calreticulin, in the case of US2 (Oresic and Tortorella 2008) and more recently US2 and US11 have been shown to associate with the translocating chain associated membrane protein 1 (TRAM1) of the ER. Indeed, the knockdown of TRAM1 in cells expressing US2 and US11 resulted in an impaired level of class I translocation (Oresic *et al.,* 2009). This study indicated that US11 may be more reliant upon TRAM1 than US2 (Oresic *et al.,* 2009). Interestingly, a more recent study has suggested that US2 and US11 show differential targeting of HLA molecules, where they show less strong targeting of HLA-C molecules, possibly as a means of ensuring infected cells are not targeted by natural killer (NK) cell lysis (Ameres *et al.,* 2013).

1.5.6. Internalisation and degradation of MHC class I molecules

Those MHC class I molecules which make it to the cell surface are also targeted by some viral immune modulating proteins. KSHV encodes two such proteins, K3 and K5. These proteins are localised primarily near the plasma membrane and are able to increase the endocytosis of surface MHC class I molecules, in a clathrin-dependant manner (Coscoy and Ganem 2000). These proteins are E3 ubiquitin ligases, thus they are able to ubiquitinate the cytosolic tail of MHC class I molecules, which provides the signal for internalisation (Hewitt *et al.,* 2002). Once internalised, Ub tagged MHC class I molecules are sorted to acidic endolysosomal vesicles of the late endocytic pathway, where they are subsequently degraded. Surprisingly this ubiquitination occurs after MHC class I export from the ER and is thought to occur late in the secretory pathway or at the plasma membrane (Hewitt *et al.,* 2002). These proteins have also been shown to differentially target HLA molecules. K5 dramatically down regulates HLA A and B, having a weak effect on HLA-C and no effect on HLA-E whereas K3 is able to down regulate all HLA types (Ishido *et al.,* 2000).

The retrovirus HIV-1 also encodes a protein, nef, which is has been shown to modulate the cell surface expression of MHC class I molecules (Williams *et al.,* 2002). Nef acts only on HLA-A and –B, so that HLA-C and –E remain present at the cell surface thus protecting infected cells from recognition and lysis by NK cells. Nef has been shown to directly bind to HLA-A2 molecules (Williams *et al.,* 2002). This interaction is weak leading to the suggestion that nef acts more like an adaptor molecule, promoting an interaction between the MHC class I and the cellular adaptor protein AP-1 (Roeth *et al.,* 2004). This interaction results in the rerouting of newly synthesised MHC class I molecules from the trans-Golgi network to lysosomal compartments for degradation.

1.5.7. Conclusion

Clearly, the down regulation of cell surface display of MHC class I complexes is important for viral survival. Those mechanisms outlined above target almost every step in the antigen processing pathway, as a means of protecting cells from $CD8^+$ T cell recognition and enabling the survival of the virus. Importantly for this research project, EBV is no exception to the immune evasion strategy. What follows is a detailed review of the relevant aspects of EBV biology, with particular focus on the different immune evasion strategies employed by EBV, and importantly for this work, the ability of this virus to evade detection by $CD8^+$ T cells during lytic infection.

1.6. **Epstein Barr Virus**

EBV is a member of more than 130 known viruses that constitute the Herpesvirus family. Herpesviruses are further subdivided into three subgroups: the α-, β, and γ-herpesviruses. These are complex viruses which have linear double stranded DNA genomes and mostly infect vertebrate host animals. Of the 130 known Herpesviruses, eight are known to primarily infect humans and, in doing so, cause a variety of diseases which range in severity from self-limiting skin lesions to cancer.

The structure of all Herpesviruses consists of a double stranded DNA genome which, for EBV is 192 kbp but can be as big as 241 kbp. This DNA genome is contained within an icosahedral capsid which is further surrounded by a thick layer of virus encoded protein rich tegument. This is then surrounded by a membrane which is derived from the plasma membrane of the host cell during virus budding, and contains multiple virus-encoded glycoproteins which are involved in virus attachment and entry in to host cells, among other functions (Brown and Newcomb 2011).

EBV is known to infect more than 90% of the human population. Infection normally occurs early in childhood and is often asymptomatic. However, if exposure is delayed until adolescence, it can be associated with the self-limiting disease, infection mononucleosis (IM). IM is characterised by a rapid expansion in $CD8^+$ T cell responses directed towards EBV antigens. Following primary infection, EBV establishes a life-long infection of the host.

Infection with EBV occurs via the oral route, through contact with infected saliva. The primary site of infection, within the oropharynx, was initially thought to be specialised epithelial cells, where it is thought to replicate and subsequently infect B cells trafficking

through the oropharynx. Some studies, however, have suggested that B cells in the oropharynx may be the primary site of infection (Karajannis *et al.*, 1997; Niedobitek *et al.*, 1997). Irrespective of the primary infected cell, EBV establishes a latent infection of B cells in which the linear genome is circularised and maintained episomally.

Following initial infection, B-cells are thought to express latency type III genes, as seen in lymohoblastoid cell lines (LCLs), these genes drive B-cell proliferation (as discussed below), or alternatively EBV may enter lytic cycle replication, producing virus which can subsequently re-infect epithelial cells (Fig 1.2). Upon entry into the blood, virus infected B-cells are thought to differentiate into memory B-cells and express latency 0 pattern of gene expression, which is thought to be the latency pattern expressed by the majority of circulating memory B cells. In this latency pattern there are no viral genes expressed, unless the B-cells divide (entering latency I), whereby EBNA-1 is expressed (described in detail below) (Hochberg *et al.*, 2004). Some of these circulating B-cells are thought to differentiate into plasma cells, entering lytic cycle replication, and release virus that can then infect other naïve B cells. Other circulating EBV infected B cells are thought to traffic back to the oropharynx, where they can undergo differentiation into plasma cells and shed virus into the saliva, or infect other epithelial cells, which then shed virus in to the saliva (Fig 1.1). Thus the need for lytic cycle in B-cells is paramount to maintain EBV infection and shed EBV into the saliva, enabling transmission to new hosts. The details of latency and lytic cycle are discussed in detail below.

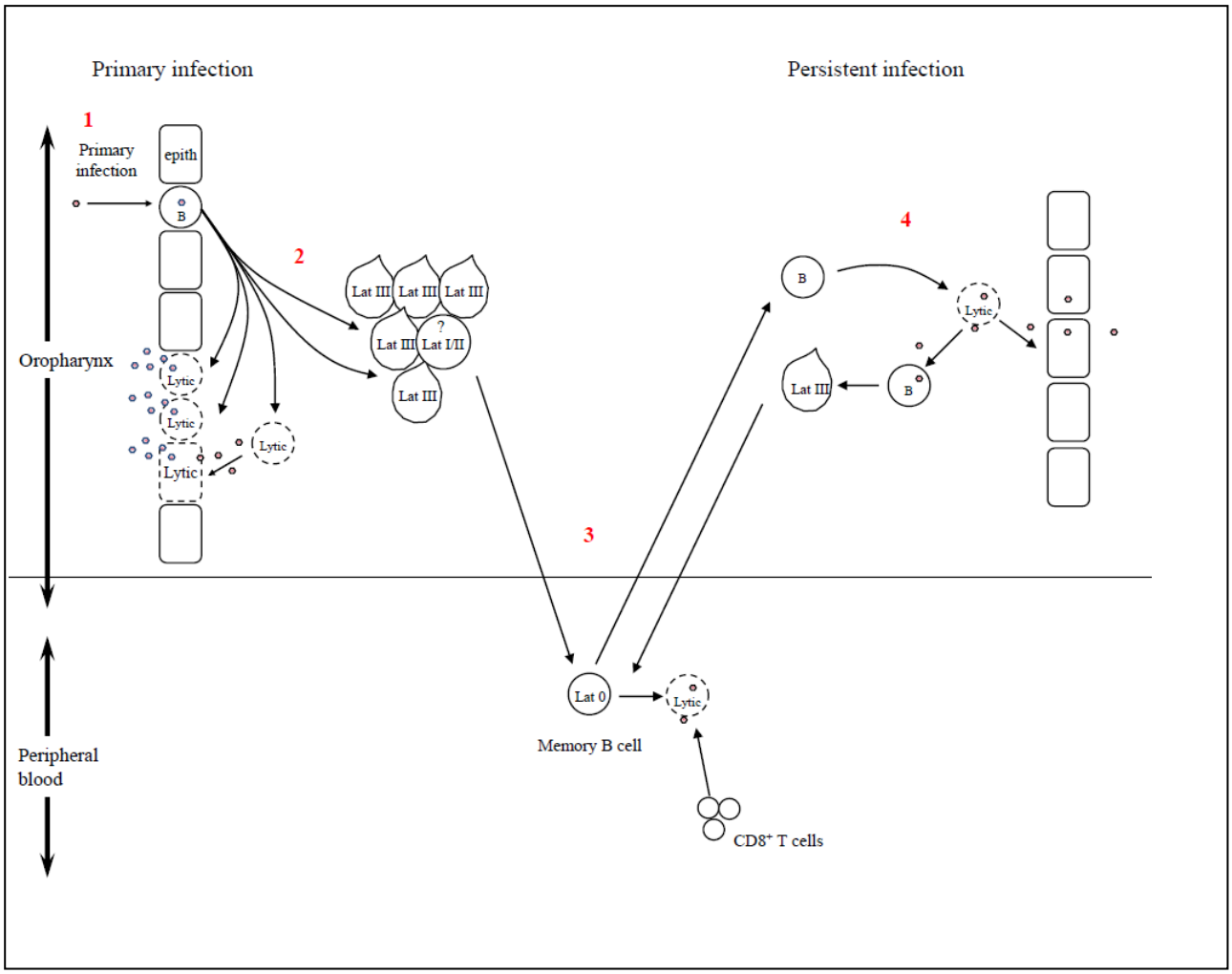


Figure 1.2 Biology of EBV infection.
(Adapted from (Cohen et al., 2000). (1) EBV initially either infects epithelial cells in the oropharynx where it replicates and then infects resting naïve B cells that traffic through the oropharynx, or it may infect resting, naïve B cells directly. (2) These B cells can undergo lytic infection and reinfect epithelial cells or B cells. Or EBV can establish a latent infection in these B cells, which eventually becomes latency 0. (3) Latency 0 infected memory B cells in the blood express no viral proteins, unless they divide. They can reactivate and undergo lytic cycle replication. These cells are subject to control by circulating CD8+ T cells. Released virus can infect other submissive cells. (4) Latency 0 infected B cells can traffic to the oropharynx where they undergo lytic replication and release virus which can be detected in the saliva. This virus can further infect other cells.

1.6.1. Latent infection

Following B cell infection, EBV is able to drive the proliferation of latently infected B cells by expressing a subset of latent growth transforming genes, establishing a form of latency known as latency III. These genes include; EBNA -1, -2, -3A, -3B, -3C and -LP; latent membrane proteins (LMP) 1, 2A and 2B; and two types of nontranslated RNA (Reviewed (Cohen 2000). The roles of these proteins have been studied using transfection of single genes into EBV negative cells and epithelial cell lines. They have also been studied in LCLs, which are established through in vitro EBV infection of B cells, using recombinant EBV to show which genes were dispensable or essential for transformation of B cells.

EBNA-1 has been shown to be expressed in all known EBV-associated tumours, which reflects its essential role in maintaining the latent viral genome in proliferating cells. EBNA-1 does this by binding to the plasmid origin of viral replication (OriP) site in the EBV episome and tethering it to the host genome, allowing the viral genome to be maintained during cell division (Yates *et al.,* 1984).

EBNA-2 plays an essential role in activating the expression of key cellular genes involved in EBV transformation of B cells (Cohen *et al.,* 1989). This protein was the first EBV gene to be identified as being essential for EBV transformation of B cells, as isolates deleted for the EBNA-2 gene (e.g. Daudi cells) were shown to be transformation deficient. EBNA-2 interacts with the cellular protein RBPJκ. This interaction facilitates the transactivating role of EBNA-2 by linking it to DNA response elements, resulting in activation and expression of cellular genes as well as LMP1 and 2 (Cohen *et al.,* 1989; Johannsen *et al.,* 1995). EBNA2B is less efficient at transformation, which has been mapped to a single amino acid change (Cancian *et al.,* 2011). The EBNA-3 proteins; -3A, -3B also bind to RBPJκ, with a higher affinity than

EBNA2, therefore preventing EBNA2 from binding to DNA, thus inhibiting the ability of EBNA2 to disregulate gene expression (Robertson *et al.,* 1996). EBNA3A and -3C are also required for B cell transformation (Tomkinson *et al.,* 1993). EBNA-3B has been found as non-essential for transformation (Tomkinson and Kieff 1992). EBNA-3 proteins also upregulate the expression of other viral and cellular genes such as CD21 (Wang *et al.,* 1990). EBNA-LP has been shown to regulate gene expression by inhibiting the EBNA2 induced activation of LMP1, LMP2 and cellular protein expression. EBNA-LP is also a classic example of the problems which have been encountered in defining genes as essential or non-essential for transformation. In the first instance this protein was shown to be non-essential, because LCLs could still be generated with viruses knocked out for LP. However, more quantitative experiments suggest that the transformation more than 1000 times more efficient in the presence of EBNA-LP.

LMP1 however, is essential for transformation of B-cells and also plays the role of an oncogenic gene, as evident in mouse studies, where the expression of LMP1 in transgenic mice leads to the formation of tumours (Wang *et al.,* 1985). LMP1 is also a functional analogue of a constitutively active form of the B cell surface molecule CD40 (Uchida *et al.,* 1999). In B-cells, the interaction of CD40 with its ligand results in binding to tumour necrosis factor receptor (TNFR)-associated factors and B cell activation and proliferation. LMP1 binds to several of these TNF-associated factors, which results in the activation of the nuclear factor-kB (NFkB) transcription factor, resulting in B-cell proliferation. In addition, LMP1 has been shown to upregulate the prosurvival, bcl-2 family members including, Bcl-2 and Mcl-2.

EBV LMP2 proteins are not required for B-cell transformation by EBV and instead function to prevent the reactivation of EBV in latently infected B cells (Miller *et al.,* 1994; Miller *et*

al., 1995), and allow B-cells to survive in the absence of normal B-cell receptor signalling (Caldwell *et al.*, 1998). Thus both LMP1 and LMP2 are tapping into B cell signalling pathways that are known to affect differentiation and survival of B cells. The nontranslated RNAs of EBV do not encode proteins but are thought to be important for oncogenesis.

EBV associated diseases tend to show one of three patterns of latency: latency 0/I, with the expression of no viral proteins or only EBNA1, upon cell division; Latency II, involving the expression of EBNA1, LMP1 and LMP2 and latency III, the expression of all latent genes.

As described previously, the entry of EBV infected B-cells into lytic cycle is an essential component of EBV biology. Lytic cycle results in the production of viral progeny, resulting in infection of naïve B-cells and shedding of virus into the saliva in order to maintain and transmit the virus. Below is a detailed description on lytic cycle replication.

1.6.2. Lytic infection

The study of EBV lytic cycle in vitro has proved difficult as there is currently no cell culture system which allows for primary lytic cycle infection to an efficiency necessary for in vitro study. As a consequence, in vitro models in which EBV is reactivated in latently infected cell lines have been used. This commonly involves the use of broad acting chemical inducers such as butyrates or the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA); among other things, these agents activate transcription of the immediate early viral gene, BZLF1. The broad range of effects of chemical inducers include increased expression of components of the antigen processing pathway, such as MHC class I, which compromises their use in studying immune responses to EBV lytic cycle. A more physiological model is available in which surface immunoglobulin crosslinking on an EBV infected cell line, AKATA, induces the initiation of lytic cycle, allowing for the examination of the expression kinetics of lytic cycle (Takada and Ono 1989).

During lytic cycle, EBV has been found to express more than 90 viral proteins, which are temporally regulated (Fig 1.3) and thus, like other herpesviruses, can be divided into three classes according to the timing of their expression: immediate early, early and late. The immediate early genes are transcribed even in the presence of protein synthesis inhibitors, and they function as transactivators of other lytic cycle viral and cellular genes. Direct and indirect targets of the immediate early genes include the early viral genes which are transcribed before viral DNA replication and their transcription is inhibited in the presence of protein synthesis inhibitors but is not inhibited by viral replication inhibitors. Finally, the late genes are those expressed following viral DNA replication, and their expression is blocked by inhibitors of viral DNA replication.

EBV expresses two immediate early proteins encoded by the BZLF1 and BRLF1 genes, both of which are required to activate the transcription of early viral genes (Feederle *et al.,* 2000; El-Guindy *et al.,* 2013). BZLF1 is a DNA binding protein with homology to cellular c-jun and c-fos proteins. BZLF1 binds to activator protein-1 (AP-1) like Z-responsive elements (ZREs) in promoter sequences (Farrell *et al.,* 1989; Chang *et al.,* 1990). Interestingly, BZLF1is able to activate its own expression, which also requires other cellular transcription factors, and this in turn transcriptionally activates the promoter of BRLF1, through direct binding to ZREs within the BRLF1 promoter (Urier *et al.,* 1989). BRLF1 can then upregulate the expression of BZLF1 (Ragoczy *et al.,* 1998). Combined, this results in rapid expression of BZLF1 and BRLF1 following reactivation of lytic cycle, which subsequently activates the expression of early and late genes by binding to response elements in the promoters of these genes. Whilst BZLF1 and BRLF1 are necessary for efficient induction of the lytic cycle cascade, there are also cellular factors involved. This is exemplified by the fact that BZLF1 will efficiently induce lytic cycle in Akata cells, but not in LCLs. This has not been extensively studied, but there is a suggestion that in addition to cellular factors, the expression of LMP1 in latency III may block the B cell differentiation required for lytic cycle (Vrzalikova *et al.,* 2011), which may explain the effects of BZLF1 in Akata compared to LCLs.

Proteins expressed during the early phase of lytic cycle play numerous roles such as protecting cells from apoptosis via the expression of the cellular bcl-2 homologue, BHRF1 (Henderson *et al.,* 1993), while some early genes function to activate the expression of other early genes. Collectively, two major roles played by the genes expressed in the early phase of lytic cycle are to mediate viral DNA replication, and to modulate the immune response directed towards EBV (discussed below). Using an in vitro assay to amplify plasmids containing the OriLyt, six early proteins have been identified which are essential for viral

DNA replication. These include the viral DNA polymerase, BALF5; BALF2, which functions as a single stranded DNA binding protein; BMRF1, which is a DNA polymerase accessory factor; the primase homologue BSLF1; a helicase homologue BBLF4 and BBLF2/3 a helicase-primase homologue (Fixman *et al.,* 1992).In addition, the immediate early gene BZLF1, which interacts with the replication proteins, is also required for binding to the OriLyt.

Those genes expressed during late phase lytic cycle predominantly encode the structural proteins of EBV. These include numerous glycoproteins, such as gp350, the major viral envelope protein encoded by BLLF1, which binds to its receptors CD21 on B cells, thereby enabling EBV attachment to submissive cells (Fingeroth *et al.,* 1984). The capsid proteins, such as the major nucleocapsid protein encoded by BcLF1, and tegument proteins such as BNRF1 are also expressed at the late stages of lytic cycle.

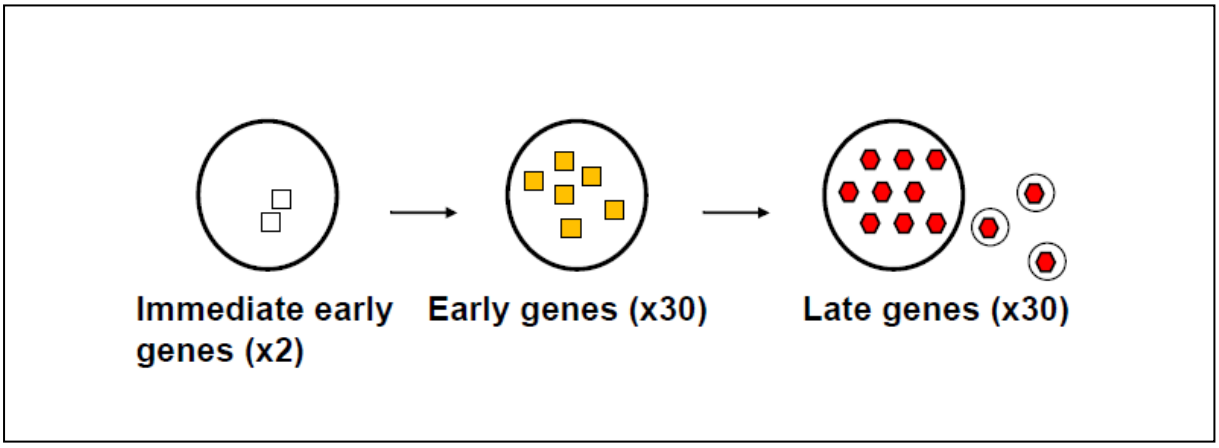


Figure 1.3 Lytic cycle infection.
Following entry of lytic cycle, proteins are expressed in a synchronous manner. This begins with the expression of immediate early genes i.e. BZLF1, followed by the expression of early gene products such as BMRF1 and some immune evasion proteins i.e. BILF1 and finally late genes such as BALF4. This sequence of events results in the production of viral progeny and can last up to 4 days.

1.6.3. The host immune response to EBV infection

Infection by EBV elicits both an innate and adaptive immune response, however the cell mediated response is considered more important for control of the virus. This is most evident in the EBV-associated diseases which arise in immunocompromised individuals, including the onset of post-transplant lymphoproliferative disorder (PTLD) which is seen in transplant recipients, following immune suppression and is the result of uncontrolled proliferation of EBV transformed B cells, in the absence of functional T cells.

Given that EBV encodes such a vast number of proteins expressed during latent and lytic phases of its biological cycle are they all equally potential targets for T cell responses, or is there immunodominance of a particular subset? The investigation of T cell responses in individuals suffering from IM, as well as healthy EBV infected carriers, has allowed for the detailed study of T cell responses mounted towards EBV during infection. During IM there is a dramatic expansion of $CD8^+$, but not $CD4^+$, T cell responses to both lytic and latent EBV antigens, although $CD8^+$ T cells specific for lytic epitopes have been shown to be the more dominant population, while later in infection this population is dominated by $CD8^+$ T cells directed towards latent antigens (Callan *et al.,* 1998; Maini *et al.,* 2000; Hislop *et al.,* 2002). Within this population of lytic epitope specific $CD8^+$ T cells, it has been shown that a pattern of immunodominance exists, where there is a higher proportion of $CD8^+$ T cells directed towards the immediate early antigens, BZLF1 and BRLF1, with up to 44% of all $CD8^+$ T cells directed toward one BZLF1 derived epitope, in one example (Callan *et al.,* 1998; Hislop *et al.,* 2002). Reactivity towards early epitopes such as BMLF1 and BMRF1 are less frequent, accounting for up to 12% of the total $CD8^+$ population (Callan *et al.,* 1998; Annels *et al.,* 2000; Hislop *et al.,* 2002), and T cells specific for late epitopes are rarely seen (Pudney et al.,

2005). Following control of IM, there is a rapid contraction in these EBV-specific $CD8^+$ T cell responses (Hislop *et al.*, 2002).

This pattern remains similar in healthy carriers, where the frequencies of EBV specific $CD8^+$ T cells are lower than seen in IM, with around 0.2-2% of $CD8^+$ T cells directed towards lytic epitopes while only 0.05-1% are directed toward the latent (Hislop *et al.*, 2007). In a similar pattern as seen in IM patients, healthy donor lytic epitope specific $CD8^+$ T cell populations are dominated by those directed towards the early and especially immediate early epitopes. This pattern of $CD8^+$ T cell immunodominance can be correlated with the efficiency with which the different epitope peptides are presented during infection (Pudney *et al.*, 2005), where immediate early epitopes and early epitopes are better presented than late epitopes. This is thought to be due, in part, to EBVs immune evasion mechanisms which enable this virus to evade $CD8^+$ T cell recognition by interfering with the antigen presentation during lytic cycle (discussed below).

Studies into the $CD4^+$ T cell response to EBV targets has been less well studied, although it has been shown that during IM, $CD4^+$ T cells predominantly recognise EBNA-3C to a greater extent than EBNA-1 or EBNA-2, while later in infection EBNA-1 appears to be the dominant target (Woodberry *et al.*, 2005). Interestingly, while there is a hierarchy of $CD8^+$ T cell responses to lytic antigens, as described above, $CD4^+$ T cell responses directed towards latent antigens appears to be more evenly distributed (Long *et al.*, 2011). The frequency of responses in healthy individuals is, as mentioned, dominated by $CD8^+$ T cells. However, $CD4^+$ T cell responses are still important as both helper cells, producing IFN-γ and some also exhibit cytotoxic effects, evident in the ability of these cells to inhibit outgrowth of virus transformed cells (Long *et al.*, 2005).

1.6.4. Immune evasion by EBV

Due to this strong immunological pressure, EBV has necessarily evolved a number of ways to avoid detection by T cells, particularly $CD8^+$ T cells. As described above, circulating EBV infected B-cells are thought to show latency-0 pattern of expression. This minimal expression of viral proteins can be considered a means of avoiding elimination by T cells, by reducing the amount of viral peptides available for presentation to T cells. In addition, some latently expressed proteins employ their own mechanism to prevent presentation to $CD8^+$ T cells, such as EBNA1, as described in section 1.6.1. However, during lytic cycle, more than 90 viral proteins are expressed, creating an extensive pool of viral epitopes available for presentation to the immune system and thus cells undergoing lytic cycle are highly vulnerable to detection and elimination by the immune system.

The study of $CD8^+$ T cell evasion by EBV during lytic cycle was initially difficult due to the lack of an adequate in vitro model. As a consequence, the inducible EBV infected cell line, AKATA, was engineered to express a rat-CD2-GFP reporter plasmid, under the control of the early lytic cycle BMRF1 promoter, now known as AKBM cells. Upon induction, AKBM cells express both rat-CD2 and GFP, thus allowing for the isolation of lytic populations (Ressing *et al.,* 2005). LCLs in culture can also spontaneously enter lytic cycle. Although, is normally a rather small subset of around 1-2% and rarely more than 5%. In addition, this is unpredictable, and the factors which dictate this are unknown. Nevertheless, using spontaneously lytic LCLs and the AKBM model described above, it has been shown that during lytic cycle there is a marked decrease in the expression of cell surface MHC class I molecules (Keating *et al.,* 2002; Ressing *et al.,* 2005). In addition to this, lytic cycle induction is also associated with impaired peptide transport by TAP (Ressing *et al.,* 2005), this, together

with the finding that the efficiency of epitope presentation of EBV antigens diminishes as lytic cycle progresses (Pudney *et al.,* 2005), suggests that EBV is able to interfere with the MHC class I antigen processing pathway.

It is now known that there are multiple lytic cycle proteins capable of downregulating MHC class I surface levels, when ectopically expressed. The first such EBV protein to be implicated was the vIL-10 encoded by BCRF1, which was reported to inhibit transcription of TAP1 (Zeidler *et al.,* 1997). However, the first EBV gene to be shown to inhibit functional recognition by $CD8^+$ T cells specific for lytic cycle gene epitopes, was BNLF2a (Hislop *et al.,* 2007).

BNLF2a is a small tail-anchored protein which is able to insert into the ER membrane via its C-terminal hydrophobic domain, a process facilitated by the cellular protein AsnaI, while the N-terminal domain of BNLF2a is responsible for TAP inhibition (Horst *et al.,* 2011). In contrast with other known viral TAP inhibitors which target either the ATP or peptide binding functions of TAP, the association of BNLF2a with TAP inhibits both the ATP and peptide functions of TAP (Hislop *et al.,* 2007). As a result there is a very efficient reduction of peptide transport and subsequent peptide loading of MHC class I molecules, thereby reducing cell surface expression of MHC class I molecules in cells expressing BNLF2a (Hislop *et al.,* 2007). The interaction of BNLF2a with TAP requires TAP2 which is thought to stabilise BNLF2a, since in cells lacking TAP expression, there is decreased levels of BNLF2a protein expression (Horst *et al.,* 2009). In this same study, ectopic expression of BNLF2a in EBV-negative cells was demonstrated to impair their functional recognition by epitope specific $CD8^+$ T cells. Importantly, recognition of EBV lytic cycle antigens was significantly enhanced in spontaneously lytic LCLs transformed with recombinant EBV lacking BNLF2a, compared to LCLs transformed with wild-type EBV. Interestingly, whilst there was a

dramatic increase in recognition of epitopes originating from immediate early and early antigens, there was little effect on recognition of late lytic epitopes (Croft *et al.,* 2009). This suggests that additional immune evasion mechanisms are in place during lytic cycle, which are also able to interfere with the processing and presentation of lytic peptides.

Very recently, in addition to aiding in the evasion of $CD8^+$ T cell detection in cells spontaneously reactivating into lytic cycle replication, BNLF2a has also been implicated in protecting EBV infected cells from $CD8^+$ T cell recognition immediately after infection of B-cells. This same study showed that BNLF2a mRNA is contained within the EBV virion, and is transcribed and active immediately after infection of B-cells, thus protecting newly infected B-cells from $CD8^+$ T cell recognition in the initial stage of infection, before the virus enters full lytic cycle replication (Jochum *et al.,* 2012; Jochum *et al.,* 2012).

More recently, the early lytic expressed protein BILF1 has been shown to reduce cell surface expression of MHC class I, and recognition by EBV lytic epitope specific $CD8^+$ T cells, upon ectopic expression (Zuo *et al.,* 2009). BILF1 is a G-protein coupled receptor which is able to bind to MHC class I molecules, requiring the C-terminal domain to do so, and subsequently increase their turnover from the cell surface and subsequent lysosomal degradation (Zuo *et al.,* 2009; Griffin *et al.,* 2013). The exact function which enables BILF1 to exert this effect on MHC class I molecules is currently unknown, however it is not dependent upon ubiquitination of MHC class I molecules, as it the case with KSHV immune modulating proteins, K3 and K5 (Zuo *et al.,* 2009). The function of G-protein signalling and MHC class I turnover by BILF1 are independent of one another, which was shown in a study using a BILF1 mutant which lacked in the signalling ability yet was still able to downregulate surface MHC class I levels (Zuo *et al.*, 2009). In addition to increasing the degradation of cell surface MHC class I molecules, BILF1 is also able to target newly synthesised MHC class I molecules which are

trafficking to the cell surface, by diverting them from the exocytic pathway. Indeed, this latter mechanism appears to be responsible for a greater reduction in cell surface MHC class I than is caused by internalisation of MHC class I (Zuo *et al.*, 2011). Importantly, B cells infected with EBV in which BILF1 has been deleted revealed that in the absence of BILF1, EBV infected B cells were better recognised by CD8+ T cells specific for lytic epitopes (Zuo *et al.*, 2011), while the ectopic expression of BILF1, albeit in a different cell type, has been shown to decrease $CD8^+$ T cell recognition (Zuo *et al.*, 2009). Moreover, the ability of BILF1 to target MHC class I molecules appears to be selective, in that it has been shown to have minimal effect on levels of HLA-C molecules. By deleting a portion of the C-terminal domain of HLA B8 HC molecules, BILF1 was unable to reduce the cell surface expression of MHC class I molecules, indicating that some or all of these residues are required for BILF1 binding to MHC class I molecules, moreover it was shown that these required residues were not present in the cytoplasmic domain of HLA-C molecules (Griffin *et al.*, 2013).

The early lytic cycle expressed protein BGLF5 has also been implicated in $CD8^+$ T cell evasion by EBV. BGLF5 was first identified as a viral exonuclease enzyme, which is required for successful viral replication and production of viral progeny (Feederle *et al.*, 2009), is now known to have a host shut-off function that decreases the synthesis of new MHC class I molecules (Rowe *et al.*, 2007; Zuo *et al.*, 2008), which is genetically separable from its DNase enzyme function, and is responsible for impaired recognition of antigen presented to $CD8^+$ T cells. The molecular mechanism of BGLF5 host shut-off was resolved by Buisson et al (2009) who demonstrated that in addition to DNase activity, BGLF5 had potent RNase activity that was dependent upon the presence of Mn++ ions (Buisson *et al.*, 2009; van Gent *et al.*, 2011). While the RNase activity of BGLF5 accounts for the host shut-off function of BGLF5, its consequences are not confined to MHC class I synthesis but extended to reduce

the levels of most host mRNA species, including MHC class II (Rowe *et al.,* 2007). More recently it has been shown that during lytic cycle replication there is a strong decrease in the synthesis of the dsDNA sensor TLR9, which is itself activated by the EBV infection. In vitro studies have shown that in the presence of Mn^{2+} BGLF5 is able to reduce the transcription of TLR9 mRNA, suggesting that BGLF5 may contribute to the decreased expression of TLR9 during lytic cycle (van Gent *et al.,* 2011). This may suggest that BGLF5 adopts a less targeted approach to interfering with $CD8^{+}$ T cell recognition of infected cells, and is also important in evading the innate immune responses which are initiated EBV upon infection.

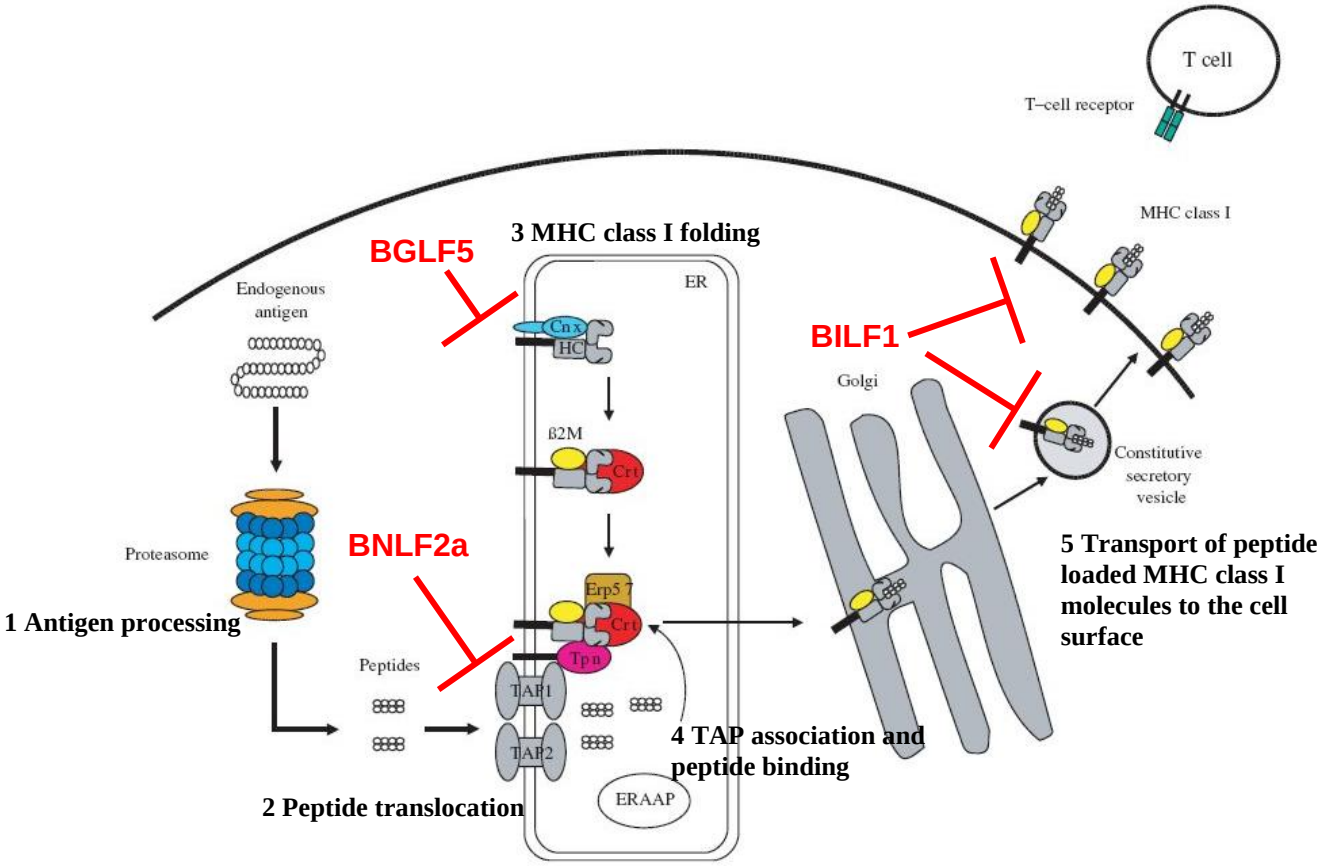


Figure 1.4. The stages during MHC class I antigen presentation which are targeted EBV immune evasion proteins.

BGLF5 increases the turnover of MHC class I mRNA. BNLF2a interferes with the function of TAP and BILF1 targets MHC class I molecules, which are en route to the cell surface and at the cell surface, for lysosomal degradation.

As mentioned previously, the BCRF1 gene of EBV encodes a vIL-10, which has a variety of effects on the immune system. These include reducing the mRNA levels of TAP1 and LMP2, a subunit of the immune proteasome, in B cells (Zeidler *et al.*, 1997). This results in a decrease in peptide transport and subsequent peptide loading and presentation of MHC class I molecules at the cell surface (Zeidler *et al.*, 1997). More recently it has been shown that vIL-10 is expressed early after infection of primary B cells (Jochum *et al.*, 2012). Although this did not result in a decrease in $CD8^+$ T cell recognition, the deletion of this gene did resulted in an increase in NK cell lysis of B cells, compared to those infected with wild type virus (Jochum *et al.*, 2012), suggesting it may impair NK cell mediated killing, thereby protecting newly infected cells from detection following infection.

In addition to vIL-10, the BART2-5p EBV microRNA (miR), which has been shown to reduce expression of the MICB protein, a stress induced natural killer cell ligand which promotes the destruction of infected cells by natural killer cells. miR-BART2-5p achieves this by binding to the 3'UTR region of the mRNA which encodes MICB, this decrease in MICB leads to protection of infected cells from killing by NK cells (Nachmani *et al.*, 2009).With the exception of these two examples there has been no other EBV encoded protein identified as being able to evade NK cell recognition, which is surprising considering the extent of proteins able to down regulate MHC class I levels. Indeed the down regulation of cell surface MHC class I expression means that cells undergoing lytic cycle replication are more vulnerable to NK lysis than latently infected cells (Pappworth *et al.*, 2007). However, this is not to say that other NK cell evasion mechanisms that active during lytic cycle replication do not exist. Some of EBVs miRs have also been identified as having potential immune evasion mechanisms. This includes some that act to inhibit apoptosis of infected cells such as miR-BART5, -BART4 and –BART15.

The evasion of $CD4^+$ T cell responses by EBV has been less extensively studied. However, in addition to the turnover of MHC class II transcripts by BGLF5, BZLF1 has also been shown to interfere with MHC class II synthesis. Studies have revealed that BZLF1 inhibits the expression of MHC class II molecules by either binding to ZRE elements in the promoter of CIITA, the transactivator of MHC class II expression (Li *et al.,* 2009), or by modulating the expression of the invariant chain, which plays a role in the loading of peptides on to MHC class II molecules and subsequent transport of these complexes (Zuo *et al.,* 2011). This confers a decrease in cell surface expression of MHC class II molecules and reduced $CD4^+$ T cell recognition (Zuo *et al.,* 2011).

The EBV encoded glycoprotein gp42 (BZLF2) has also been implicated in the evasion of $CD4^+$ T cell recognition. Gp42 exists as a type II membrane protein and as a truncated soluble protein, both of which bind MHC class II molecules, at various stages of maturation (Ressing *et al.,* 2005). This complex results in a block in the interaction of MHC class II:peptide complexes with TCRs and thus inhibits $CD4^+$ T cell recognition (Ressing *et al.,* 2003; Ressing *et al.,* 2005).

1.7. **Scope of thesis**

The ability of EBV to modulate the T cell response is clearly an important one, which allows for its lifelong persistence in a healthy host. Equally, the ability of the host to respond to infection is of paramount importance in order to control EBV infection and reactivation. Given the diverse range of mechanisms that EBV has evolved in order to interfere with the antigen presentation pathway, this research project aimed to assess the role that three of these proteins (BNLF2a, BILF1 and BGLF5) play, in the context of the whole virus, in evading $CD8^+$ T cell recognition by interfering with the antigen presentation pathway at the different phases of lytic cycle (Fig 1.4), and to investigate the relative contribution each make towards this interference.

CHAPTER 2

MATERIALS AND METHODS

2.1. Tissue culture

2.1.1. Tissue culture media and reagents

RPMI-1640 supplemented with 2mM L-glutamine (sigma) was stored at 4°C.

DMEM- Stored at 4°C (Sigma)

Foetal calf serum (FCS) (PAA) was stored in 50ml aliquots at -20°C

Human serum (HuS) (PAA) was free from virus and mycoplasma, and derived from a male type AB, and was stored in 50ml aliquots as -20C.

Penicillin-streptomycin (Gibco) containing 500 IU/ml penicillin and 5000ug/ml streptomycin. Stored as 100x stock and stored at -4C.

Trypsin-express (Gibco) was used as per manufacturer's recommendations.

Phosphate buffered saline (PBS) Made by dissolving 1x PBS tablet (Oxoid) per 100ml of water which had been filtered and ions removed. 500ml aliquots were then sterilised by autoclaving for 20 minutes at 121C.

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

Monkey Leukocyte antigen-144 supernatant (MLA). Derived from the supernatant of cultured MLA-144 cells. Supernatant was filtered and stored in 60ml aliquots at -20°C.

Lymphoprep was purchased in 500ml bottles (PAA)

Dimethyl sulphoxide (DMSO)

Opti-MEM- Purchased from Gibco

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 -20C.

2.1.3. Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was collected from donors into a syringe with heparin and then diluted with 1 part RPMI-1640. Lymphocytes were then separated by layering 35ml blood/RPMI onto 15ml lymphoprep and centrifuged at 1800rpm with no break. Cells were then washed and either used immediately for experiments or cryopreserved. All experiments were approved by an ethics committee, and donors provided consent.

2.1.4. Isolation human of B cells

B cells were selected from donor PBMCs using CD19 Pan B Dynabeads (Invitrogen), at a concentration of 4 beads per B cell (under the assumption that B cells account for 5% of total PBMCs). Cells were resuspended in 1ml of RPMI-1640 + 10% FCS per $1x10^7$ beads, and

incubated at 4ºC for 30mins on a roller. Cells were then washed 5 times in RPMI-1640 + 10% FCS on a magnetic rack, to retain the CD19 positive cells. Cells were then resuspended in 500ul RPMI-1640 + 1% FCS with 20ul of anti-CD19 detachabeads (Invitrogen) and incubated for 45 minutes, on a roller, at room temperature. Detachabeads were then removed using a magnetic rack, resulting in the elution of purified B cells.

2.1.5. Generation of LCLs

Purified B cells ($5x10^6$) were pelleted and resuspended in a high titre of recombinant EBV at a multiplicity of infection (M.O.I) of 50 using wild-type 2089-, ΔBILF1-, ΔBNLF2a, ΔBGLF5- or ΔBZLF1-virus. Cells were incubated with virus for 30 minutes on ice and then excess virus was washed off with RPMI-1640. Cells were resuspended in RPMI-1640 + 10% FCS and incubated in one well of a 24-well tissue culture plate at 37C, 5% CO_2 . Alternatively, to generate B95.8 virus transformed LCLs, $5x10^6$ purified B cells were incubated with 5ml of B95.8 cell line supernantant overnight at 37ºC in one well of a 6-well plate. B cell transformation could be seen within one week. Cells were expanded until they could be maintained in a $25cm^2$ tissue culture flask (Corning) in 10ml RPMI-1640 + 10% FCS.

2.1.6. Limiting dilution T cell cloning

T cell clones were established using either frozen PBMCs from either healthy donors or IM patients. These were thawed into T cell cloning media (RPMI-1640 + 10% FCS + 1% HuS + 30% MLA + 50IU/ml penicillin + 50ug/ml streptomycin + 5-IU/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) allogenic feeder PBMCs (10^6/ml). These were generated from fresh 'cones' (Birmingham national blood service) and then activated with phytohaemagglutinin (PHA 10ug/ml) overnight in standard media. Alongside feeder cells, irradiated (4000 rads) autologous LCLs (10^5/ml) were also used.

Plates were incubated at 37ºC, 5% CO_2 for one week, after which they were given fresh cloning media. One week after this, any clones which grew were expanded further in to 24-well plates corning, in the presence of irradiated autologous LCLs (10^5/ml) and allogenic feeder PBMCs (10^6/ml). After this, clones were maintained by feeding twice per week with fresh T cell cloning media (see section 2.2.6) by removing 1ml of media and replacing with fresh media.

2.1.7. 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. Following this, cells were washed with cold MACs buffer (PBS with 0.5% (w/vol) BSA and 2.5mM EDTA). Cells were then resuspended in 80µl of cold standard media (RPMI-1640 + 10% FCS) and 20µl of CD45-conjugated IFNγ-catch reagent (Miltenyi Biotec). From here, the manufacturer's protocol was followed. Collected cells were then plated out using limiting dilution (section 2.1.6).

2.1.8. Cryopreservation

Cells to be preserved were pelleted and resuspended in freezing media (RPMI-1640 + 10% DMSO + 20% FCS) and transferred into sterile 1ml cryovials (Nunc). These were then stored in a 'Mr Frosty' at -80ºC overnight, allowing for a slow decrease in temperature (1ºC/minute). The next day, cryovials were transferred into liquid nitrogen freezers.

2.1.9. Revival of cryopreserved cells

To revive cells, 1ml cryrovials were placed into a 37ºC water bath for thawing. Following this cells were washed with standard media (RPMI-1640 + 10% FCS), resuspended in appropriate media and transferred into an appropriate plate or flask, for culturing in a 37ºC, 5% CO_2 incubator.

2.1.10. Mycoplasma testing

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

2.2. **Generation of shRNA lentiviruses and transduction of lytic LCLs**

Screening of candidate siRNAs

Candidate siRNAs were generated by sigma aldrich, these are shown in Table 2.1. Candidate siRNAs were screened using 293 cells which had been transfected with a plasmid expressing the target knockdown gene. On day one 293 cells were plated into one 24cm^2 flask, per panel of siRNAs, and incubated at 37ºC, 5% CO_2 overnight. The next day, cells were transfected with plasmids expressing BNLF2a, BILF1-HA or BGLF5 using lipofectamine. To do this lipofectamine-2000 was incubated with OPTI-MEM for 5 minutes. Following this, 1ug of plasmid was added. This was incubated at room temperature for 20 minutes, after which, this mix was topped up with of OPTI-MEM and layered over 293 cells, these were left for 6 hours and then topped up with DMEM media, these are incubated over night at 37ºC, 5% CO_2. The following morning, the transfected 293 cells are washed in PBS and resuspended using 1ml trypsin. Cells are then resuspended in 8ml of DMEM media and aliquoted into 8 wells of a 24 well plate. This ensures that all wells have an equal proportion of cells expressing the target protein. Cells are then incubated at 37ºC, 5% FCS for 8 hours. Following this, cells are transfected with a panel of siRNAs (see Table 2.1) for each target gene, including a control siRNA for each screen.

siRNAs were transfected using 1ul oliofectamine, which was incubated with 2ul of OPTI-MEM per well, for 5 minutes at room temperature. Following this the required (50-250uM) concentration of siNRA diluted in OPTI-MEM was added and this mix was incubated at room temperature for 20 minutes. After this, 500ul OPTI-MEM was added and this was layered onto one well of transfected 293 cells. Each individual siRNA was screened in duplicate alongside a control. 24 hours later cells were harvested and lysed for use in western blot

analysis to measure the level of protein knockdown. Three different siRNAs were selected which showed successful knockdown of BNLF2a, BILF1 or BGLF5.

These sequences were used to generate shRNA constructs for each individual knockdown (Table 2.1). These constructs also expressed a fluorescent tag a puromycin resistance gene, to allow for cell sorting and enrichment following transduction.

Generation of shRNA lentiviruses

shRNA lentiviruses were generated using FT293 cells. These cells were split on day one in 30mls DMEM media and incubated in a 75cm^2 flask, to achieve approximately 70-80% confluencey the following day. On day 2, DMEM media was removed and replaced with 5mls of DMEM media. Transfection mixes were then made, firstly 40ul of lipofectamine per one flask of FT293 cells, was incubated with 1.5ml of optimem for 5 minutes at room temperature. During this time an aliquot of 4ug of shRNA-vector plasmid, 2ug envelope plasmid-pMD2G (VSV-G envelope) and 6ug packaging plasmid-psPAx2 (gag-pol) in 1.5ml of OPTI-MEM per flask, was made up. This was then mixed with the lipofectamine and incubated at room temperature for 20 minutes. Following this, the 3ml mixture was added to the 5ml of fresh DMEM media in a 75cm^2 flask and incubated in a 37ºC, 5% CO_2 incubator overnight. On day 3, this media/transfection mix was removed and replaced with 10ml of fresh DMEM media. On day 5, virus was harvested.

Transduction of B95.8-LCLs

Following the generation of virus using FT293 cells, cell supernatant containing virus was harvested and filtered. After filtration, supernatant was placed into a 15ml tube and concentrated using ultracentrifugation whereby supernatants are centrifuged in metal buckets, using a SW40-rotar at 19500rpm, 16ºC for 3 hours. Concentrated virus was then titrated to check the quality of the virus and one concentrated aliquot per flask was used to transduce $2x10^6$ B95.8 LCLs.

To transduce B95.8 LCLs lytic LCLs were selected on the basis of BZLF1 expression, which was assessed using intracellular BZLF1 staining and flow cytometry. These cells were then washed and aliquoted into $2x10^6$ per 15ml tube (Falcon) for each virus transduction, these were pelleted and the supernatant removed. Concentrated virus was then used to resuspend these cells, after which the virus/cell mix was centrifuged at 19000rpm for 30 minutes at 32ºC. Cells were then resuspended directly into the supernatant and 1ml of standard media was added. This was then incubated in one well of a 24-well plate in a 37ºC, 5%CO_2 incubator. The next day, 1ml of standard media was added and 24 hours after this, the level of transduction was assessed using flow cytometry. If cells were not more than 90% transduced, firstly puromycin is used to enrich the transduced population (see 2.1.13). If this was not successful, transduced cells were sorted by selecting for successfully transduced LCLs which were expressing the appropriate fluorescent tag (fluorescence activated cell sorting). Cells were then maintained in RPMI-1640 + 10% FCS and used in T cell recognition assays.

2.3. **Culturing cells**

EBV transformed LCLs

Established LCLs were maintained in standard media (RPMI-1640 + 10% 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.

shRNA-lentivirus transduced LCLs

Following transduction, LCLs were maintained in standard media. If transduced cells needed to be further enriched, puromycin was added to media (100ug/ml); this was removed once cells were efficiently enriched.

B95.8 virus producing cell line

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

293 and FT293 cells

293 and FT293 cells were maintained in 75cm^2 flasks in 18ml of DMEM media (DMEM + 10% FCS + 50IU/ml penicillin + 50ug/ml streptomycin), with the addition of geneticin for FT293 cells. 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 minute at room temperature. After, 10mls of DMEM media was added and cells were split as required.

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.

CD8+ T cell clones

Established clones were maintained in 24-well plates (2ml/well) with T cell cloning media (RPMI-1640 + 10% FCS + 1% HuS + 30% MLA + 50IU/ml penicillin + 50ug/ml streptomycin + 5-IU/ml IL-2). Cells were fed twice a week by removing 1ml of media and replacing this with fresh media. If clones needed to be restimulated 200ul of T cells were removed from cluture and mixed with allogenic feeder PBMCs (10^6) and autologous LCLs (10^5) in T cell cloning media. After one week these were fed with 1ml media and maintained as normal.

2.4. **Immunological assays**

2.4.1. Chromium release assays

Chromium release assays were used to assess the cytotoxic effects of potential CD8+ T cell clones on target cell lines which had been loaded with Cr^{51} and either infected with vaccinia constructs or sensitised with synthetic peptides.

Target LCLs were infected with vaccinia at an MOI of 10 for one hour at 37ºC, 5%CO_2, with continuous resuspension of cells. To this, 2ml of standard media was added and this was incubated overnight. Following this, cells were pelleted, the supernatant removed and 15ul of Cr-sodium-chromate (Cr^{51}) (Perkin Lemer, 50uCi) was added. At this stage, peptide (5ug/ml) was added to those cells which required peptide sensitisation. Cells were incubated at 37ºC, 5% CO_2 for 1hour 30minutes and then washed twice with standard media. During the incubation of target cells, CD8+ T cells were plated into 96-well plates in 100ul standard media (250cells/100ul) to achieve a target to effector ratio of 1:10. Washed target cells were added to T cells at $2.5x10^3$ cells per well in 100ul standard media. This was carried out in triplicate for each combination. To control for background release of Cr^{51}, the spontaneous release of Cr^{51} from target LCLs in the absence of T cells was measured, as a control for maximum release, target cells were incubated with standard 100ul of sodium dodecyl sulphate (SDS). Plates were then centrifuged at 900rpm for 5 minutes and then incubated for 5 hours at 37ºC, 5%CO_2. Plates were then centrifuged at 1000rpm for 5 minutes and 100ul of supernatant from each well was harvested in to LP2 tubes (Luckmann). Due to the use of vaccinia, these tubes were decontaminated overnight in the presence of paraformaldehyde fumes. Following this, the Cr^{51} release was then counted in a gamma-radiation counter (Cobra

II Auto-gamma, Packard). For all experiments the percentage of specific lysis was calculated using the following equation:

(Release by target cells in the presence of T cell-spontaneous release)/ (Maximum release-Spontaneous release) x 100

2.4.2. IFN-γ enzyme linked immunosorbant assay (ELISA)

To test the ability of CD8+ T cell clones to recognise targets, IFN-g ELISA was used. Firstly, target LCLs, either untreated or sensitised with appropriate peptide, were counted to give $100x10^3$ cells per well (target to effector ratio of 10:1), washed twice in standard media and resuspended in 100ul of standard media per well of a v-bottom 96-well plate. Effector $CD8^+$ T cells were then counted and washed in standard media to give $10x10^3$ cells per 100ul which was added to target cells. All target:effector combinations were carried out in triplicate. As controls, IFN-g release by T cells in the absence of target cells was measured (T cell alone), and as a positive control target LCLs were sensitised with peptide by incubating target LCLs with 5ug/ml of peptide for 2 hours and then washing three times in standard media, before adding to 96-well plate. Cells were then incubated overnight at 37ºC, 5% CO_2, at the same time, MaxiSorp 96-well plates (Nunc) were coated with 50ul per well of anti-human IFN-γ antibody (0.75ng/ml) (Thermo Scientific) in coating buffer (0.1M Na_2HPO_4, pH 9) and incubated overnight at 4ºC.

Following this, MaxiSorp plates were washed with wash buffer (PBS-0.05% tween-20) and then blocked with 200ul of blocking buffer (PBS-0.05% tween-20 + 10% BSA) for two hours at room temperature. Following this, plates were washed four times with wash buffer and then 100ul of cell supernatant from the overnight assay was harvested and added to the MaxiSorp

plates. 100ul of an IFN-g standard was also added, in triplicate, this was done using doubling dilutions (2000pg/ml – 31.25pg/ml) of recombinant IFNγ (peprotech). Plates were then incubated at room temperature for 3-4 hours after which, plates were washed four times with wash buffer. 50ul of biotinylated anti-human IFN-γ (3.75ng/ml) (Thermo Scientific), diluted in blocking buffer, was added to each well and plates were incubated for 1 hour at room temperature. Following this, plates were washed 4 times in wash buffer and then 50ul 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 100ul 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, 100ul 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 IFN-g standard curve, the level of IFN-g release could be calculated as IFN-g release in pg/ml.

2.5. **Detection of protein expression**

2.5.1. Intracellular flow cytometry

In order to detect the presence of EBV lytic proteins, including, BZLF1, BRLF1, BHRF1 and BALF4 in transformed LCLs, we used intracellular flow cytometry. A 1ml aliquot of cells was taken and washed twice with PBS-5%HINGS and then fixed using 100ul of Intracellular Fixation Buffer (ebiosciences) for one hour, on ice. Cells were then permeablised by adding 100ul of 0.02% Triton-X-100 (final concentration of 0.1%), on ice for 30 minutes. Cells were washed twice with PBS-5%HINGS. Following this, 100ul of appropriate primary antibody (see Table 2.1) diluted in PBS-5%HINGS+2% HuS, was added and incubated for one hour at 37ºC. Cells were then washed twice with PBS-5%HINGS. After this, 50ul of secondary either FITC or R-phycoerythrin (RPE) conjugated goat anti-mouse IgG_1-specific secondary antibody (Table 2.1) was added (diluted in PBS-5%HINGS), and incubated for one hour at 37ºC. Cells were then washed three times in PBS-5%HINGS and resuspended in 100ul of IC fixative before being analysed on a flow cytometer.

2.5.2. Flow cytometry

To detect the expression of GFP, CFP, YFP and FP635, transduced cells ($100x10^3$) were washed three times in PBS-FCS and then ran on an using LSR-II or accuri (BD biosciences) and then analysed using Flow Jo (Tree Star).

2.5.3. Western blotting

Cell lysate

For each sample, 10^6 cells were washed and pelleted in PBS by centrifugation and then resuspended in 100ul lysis buffer solution. Lysates were then sonicated for 30s at 30-40%. Samples were either used immediately or stored at -80ºC.

SDS-PAGE

Proteins were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using a mini gel tank. Pre-made resolving gels at 9-12% acrylamide were used. This was done by placing precast gels into a running-tank and submerging them in running buffer (0.192M glycine, 0.025M Tris, 0.1% SDS as pH 8.3). Following this, cell lysates were boiled at 90°C for 5 minutes And then 25ul ($25x10^3$ cells) was loaded into each lane, alongside this, 6ul of SeeBlue Plus2 pre-stained marker (Invitrogen) was added to one well. The gels were then electrophoresed at 60v.

Blotting of SDS-PAGE gels

Resolved proteins were then transferred onto nitrocellulose membranes using transfer chambers as per manufacturer's protocol. This was done by submerging the chamber in blotting buffer, inserting this into a gel tank and surrounding this with water, under a voltage of 95 for 3 hours.

Staining of nitrocellulose membranes

Membranes were blocked for one hour at room temperature using 5% skimmed milk powder dissolved in PBS-Tween 20 (0.05% vol/vol). Specific proteins were then detected by incubation with primary antibodies for BZLF1 (murine monoclonal antibody (MAb) BZ.1, final concentration of 0.5ug/ml), BRLF1 (murine Mab clone 8C12, final concentration 2.5ug/ml), BMLF1 (rabbit serum to EBV BSLF2/BMLF1-encoded SM, clone EB2, used 1/6000), BMRF1 (murine MAb clone OT14-E, used at 1/2000), BALF2 (murine MAb clone OT13B, used at 1/5000), BNLF2a (rat hybridoma supernatant directed to the N-terminus of BNLF2a, clone 5B9, used at 1/1000), diluted in 5% skimmed milk powder dissolved in PBS-Tween 20 (0.05% vol/vol), for two hours at room temperature. Following this, membranes were washed every 15 minutes for one hour, with PBS-Tween.

Bound antibodies were detected by incubating membranes with appropriate secondary horseradish peroxidase (HRP)-conjugated antibodies for one hour at room temperature. Bound secondary antibodies were then detected using enhanced chemiluminescence.

2.6. **Molecular techniques**

2.6.1. 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 generation of the plasmids used for lentivirus production, stable 2 competent bacteria were used. For all other plasmids used in this work, DH5α competent bacteria were used.

2.6.2. Generation of plasmid DNA by bacterial transformation

For generation of plasmids, competent bacteria (either stable-3, or DH5α) were transformed using purified plasmid DNA (50-100ng). This was added to 200ul of competent bacteria in a 1.5ml eppendorf and incubated on ice for 30 minutes. Following this, bacteria was heat shocked for 90 seconds at 42ºC and then placed briefly on ice. 800ul of LB broth was then added and samples were incubated in a shaker at 37ºC for one hour. For each transformation the bacteria were plated by spreading 200ul and 20ul onto two different agar plates (containing the appropriate antibiotic), and a final plate of concentrated bacteria. Plates were then incubated overnight at 37ºC.

Purification of plasmid DNA

Following plating of transformed bacteria, individual colonies were picked and inoculated into a 15ml falcon tube containing 3ml of LB broth, containing appropriate antibiotic. These were incubated in a 37ºC shaker overnight. The next day 1/1000 of this was inoculated into 200ml of LB agar, containing antibiotic, in a 1L conical flask and this was incubated overnight in a 37ºC shaker. The following day 200ml of the bacterial culture was transferred into large containers and pelleted by centrifugation. Following this, plasmid DNA was extracted using a QIAgen Maxi Prep kit, as per manufacturers protocol (QIAgen). DNA was eluted in 200ul of nuclease free water. DNA concentration was quantified using a Nanodrop machine (Thermo Scientific). All DNA was diluted, in nuclease free water, to a concentration of 100ug/ul and stored at -20ºC.

2.6.3. Quantification of EBV transcripts

RNA isolation

Up to $5x10^6$ cells were washed in PBS by centrifuging at 1600rpm for 5 minutes, cells were then resuspended in 1ml PBS, transferred into a 1.5ml eppendorf and pelleted. Cellular RNA was then extracted and purified using RNAeasy Nugen kit, as per manufacturer's protocol. RNA was quantified using a Nanodrop machine (Thermo Scientific), and stored at -80ºC until needed.

DNase treatment of isolated RNA

Since the majority of EBV lytic transcripts are unspliced, an extra step of DNase treatment was carried out, in order to remove any contaminating DNA. This was done using a Turbo DNA-free kit (Ambion), as per manufacture's protocol. 1ug of isolated RNA was used, per reaction. RNA was then stored at -80ºC until needed.

cDNA synthesis

cDNA synthesis was carried out using on 10ul of RNA (0.05ug/ul) in a 0.5ml PCR tube. RNA was denatured at 90ºC for 3 minutes and then plunged into ice. Following this, 4ul of qScript mastermix was added with 6ul of water and incubated as per manufacturer's protocol. qScript master mix, as per manufacturer's protocol. cDNA was then diluted to 5ng/ul and stored at -20ºC until required.

qRT-PCR was performed using an Applied Biosystem 7500 machine. Reactions were carried out in 96-well plates and aliquoted in a sterile PCR room. Each reaction was carried out in duplicate, alongside a non-template control and no reverse transcriptase reaction. Each well consisted of:

5ul cDNA (5ng/ul)

2.5ul forward primer

2.5ul reverse primer

1ul FAM labelled probe

0.5ul VIC labelled GADPDH assay

1ul water

A standard control was included to allow relative levels of transcript to be calculate, this was either induced AKBM or lytic LCLs. cDNA from these cells was generated as above and then diluted to achieve 5000pg/ul, 1000pg/ul, 200pg/ul, 40pg/ml and 8pg/ul.

Samples were run as follows:

50ºC for 2 minutes

95ºC for 10 minutes

40 cycles of: 95 ºC for 15 seconds (denature) then 1minute at 60 ºC (primer anneal and extend). Data was then analysed using Applied Biosystems 7500 software. Transcript numbers were normalised using GAPDH and expressed as transcript level relative to control (or percentage of maximal expression), for each gene.

Table 2.1 siRNA sequences generated by Sigma Aldrich

Gene Target	siRNA name	Sequence 5'-3'
BNLF2a	-036	CACAGAGUACCACCAGGAG
	-123	CGGGCAGGCCGCAGGCAGA
	-140	GAGGACUGCUGCUCUAGCA
BILF1	-251	GUGAAGGUGACGUUGCAUA
	-456	CCAUGGUAAUGAGGAGGAU
	-664	CGAGAACUCCUGAAUCAUU
BGLF5	-363	GUGGAUUGAUGAAGAUGUU
	-541	GCGCUUACGGACAUCUUUA
	-881	CAGAUGAGCUUACAGACAA
	-096	CACGUACGAGCAGAGAACA

CHAPTER 3

GENERATION AND CHARACTERISATION OF NOVEL CYTOTOXIC $CD8^+$ T CELLS SPECIFIC FOR EBV LYTIC PROTEINS

As reviewed in chapter 1, $CD8^+$ T cells play a key role in controlling EBV infection. The ability of EBV to survive in the face of this $CD8^+$ T cell response is thought to be due, in part, to the action of EBV's immune evasion proteins BNLF2a, BILF1 and BGLF5, which interfere with the processing and presentation of viral epitopes during lytic cycle. The overall aim of this Research project was to determine the relative contribution that BNLF2a, BILF1 and BGLF5 make towards interfering with antigen presentation during immediate early (IE), early (E) and late (L) phases of lytic cycle.

Briefly, this was done by assessing the ability of IE, E and L lytic epitope specific $CD8^+$ T cells to recognise EBV infected cells which lacked the expression of BNLF2a, BILF1 or BGLF5. In order to carry out this work, it was important that the $CD8^+$ T cell effectors used encompassed a wide range of specificities to epitopes originating from antigens expressed at all stages of lytic cycle (IE, E and L). For this reason, a selection of $CD8^+$ T cell clones needed to be generated. Ideally these would include known and new specificities but, more importantly for the chosen experimental strategy, was a need to generate a set of $CD8^+$ T cells restricted through the same HLA allele, with specificities directed towards lytic epitopes from all stages of lytic cycle (IE, E, and L).

To date there have been a number of EBV lytic antigens to which $CD8^+$ T cell responses have been identified These span an extensive repertoire of specificities and are restricted through different HLA alleles. This results chapter documents the experiments which led to the

generation of $CD8^+$ T cell clones directed towards EBV lytic antigens. These clones included both known and novel specificities. Three different strategies were employed: i) IFN-γ capture, ii) Identification of novel peptides using mass spectrometry analysis of MHC class I complexes expressed by EBV infected B cells and, iii) limiting dilution using IM donor PBMCs.

3.1. **Generation of known CD8$^+$ T cell clones using IFN-γ capture**

The first experimental strategy used for generating EBV lytic epitope specific CD8$^+$ T cell clones was IFN-γ capture, as described in chapter 2.1.7. A schematic description of this method is shown in Figure 3.1. Briefly, PBMCs from donors positive for common HLA-alleles (Table 3.1) were stimulated with peptides of previously identified CD8$^+$ T cell specificities, which would be presented by particular HLA alleles of each donor. Any T cells specific to the peptides used were stimulated and thus released IFN-γ. These reactive T cells were then selected on their IFN-γ production, and plated out to achieve one T cell clone per well of a 96 well plate. These were expanded and then tested for their specificity using autologous LCLs sensitised with the appropriate peptide. Any T cells which displayed specificity to only one peptide were further expanded and used as effectors in subsequent T cell recognition assays.

This approach allowed us to generate CD8$^+$ T cell clones of known specificity. Both lab and IM donor PBMCs were used as a source of CD8$^+$ T cells (Table 3.1). IM donor PBMCs were preferentially used to generate CD8$^+$ T cells specific to L lytic antigens, as these donors undergo an expansion of CD8$^+$ T cell responses to EBV lytic antigens, thus are more likely to show L lytic antigen specific CD8$^+$ T cell responses. This method of T cell cloning generated numerous clones, as shown in Table 3.2. For practical reasons a maximum of 100 clones were screened from each peptide stimulation.

Those clones generated showed specificities representing each of the three stages of lytic cycle. These included more than 100 clones which recognise the IE antigens BRLF1 and BZLF1, restricted through HLA-A2 and B8 respectively. Numerous HLA-A2 restricted T cells specific to peptides from the E antigens BMRF1 and BMLF1, and a selection of CD8$^+$ T

cells which recognise L late antigens; BALF4 and BNRF1, restricted through HLA-A2. The generation of a complete panel of HLA-A2 restricted T cells is particularly important since this allows us to exclude the influence of HLA type when investigating the effects BNLF2a, BILF1 and BGLF5 have on antigen presentation at IE, E and L stages of lytic cycle.

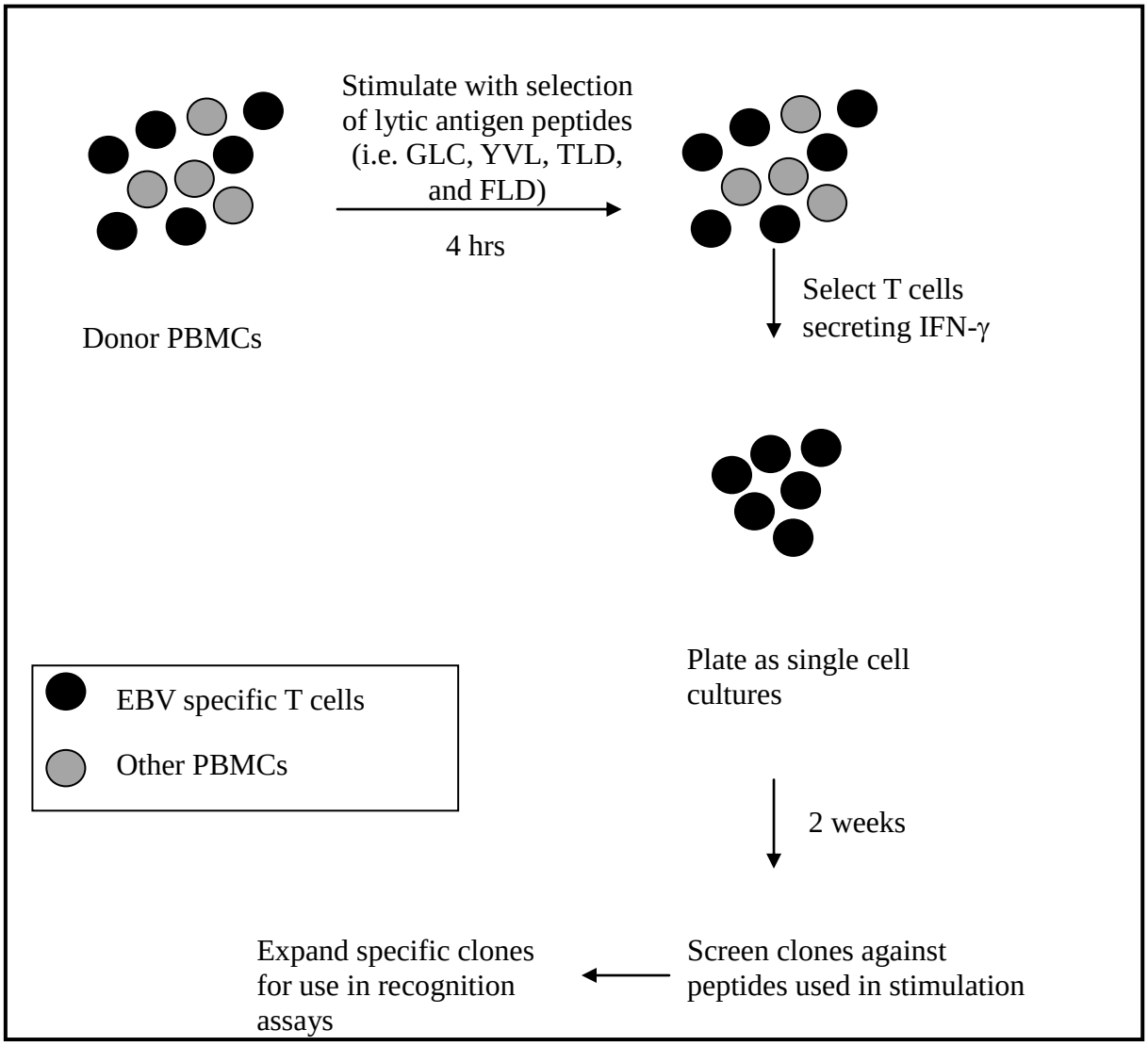


Figure 3.1 Schematic demonstrating the method if IFN-γ capture.

Donor PBMCs are simulated with a selection of known peptides for 4 hours. Cells which recognise peptide and release IFN-γ are selected using beads. These are then plated out and cultured with feeder cells for two weeks. Any T cells which expand are screened against peptides used in the first instance. Any reactive clones are further expanded and used in subsequent assays.

Table 3.1. HLA types of donors used for cloning.

Donor	HLA A		HLA B		HLA C	
IM226	2	-	50	40	3	6
IM225	2	3	7	27	2	7
IM235	2	3	7	40	3	7
LD13/18*	2	29	8	40	3	-

* Donors used in IFN-γ capture T cell cloning only. All other donors were used in both IFN- γ capture and limiting dilution methods of T cell cloning.

Table 3.2. Restrictions and peptide specificities of T cell clones generated using IFN-γ capture

Phase	Protein	Epitope sequence	HLA restriction	Number of clones generated
IE	BZLF1	RAKFKQLL	B8	>50
	BRLF1	YVLDHLIVV	A2	>50
E	BMRF1	TLDYKPLSV	A2	20
	BMLF1	GLCTLVAML	A2	>50
L	BALF4	FLDKGTYTL	A2	25
	BNRF1	WQWEHIPPA	A2	>50

3.2. **Identification of novel epitopes using mass spectrometry**

Despite successfully generating more than 250 characterised clones with useful specificities and HLA restrictions, as shown in Table 3.2, isolation of T cell clones with some other expected specificity was unsuccessful. Notably, no HLA-B7 restricted T cells recognising the late BILF2 antigen were isolated; likewise no T cell clones of the same restriction which recognise the early antigen, BALF2, were obtained. In order to generate more $CD8^+$ T cells with novel specificities, which would allow for a more extensive examination of the effects of BNLF2a, BILF1 and BGLF5 on antigen presentation during lytic cycle, a different approach was adopted. This involved using mass spectrometry to identify MHC class I presented peptides in EBV-infected cells. This element of work was done in a close collaboration with Dr Nathan Croft during a study visit that I made to the Bio21 institute in Melbourne, Australia. A schematic illustrating the experimental approach used is shown in Figure 3.2. Briefly, lytic EBV infected B-cells were lysed and incubated with an anti-MHC class I antibody (W6/32), in order to bind and isolate MHC class I:peptide complexes. The peptides in this complex were then eluted and purified using high performance liquid chromatography (HPLC). These peptides then analysed using mass spectrometry to identify the peptide sequences.

The cells used for these experiments were from an LCL in which around 5% of the cells were spontaneously undergoing lytic cycle replication. The LCL used was established from a HLA-A2 and HLA-B27 positive healthy donor by transformation of isolated B cells with EBV derived from the B95.8 producer line. Following elution and analysis of peptides using in-house analysis software it was possible to identify potential EBV epitopes and their protein of origin (Table 3.3), as well as identifying modifications to these peptides. This software also

generates a confidence score, which reflects the how often the individual peptides were detected.

Of the results generated, only those with a confidence score of more than 95% are normally used. These originated from proteins in the IE and E stages of lytic cycle. An example of novel peptides identified is shown in Table 3.3. It should be noted that some known HLA-A2 restricted peptides were also identified; however these occurred with a lower frequency score than those peptides shown in Table 3.3. The majority of peptides identified originated from latent proteins and since for this work it is of particular importance to generate $CD8^+$ T cells specific to late lytic proteins, this method was not ideal. In addition, the HLA restriction of these peptides would need to be predicted, in order to clone the T cells. Moreover, the presence of a peptide at the cell surface of infected cells does not guarantee the presence of reactive T cells. Therefore, this approach was deemed too time-consuming for this particular work and thus was not followed up. However, this method of identifying potential T cell targets could be used elsewhere, particularly as potential modifications are also identified. These peptides may be overlooked when using overlapping peptides, based on the B95.8 EBV sequence, to identify T cell specificities.

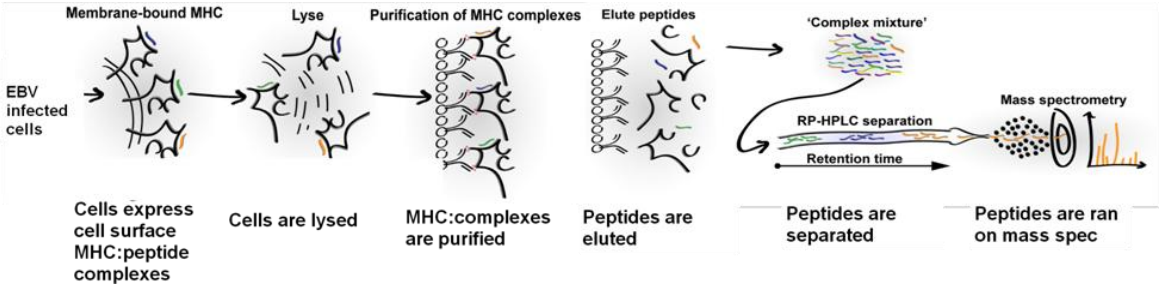


Figure 3.2 Schematic of peptide elution and mass spectrometry analysis of EBV infected cells.

Adapted from Dr Nathan Croft. EBV infected cells were lysed and the MHC:peptide complexes purified using anti MHC class I antibody. Peptides were then eluted using HPLC and their sequence analysed using mass spectrometry.

Table 3.3 Peptides identified using mass spectrometry analysis of EBV infected LCLs.

Peptide sequence	EBV protein of origin	Modifications	Confidence score
GQQLADIGVPQ	BZLF1 (IE)	Oxidation(P)@10	98
APVSTIAPSV	BPLF1 (L)	Oxidation(P)@2; Thr->Ala@5	97
YLRQVATEGL	BALF3 (E)	-	97
RLATVLPGLEV	BALF2 (E)	-	96

Thr-Threonine

Al- Alanine

P- Proline

@- amino acid position of modification

3.3. **Generation of $CD8^+$ T cells specific to EBV lytic antigens**

Although IFN-γ cloning was a successful method for generating $CD8^+$ T cells of known specificities, the panel obtained (Table 3.2) lacked any HLA restrictions beyond HLA-A2. For this reason the method of limiting dilution was used, in the hope that it would allow for the generation of a more extensive panel of $CD8^+$ T cell clones with different restrictions and ideally, a panel of T cells restricted through the same HLA allele, with relativities to IE, E and L lytic antigens.

Three sets of IM donor PBMCs were used as a source of T cells. To set up a limiting dilution cloning, blood specimens were collected from each donor, from which PBMCs were isolated (as described in chapter 2). These were then used in limiting dilution assays as described in chapter 2.1.6. Briefly, PBMCs were plated out in 96 well plates in such a way to statistically allow for 0.3, 3 or 30 PBMCs per well. With the hypothesis that 0.3 cells per well will result in monoclonal T cell populations. These were incubated with feeder cells, autologous LCLs and cytokines required for $CD8^+$ T cell growth. After two weeks, any wells in which there appeared to be T cell growth were selected and expanded from 96 to 24 well plates. Selected T cells were then screened against a panel of EBV lytic antigens. A schematic of this screening technique is shown in Figure 3.3. Briefly, recombinant vaccinia viruses expressing individual EBV lytic antigens were used to infect autologous donor LCLs. These were then loaded with Cr^{51} and used in a cytotoxicity assay, whereby they were incubated with the T cells to be screened. T cells which recognise the antigens being presented by LCLs lyse these Cr loaded LCLs, releasing the Cr^{51} into the supernatant. By measuring the levels of Cr^{51} release, it is possible to identify the antigens which the T cells recognise.

In the first instance, T cells were screened in a cytotoxicity assay against autologous LCLs infected with pairs of vaccinia expressing EBV lytic antigens i.e. BRLF1 and BMLF1 (Fig 3.4). This was done to minimise the number of targets and therefore, the number of T cells required. It should be noted that all antigens used in this assay were expressed from a recombinant vaccinia, with the exception of BZLF1. It was not possible to generate a vaccinia expressing BZLF1 due the toxicity associated with BZLF1 over-expression. Instead, synthetic overlapping peptides covering the amino acid sequence of BZLF1 were used. These were 15 residues in length, overlapping each other by 10 residues. These were used to sensitise autologous LCLs, which were subsequently used as targets in a cytotoxicity assay.

In total, more than 750 clones, form three different limiting dilution cloning experiments were screened using cytotoxicity assays, against 10 autologous targets which were infected with different pairs of vaccinia virus expressing lytic antigens. This allowed for the testing of T cell reactivity against a total of 21 EBV lytic antigens. An illustrative result for this screening strategy for three T cell clones from donor IM225 (c51, c97 and c155) are described below. Each of these clones recognised one target expressing a pair of lytic antigens or the pool of BZLF1 peptides (Fig 3.4). In all three cases there were low levels of background lysis of other autologous LCL targets. Thus, each $CD8^+$ T cell clone recognised a peptide which originated from one of the antigen pairs, or in the case of c97 to a peptide from the BZLF1 antigen.

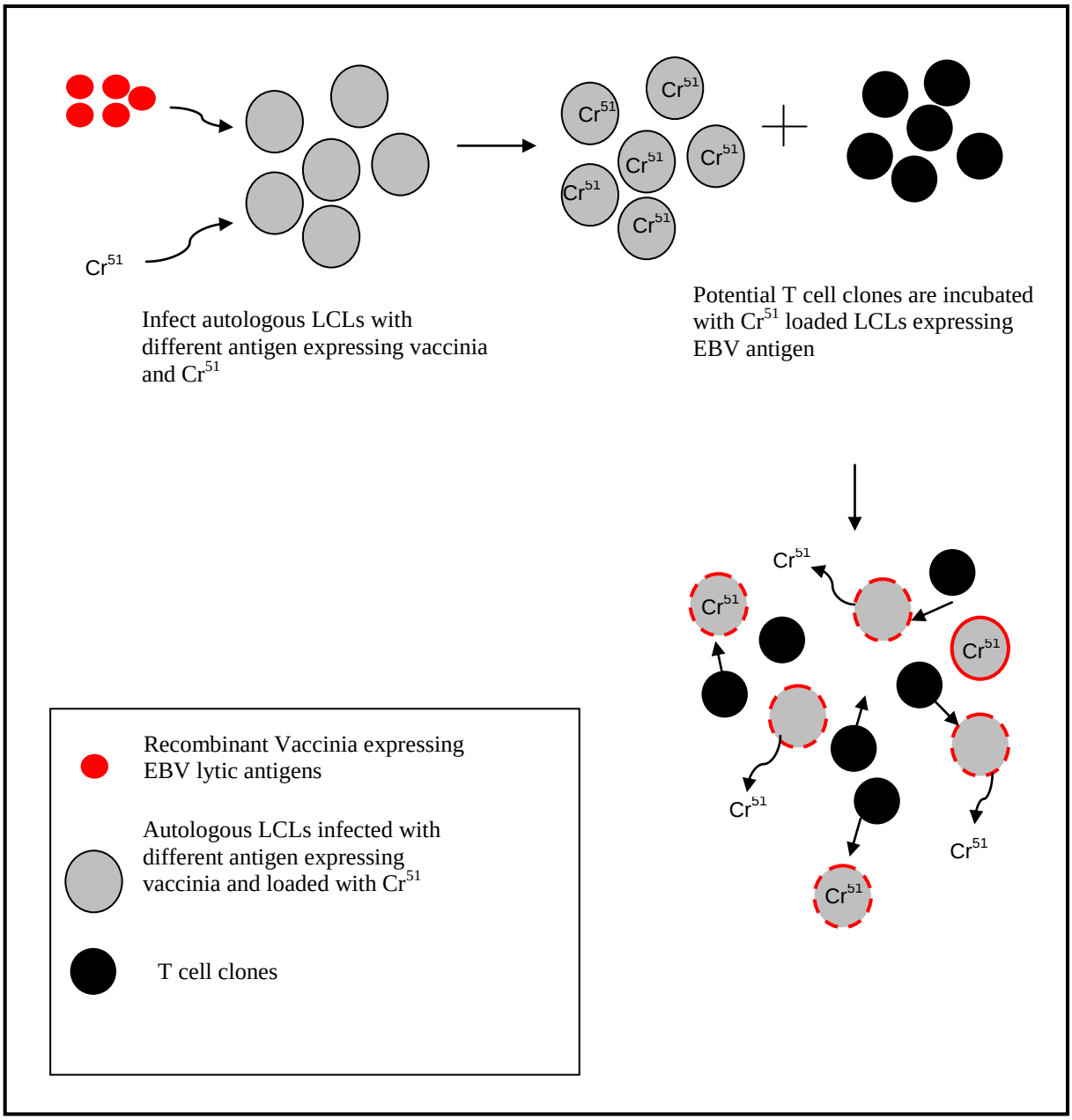


Figure 3.3 Schematic illustrating the cytotoxicity assays used for screening potential T cells clones.

Following the plating of IM donor PBMCs into 1 cell per well, in a 96 well plate, and incubation of cells with feeder cells. Any T cells that grow are screened using a cytotoxicity assay. Autologous LCLs are infected with recombinant vaccinia expressing different EBV lytic antigens. These LCLs are then loaded with Cr^{51} and incubated with the selected T cell clones. T cells which recognise the antigen expressed by the LCLs lyse these cells, which releases the Cr^{51} into the supernatant. The level of Cr^{51} release is then measured, thus indicating the antigen which T cells recognise.

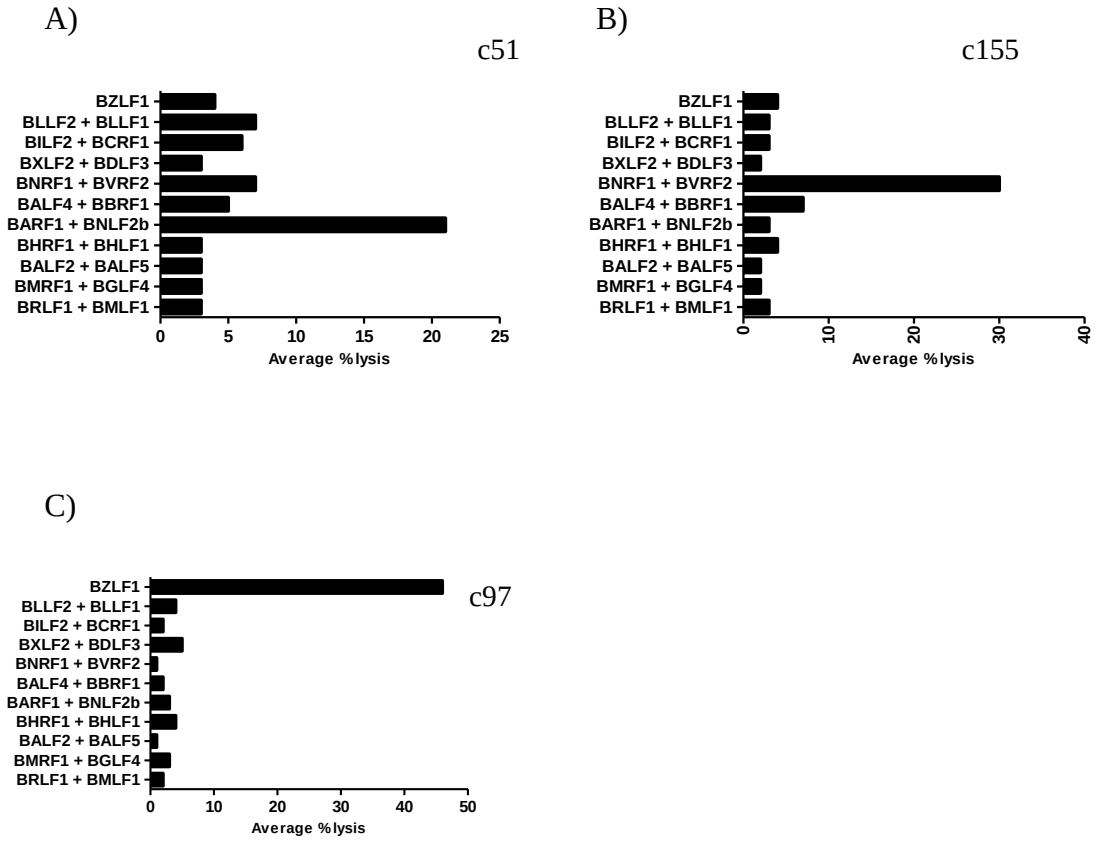


Figure 3.4 Specificity of EBV lytic antigen-specific clones derived from donor IM225.

Three representative clones, c51 (A), c155 (B) and c97 (C) were used in cytotoxicity assays against autologous LCLs infected with pairs of recombinant vaccinia viruses expressing EBV lytic antigens. For BZLF1; overlapping 15-mer peptides for the protein were used. Results are shown as average % lysis.

Having identified T cell clones with significant reactivities, the next steps were to narrow down the lytic antigen specificity, determine the HLA restriction and identify the specific epitope. Where a T cell clone recognised targets infected with a pair of vaccinia viruses (c51 and c 155) the next experiment involved screening these T cells against autologous LCLs infected with the component individual recombinant vaccinia viruses. As shown in Figure 3.5a, c155 was shown to recognise only BNLF2b and c155 recognised only BNRF1 (Fig 3.5b). Where a T cell clone recognised the pool of BZLF1 peptides, the specificity was narrowed down by screening against target LCLs stimulated with separate pools of peptides from the N-terminus or C-terminus of BZLF1; screening c97 in this way showed reactivity to the N-terminus peptide pool only (Fig 3.5c).

Before proceeding to find the peptides to which these T cells were specific, experiments were carried out to determine the HLA restrictions of these clones. This allows for the prediction of possible peptides, since different HLA alleles have different anchor residue requirements. To identify the HLA molecules responsible for presenting peptide to these clones, a panel of partially HLA matched LCLs which express different combinations of HLA molecules were infected with vaccinia expressing antigen or sensitised with BZLF1 N-terminus peptide, and tested for their ability to stimulate responses from clones c51, c155 or c97, in cytotoxicity assays. Donor LCLs used to reveal the HLA-restriction element for each of the T cell clone are shown in Table 3.4, where the boxed alleles are those shared with donor IM225. Expression of the relevant target antigen for each T cell clone in this panel of partially matched allogeneic LCLs enables the use of this panel of LCLs as targets in cytotoxicity assays to reveal the HLA restriction.

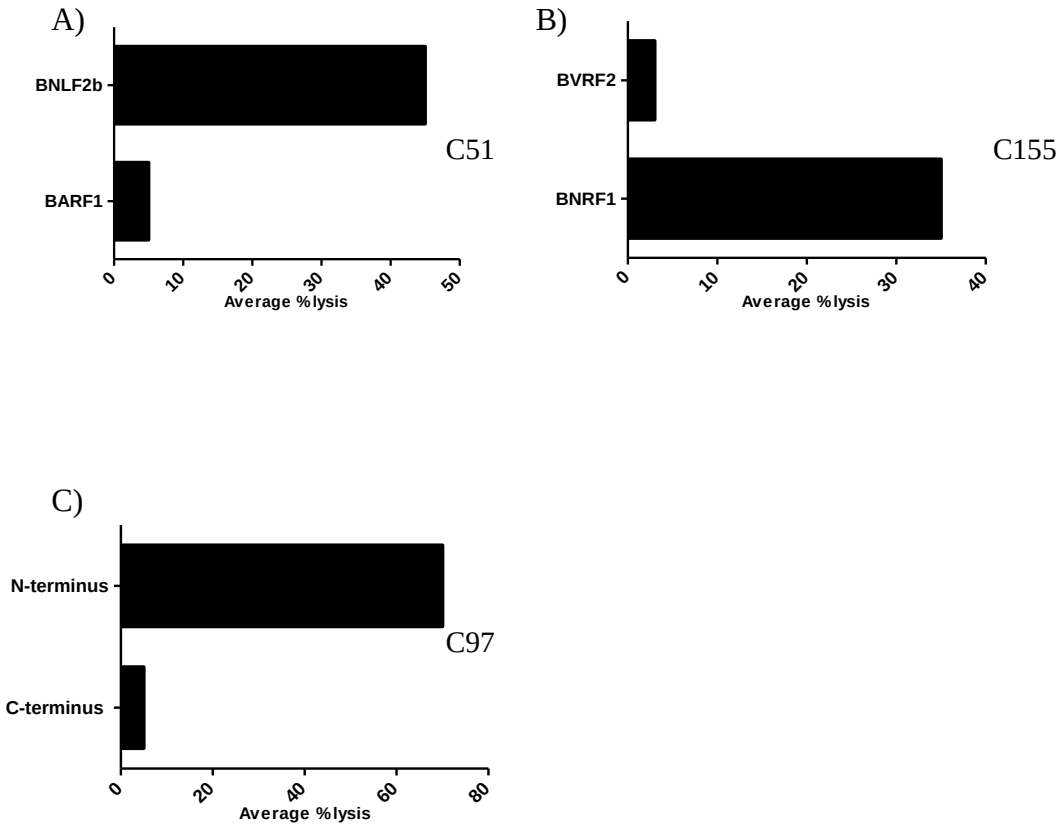


Figure 3.5 Individual antigens recognised by c51, 97 and 155.

Clones c51 (A), c155 (B) and c97 (C) were screened in a cytotoxicity assay against autologous LCLs infected with single recombinant vaccinia viruses expressing proteins BARF1, BNLF2b for c51, BNRF1 and BVRF2 for c155 and the N- or C- terminus of BZLF1 protein in the case of c97. Results are shown as average % lysis.

Table 3.4 Class I HLA types of LCLs used to determine the HLA restriction of CD8+ T cell clones c51, c155 and c97.

*Black squares represent HLA alleles expressed by both IM225 and those LCLs used in the panel.

Not determines (ND)

Donor	HLA A		HLA B		HLA C	
IM225	2	3	7	27	2	7
(14,7)	2	32.01	44	ND	5.01	ND
(22, 23)	1	3	8	35	4	7
(19, 18)	3	31	7	16	W3	7
(18, 20)	2	24.02	27	35.01	2	4
IM83	2	ND	45.01	51	2	ND
(8, 12)	1.01	2	44.02	57	5.01	7

As shown in Figure 3.6a, after LCLs were infected with BNLF2b expressing vaccinia, c51 lysed LCL LD19,18 and the autologous IM225 LCL. These LCLs both possess HLA-A3, -B7 and -C7 alleles. The lack of lysis of LCLs from donor LD22,23 ruled out the possibility of these clones being restricted through HLA-A3 and no lysis of LD22,23 or LD8,12 LCLs ruled out the restriction being through HLA-C7. Thus c51 recognises an epitope presented by HLA-B7. When the BNRF1-c155 effectors were similarly assayed on the same panel of LCLs but expressing BNRF1, the result was the identical; revealing that this clone was also restricted through HLA-B7. Likewise, when the same panel of LCLs were sensitised with the N-terminus of BZLF1, and used in a cytotoxicity assay with c97, a similar result was seen. Thus c51, c155 and c97 are all restricted through HLA-B7. This is of importance for this work, since it represents a set of $CD8^+$ T cells recognising antigens form each of the three phases of lytic cycle (IE, E and L) and restricted through the same common HLA allele.

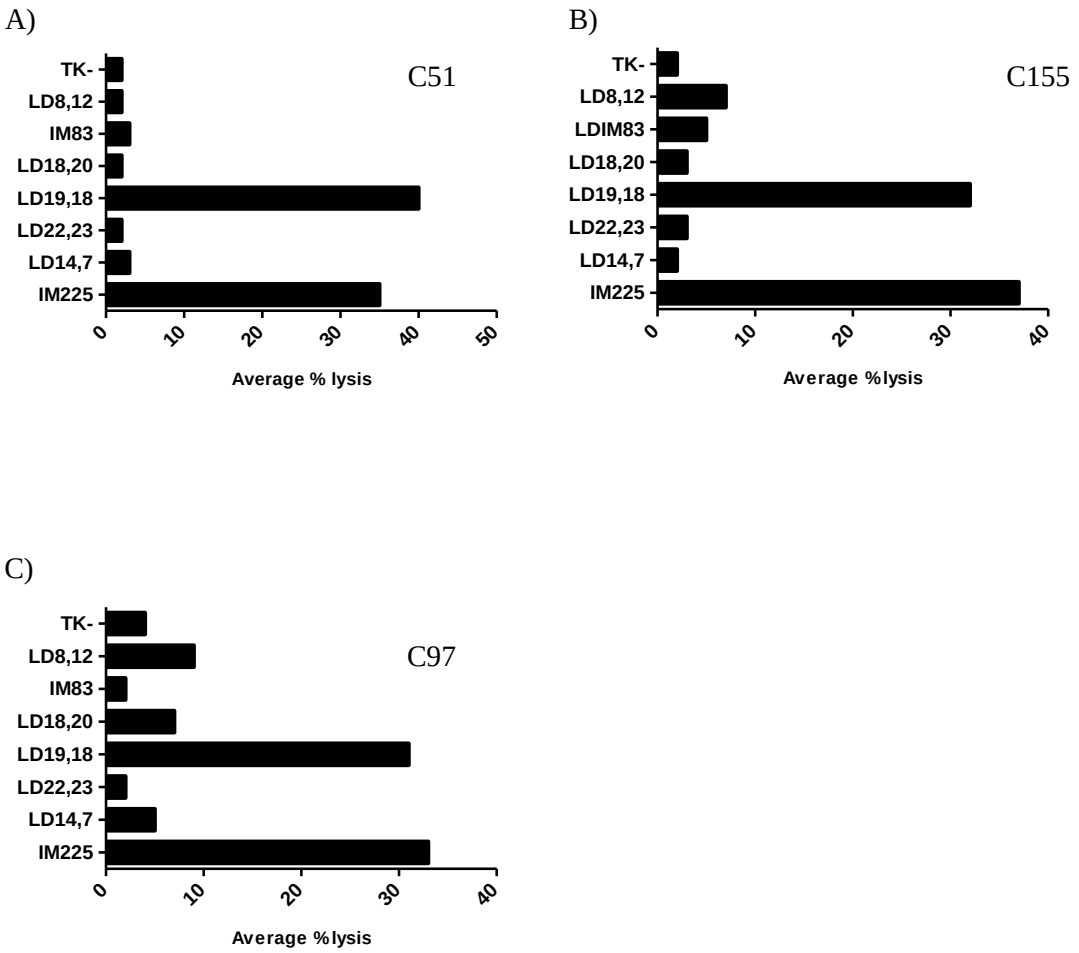


Figure 3.6 HLA restriction of BNLF2b spcific c51, BNRF1 specific c155 and BZLF1 specific c97 T cells.

(A, B) c51 and c155 T cells were used in cytotoxicity assays against autologous LCLs and LCLs from donors LD14,7, LD22,23, LD19,18, LD18,20, IM83 and LD8,12 which were infected with vaccinia expressing BNLF2b or BNRF1 respectively. As a negative control an empty vaccinia (TK-) was used to infect the same panel of LCLs (C) The same panel of LCLs were loaded with synthetic peptides spanning the N-terminus of BZLF1. These were used to probe recognition by c97. Results are shown as average % lysis.

Following identification of the HLA restriction element and EBV-antigen specificity of these clones, their epitope specificity was then investigated. To determine the specificity of c97, BZLF1 N-terminus peptides were divided into five pools of peptides, and these were used to sensitise autologous LCLs. Using peptides as opposed to vaccinia viruses allowed for the use of IFN-γ ELISA as a read out of T cell recognition, which is considered more sensitive than cytotoxicity assays. As controls, IFN-γ release was measured from c97 alone and from c97 co-cultured with LCLs sensitised with an equivalent dilution of DMSO, in which peptides are dissolved. As Figure 3.7a shows, IFN-γ release was highest when LCLs were sensitised with pool 1 peptides, a level comparable to that seen when LCLs were sensitised with the N-terminus peptides of BZLF1. This demonstrates the specificity of c97 is for a peptide located in pool 1 of BZLF1 N-terminus. Target LCLs were then similarly sensitised with individual synthetic peptides from pool 1 and used as targets in an IFN-γ ELISA. As Figure 3.7b shows, c97 is specific for a 15-mer peptide DPYQVPFVQAFDQAT. HLA-B7 restricted peptides characteristically contain a proline at position two and are typically 9-11 residues in length. A series of peptides of 9-12 residues long, containing one of the two prolines at position 2, were therefore synthesised to more finely map the specific epitope within the 15-mer DPYQVPFVQAFDQAT. Autologous LCLs were sensitised with these peptides and used to probe the recognition by c97 in an IFNγ ELISA (Fig 3.7c). The 11-mer peptide DPYQVPFVQAF showed the most effective sensitisation c97 T cells, with significant, but less efficient sensitisation achieved with longer and shorter peptides containing the DPY proline anchor sequence.

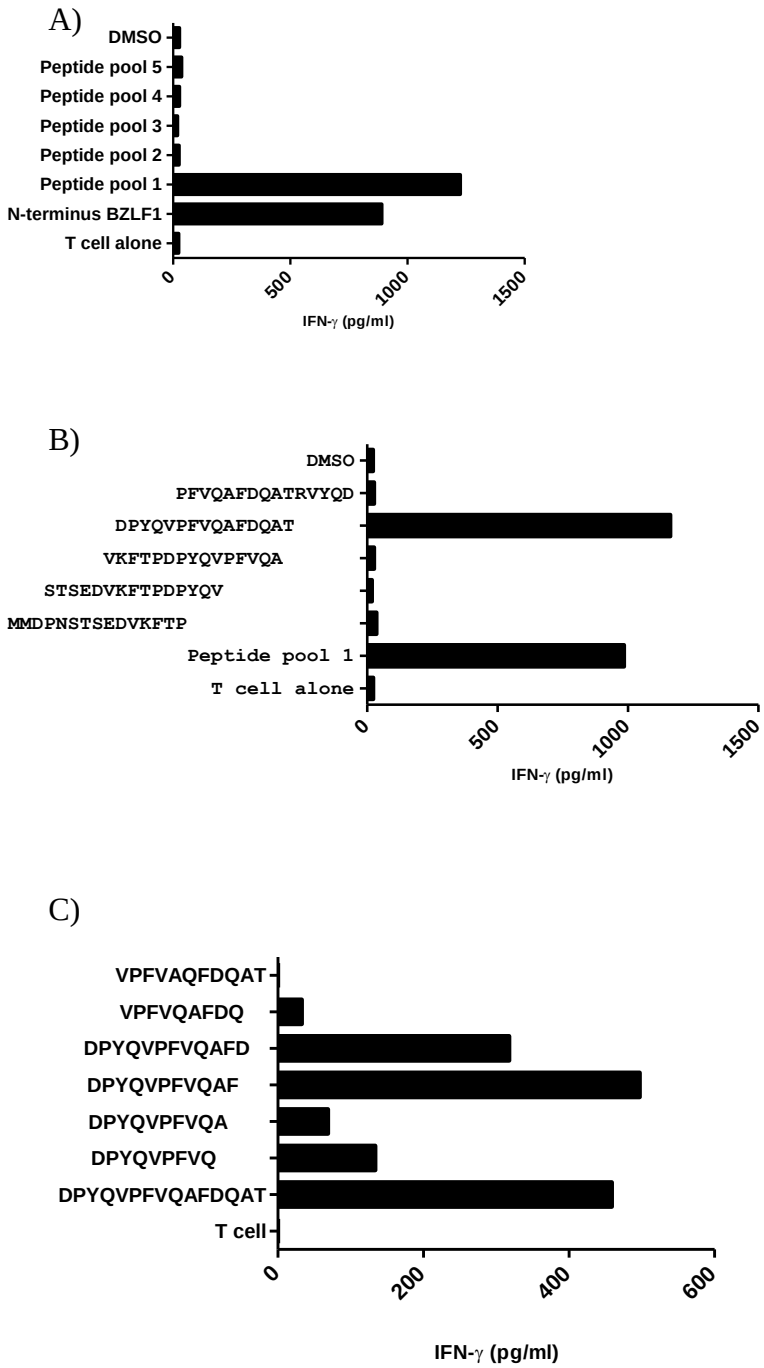


Figure 3.7 Peptide specificity of c97.

(A) Peptides spanning the N-terminus of BZLF1 protein were divided into five peptide pools. These were used to sensitise autologous LCLs which were used to probe the recognition of c97. (B) Pool 1 of N-terminus BZLF1 was further divided into single 15-mer peptides. Which were used to sensitise autologous LCLs. These were then used to probe the recognition of c97. C) The 15-mer peptide was further minimised and used to sensitise autologous LCLs. All results are shown as pg/ml of IFN-γ release.

To determine the peptide specificity of clone c51, a similar approach was taken. Since BNLF2b is a relatively small protein of only 98 amino acids in length, synthetic peptides were generated (synthesised by peptide .0), which spanned the entire BNLF2b protein sequence. These peptides were 15-amino acids in length and overlapped by 10 amino acids. These were first divided into four pools and used to sensitise autologous LCLs for use as targets in a cytotoxicity assay. Clone c51 showed 50% specific lysis against LCLs sensitised with pool 1, which was comparable to the lysis of target LCLs infected with BNLF2b recombinant vaccinia virus, while targets incubated with peptide pools 2, 3, 4, or with DMSO solvent alone all showed less than 5% specific lysis (Fig3.8a). Autologous LCLs were then sensitised with the individual component peptides of pool 1. As Figure 3.8b shows, c51 specifically lysed only those LCLs sensitised with the MRPGRPLAGYATLR peptide. To more finely map the target epitope of c51, shorter synthetic peptides of MRPGRPLAGYATLR were synthesised based around a proline residue located at amino acid position two (Fig 3.8b). c51 specifically lysed LCLs sensitised with peptides RPGRPLAGFYA and RPGRPLAGFYATL to similar extents, suggesting that the minimal epitope for c51 is RPGRPLAGFYA (Fig 3.8c). To determine which of these two peptides c51 was specific for, peptides RPGRPLAGFYA and RPGRPLAGFYATL were diluted from 10^{-6}M to 10^{-10}M. These diluted peptides were then used to sensitise target LCLs in an IFN-γ ELISA. Clone 51 showed more specific recognition of RPGRPLAGFYA at the lower concentrations (Fig3.8d), suggesting RPGRPLAGFYA is the HLA-B7 restricted minimal epitope of c51.

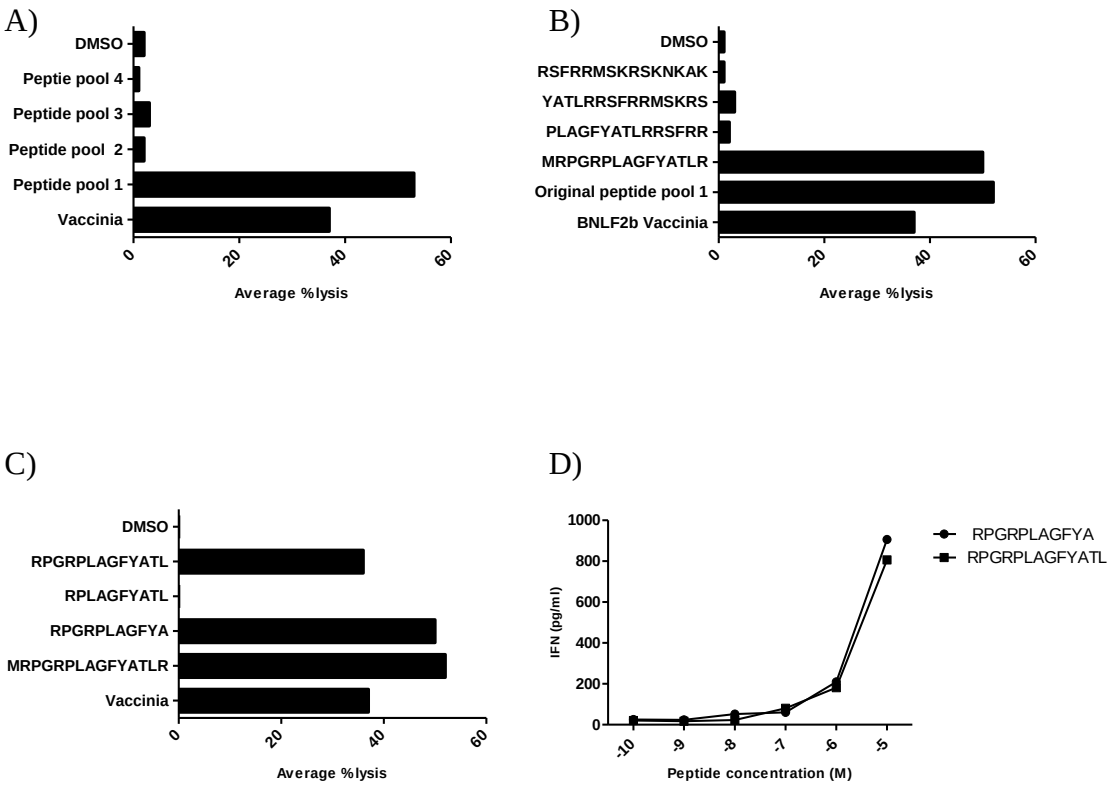


Figure 3.8. Minimal epitope mapping of HLA B7 restricted BNLF2b-c51.

(A) 15-mer BNLF2b peptides spanning the whole protein were divided into four pools. These were used to sensitise autologous LCLs, which were used to probe recognition by c51 in a cytotoxicity assay. Autologous LCLs were also infected with vaccinia expressing BNLF2b, as a positive control and an equivalent concentration of DMSO was used as a negative control. Results are shown as average % lysis. (B) Peptide pool 1 was further divided into individual peptides. These were used in a cytotoxicity assay to sensitise autologous LCLs, an equivalent concentration of DMSO was used as a negative control. Autologous LCLs were infected with vaccinia expressing BNLF2b, and original peptide pool 1 was included as positive controls. Results are shown as average % lysis. (C) Peptide 1 was minimised from its original 15mer length. These shorter peptides were used to sensitise autologous LCLs. The full length 15-mer peptide 1 and LCL infected with vaccinia expressing BNLF2b were also used as positive controls. An equivalent concentration of DMSO was used as a negative control. (D) Lytic cycle defective HLA-B7 positive LCLs were sensitised with peptides RPGRPLAGFYATL and RPGRPLAGFYA in decreasing concentrations. These were used in an ELISA. Results are shown as IFN-γ release (pg/ml).

To determine the minimal epitope of BNRF1- c155, a different technique was used. Since the BNRF1 protein is a large protein of 1318 amino acids in length, it would have required the synthesis and screening of more than 200 15-mer peptides. Therefore, a more cost-effective screening strategy was devised. Four overlapping fragments of BNRF1 were amplified from the BNRF1 DNA sequence (Fig.3.9a) and inserted into a pCDNA3.1-TOPO vector. These were used to transfect HLA-B7 positive 293-cells, for use as targets for c155 in an IFN-γ ELISA. As shown in Figure 3.9b, c155 recognised cells expressing fragment 4 of BNRF1, to a level comparable to that of BNRF1-vaccinia infected 293 cells. 20-mer overlapping peptides were then synthesised to span the length of fragment 4. These were divided in to six pools and used to sensitise 293 cells. C155 recognised a peptide contained in pool 4 of fragment 4 (Fig.3.9c). Pool 4 was then further divided into individual peptides and these were used to sensitise 293 cells, whereupon c155 was found to recognise AMNYPRNPTEQGNIAGLCSR to a similarly high level as pool 4 peptides, while other individual peptides were not recognised above background levels (Fig 3.9d). Smaller synthetic peptides were then generated by deleting one amino acid in the sequence, based around a proline residue at position 2. Titration of the 6 individual peptides and assaying IFN-γ release from co-incubated c155 T cells, revealed the 11-mer peptide YPRNPTEQGNI to be the most potent, with LCLs sensitised with as little as 10^{-9} M of peptide inducing IFN-γ release (Fig. 3.9e). A summary of the T cells generated using limiting dilution is shown it Table 3.4.

Overall, the above T cell cloning strategies generated two panels of lytic antigen specific $CD8^+$ T cell clones, one panel restricted through HLA-A2 and the other through HLA-B7.

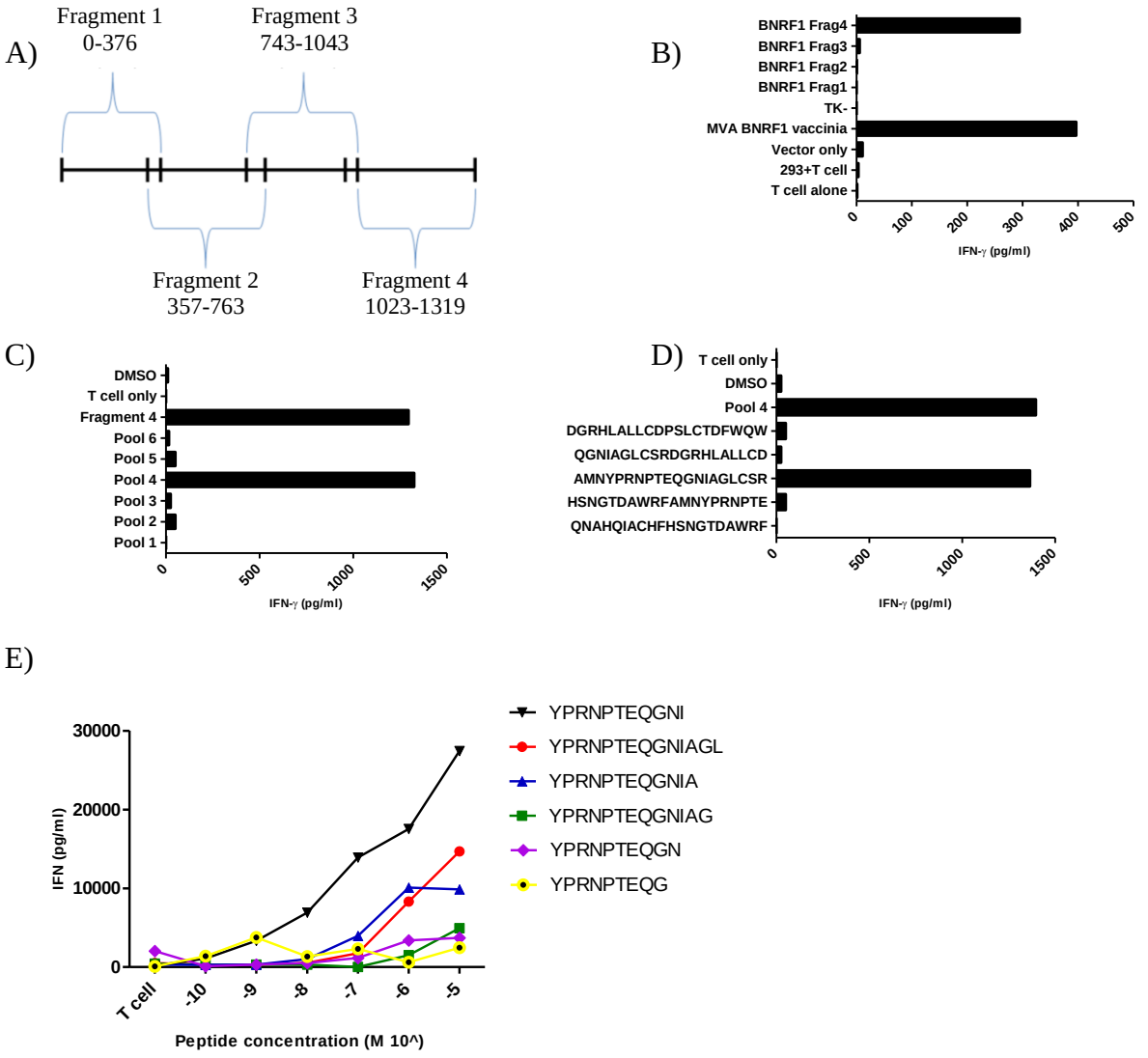


Figure 3.9. Minimal epitope mapping of HLA B7 restricted BNRF1-c155.

(A) Diagram shows the length (amino acids) and position of each fragment amplified from BNRF1 DNA. (B) BNRF1 fragments were and transfected into 293 cells, which were subsequently used as targets for c155, recognition was measured as IFN-γ release using ELISA. Autologous LCLs infected with BNRF1 expressing vaccinia were used as a positive control. T cell alone and T cell with 293 cells transfected with empty vector were used as negative controls. Results are shown as IFN-γ release pg/ml. (C) 20-mer overlapping peptides designed to cover the region of BNRF1 fragment 4 were split into 6 pools. These were used to sensitise autologous LCLs which were used to probe the specificity of c155. (D) Peptide pool 4 was divided into single 20-mer peptides. Autologous LCLs were sensitised with these, pool 4 was included as a positive control and DMSO and T cell alone as a negative controls. Results are shown as IFN- γ release pg/ml. (E) Shorter derivatives of AMNYPRNPTEQGNIAGLCSR were designed and used to sensitise lytic negative HLA-B7 LCLs, at decreasing concentrations. Results are shown as pg/ml of IFNγ.

Table 3.5 CD8$^+$ T cell clones generated using limiting dilution

Phase	Protein	Epitope sequence	HLA restriction	Cloning technique and number of clones generated
IE	BZLF1	DPYQVPFVQAF*	B7	1
	BRLF1	YVLDHLIVV	A2	>50
E	BMRF1	TLDYKPLSV	A2	>50
		LAY	C3	1
		YRS	C6	1
	BMLF1	GLCTLVAML	A2	>50
	BNLF2b	RPGRPLAGFYA*	B7	1
L	BNRF1	YPRNPTWQGNI*	B7	1

* Novel CD8+ T cell specificities identified during this work

3.4. **Identification of BNRF1 as a new latent antigen**

Although the specificity of those T cell clones generated above had been determined, this project required T cells that are potent enough to efficiently recognise endogenously processed lytic antigens that are presented in a minor subpopulation, often less than 2%, of LCLs undergoing lytic cycle replication. To assess their usefulness in this context, all clones were validated on wild-type (B95.8-EBV transformed) LCLs in which around 2% were undergoing lytic cycle, as determined by BZLF1 expression (Fig 3.10), and on LCLs generated with ΔBZLF1 EBV, which are defective for lytic virus replication. As shown in Figure 3.11a and Figure 3.11b, BZLF1-c97 and BNLF2b-c51 showed relatively high levels of recognition of B95.8 LCLs, compared to peptide sensitised ΔBZLF1-LCLs, thus they were deemed potent enough for use in this research project. In the course of these validation experiments, an interesting phenomenon was observed, where BNRF1-c155 showed good recognition of ΔBZLF1 LCLs, as well as peptide sensitised and B95.8 lytic LCLs (Fig 3.11c). This was surprising since BNRF1 is reported as being expressed only in late stages of lytic cycle, thus this finding was further investigated.

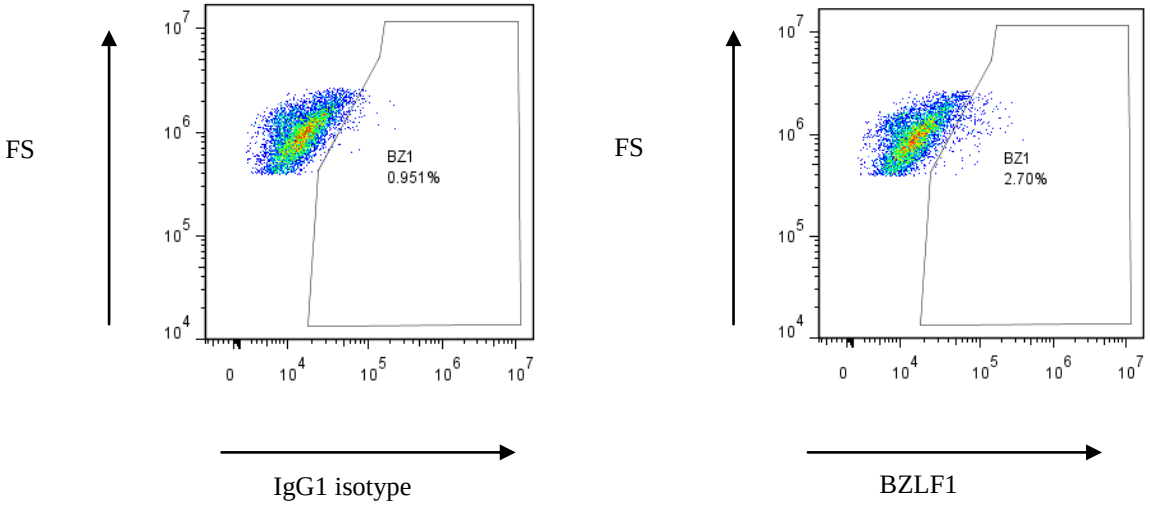
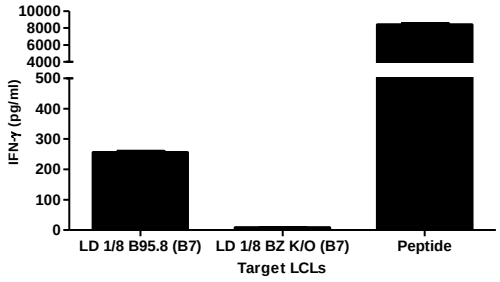


Figure 3.10 BZLF1 expression levels in B95.8 transformed lab donor LCLs.

Lab donor LCLs were fixed, permeablised and then stained with antibody for either BZLF1 or an isotype control (IgG1). Cells were analysed by flow cytometry. Dotplots show forward scatter (FS) versus BZLF1 or isotype control staining.

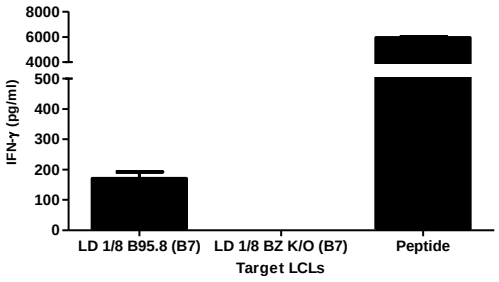
A)

C97 recognition of DPY (BZLF1 HLA-B7)



B)

C51 recognition of RPG (BNLF2b HLA-B7)



C)

C155 recognition of YPR (BNRF1 HLA-B7)

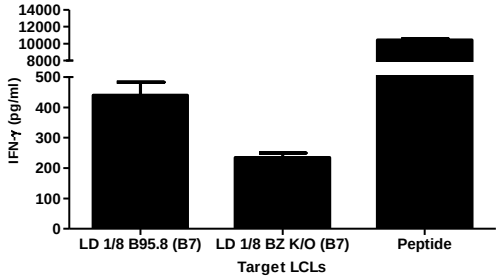


Figure 3.11 Recognition of endogenously expressed epitopes.

C97 (A), c51 (B) and c155 (C) were used as effectors in an IFN-γ ELISA T cell recognition assay against B95.8 and ΔBZLF1 transformed LCL targets. As a positive control ΔBZLF1 LCLs were sensitised with peptide. All results are shown as IFN-γ release (pg/ml).

The first stage in proving or disproving this new finding was to determine if the above result was consistent using other lab donor LCLs. To do this, two sets of B95.8 and ΔBZLF1 transformed LCLs were used, from two different lab donors, which expressed both HLA-B7 and HLA-A2 alleles. These were used as target cells in an IFN-γ ELISA T cell recognition assay against c155. As shown in Fig 3.12a the recognition of ΔBZLF1 LCLs by c155 was comparable to the recognition of their B95.8-LCL counterparts. This was the same for both donors LD13,1 and LD7,19.

Next, to confirm that ΔBZLF1 LCLs were in fact devoid of lytic cycle expression, the same assay was performed, this time using a $CD8^+$ T cell which recognised an epitope (YVL) which originates from the IE protein BRLF1. Using this T cell there was no recognition of the ΔBZLF1 LCLs but good recognition of the B95.8 LCLs (Fig 3.12b). This confirms that the ΔBZLF1 LCLs were not undergoing lytic cycle replication and that BNRF1 does appear to be expressed in latent LCLs. To further support these data, the levels of BNRF1 and BRLF1 mRNA expression was measured using qRT-PCR analysis. (Fig 3.12d). As shown, there was no detectable transcript of BRLF1 in either LD13,1 or LD7,19 ΔBZLF1 LCLs, confirming that these cells are not entering lytic cycle. The levels of other transcripts were also measured to confirm these cells were truly latent (data not shown). However, the level of BNRF1 transcript in ΔBZLF1 LCLs from both donors was higher than background, and comparable to that seen in LD7,19 B95.8 LCLs (Fig 3.12c). Taken together these data confirm the expression of BNRF1 in ΔBZLF1 LCLs. These experiments were repeated using different donor LCLs and a HLA-A2 restricted, BNRF1 specific clone, which gave the same result (data not shown).

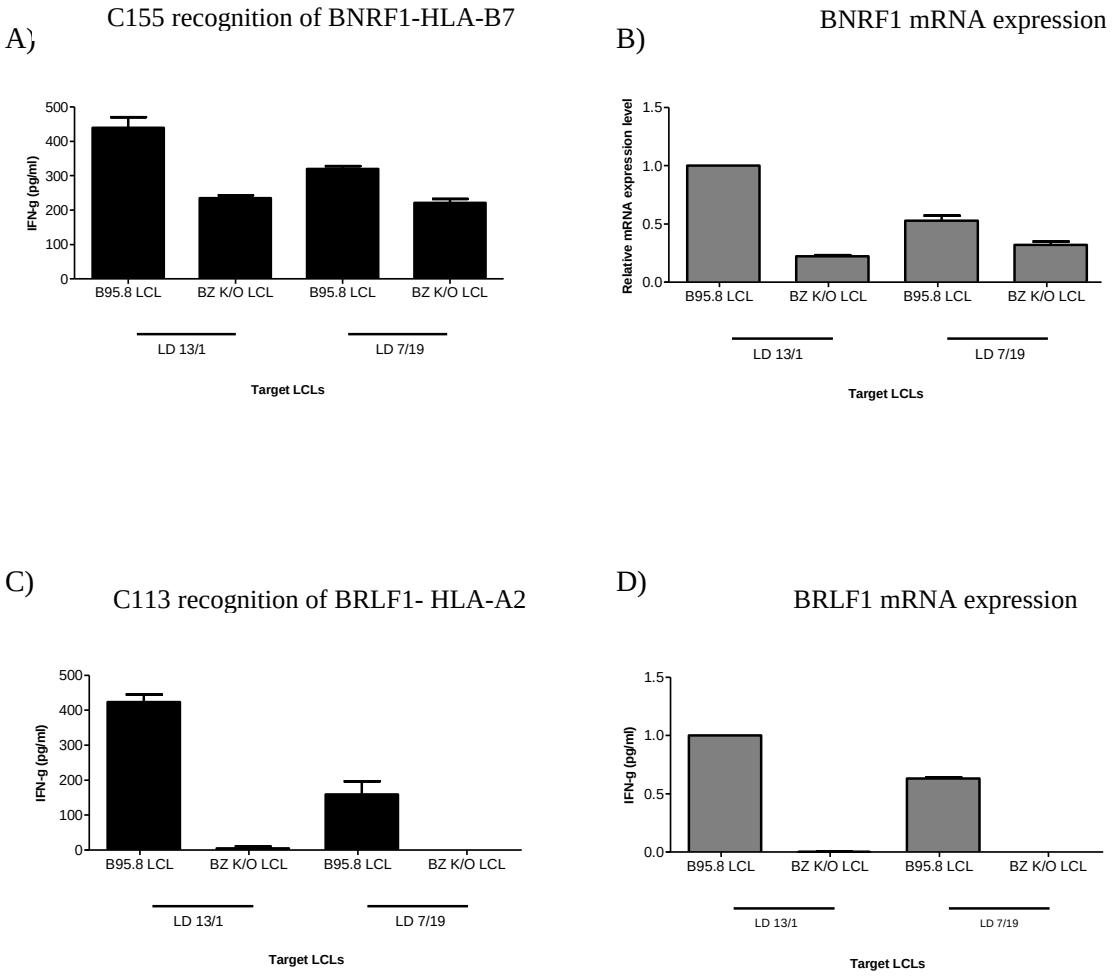


Figure 3.12 Recognition of BNRF1 in lytic incompetent cells.

(A) Two pairs of B95.8 and ΔBZLF1 LCLs from HLA-B7 positive donors LD13,1 and LD7,19 were used as targets c155 in and IFN-γ ELISA. Results shown as IFN-γ pg/ml (B) The level of BNRF1 mRNA in LD13,1 and LD7,19 was measured using qRT-PCR analysis. Results are shown as mRNA relative to LD13,1 BNRF1 mRNA levels. (C) B95.8 and ΔBZLF1 LCLs from donors LD13,1 and LD7,19 were used as targets in and IFN-γ ELISA to probe for recognition by a HLA-A2 restricted BRLF1-specific clone (c133). Results are shown as IFN-γ pg/ml. (D) The level of BRLF1 mRNA expression was measured in these cells using qRT-PCR analysis. Results are shown as levels relative to levels in LD13/1.

3.5. **Discussion**

Since the overall aim of this research project was to determine the effect of EBVs immune evasion proteins BNLF2a, BILF1 and BGLF5 on antigen processing and presentation at the IE, E and L stages of EBV lytic cycle, it was important to have a variety of $CD8^+$ T cell effectors which recognise antigens expressed in all of three phases of lytic cycle. The novel HLA-B7 and known HLA-A2 restricted $CD8^+$ T cell clones which were generated were of particular importance as they represent two panels of T cells with specificities directed towards IE, E and L epitopes. This allowed for a more extensive investigation of the HLA specificity of the immune evasion proteins could be addressed.

Although the use of mass spectrometry identification of epitopes presented by EBV infected cells was not useful for this Research project, it does raise some interesting points. The fact that no late epitopes were detected could indicate that, in support of previous findings, late epitopes are less well presented by B95.8-LCLs. It would be interesting to carry this work out using LCLs lacking in the expression of BNLF2a, BILF1 and BGLF5 to determine if these knockdown LCLs show better presentation of L epitopes.

The novel finding of BNRF1 expression in lytic incompetent LCLs has important implications in the field of EBV biology. It has recently been found that CD8+ T cell responses directed towards BNRF1, in healthy individuals and IM patients, accounts for a dominant response (Abbott et al in review). To extend this work further, it would be an advantage to investigate the expression of this protein in tightly latent EBV infected cells, such as in NK/T cell lymphomas. This would confirm whether the expression of BNRF1 protein in latency is a phenomenon of ΔBZLF1 LCLs only. Since the majority of EBV

malignancies are typically latent, this could represent an important target for treatment or vaccine design.

CHAPTER 4

USING RECOMBINANT EBV TO INVESTIGATE THE FUNCTIONAL IMPOTRANCE OF PUTATIVE IMMUNE EVASION GENES

As described in chapter one, the detection and clearance of virally infected cells by the host immune system relies upon $CD8^+$ T cell recognition of viral peptides presented by MHC class I molecules. EBV infected cells undergoing lytic cycle replication are particularly vulnerable to recognition by these T cells, since lytic cycle involves the expression of more than 90 viral genes, creating an extensive pool of viral antigens for presentation to the immune system. As a consequence of this, EBV, like other viruses, has evolved to express multiple immune evasion genes during lytic cycle, which are able to interfere with MHC class I processing and presentation. The importance of these immune evasion mechanisms during lytic cycle is implied by the potency and magnitude of the $CD8^+$ T cell responses of healthy infected individuals, most of whom demonstrate continuous production of infectious EBV. A number of candidate immune evasion genes have been identified using gene-expression model systems. Amongst these genes are BNLF2a, BILF1 and BGLF5. It is presumed, but not formally shown that they are all operative and cooperate in during lytic cycle. This issue will be the main focus of this thesis.

To demonstrate the functional significance of BNLF2a, BILF1 and BGLF5 in the context of EBV lytic cycle, it was decided first to take advantage of the available recombinant EBVs deleted for BNLF2a, BILF1 and BGLF5 expression. The panel of $CD8^+$ T cell clones generated in chapter 3 allowed for the investigation of the sensitivity of recombinant infected cells, undergoing lytic cycle reactivation, to recognition by these T cells. To this end, ΔBNLF2a-, ΔBILF1- and ΔBGLF5-transformed LCLs along with their wt-2089 and

ΔBZLF1-counterparts were used as targets for $CD8^+$ T cells specific for IE, E and L lytic epitopes. This allows for the assessment of the influence of BNLF2a, BILF1 and BGLF5 on presentation of lytic epitopes during the IE, E and L phases of lytic cycle. The proposed experiments presented technical difficulties that required carefully designed controls and optimisations, outlined below.

4.1. **Experimental strategy and methods**

LCLs were generated using wt2089, ΔBNLF2a-, ΔBILF1- ΔBGLF5- and ΔBZLF1-virus to transform donor B cells, as described in Chapter 2 1.5. LCLs were generated from numerous laboratory donors in order to span a variety of HLA class I alleles, these are summarised in Table 4.1. These LCLs were then used as target cells in $CD8^+$ T cell recognition assays.

A selection of $CD8^+$ T cell clones generated from the work outlined in chapter 3 were used, these are summarised in Table 4.2. This Table shows their antigen and epitope specificity, the phase of lytic cycle in which the antigen is expressed and the HLA class I restriction of each clone, along with the number of clones used from each specificity.

Table 4.1 HLA alleles of donors used to generate wt2089, ΔBNLF2a-, ΔBILF1- ΔBGLF5- and ΔBZLF1-LCLs.

Donor number	HLA A		HLA B		HLA C	
1	2	24	27	35	2	4
2	2	29	8	40	3	ND

ND-Not determined

4.2. **Quantification of spontaneous lytic antigen expression in knockout LCLs**

T cell recognition assays outlined in this chapter relied upon target LCLs (wt2089-, ΔBNLF2a-, ΔBILF1- ΔBGLF5-virus transformed) spontaneously entering lytic cycle. The proportion of an LCL culture which will undergo spontaneous EBV-lytic cycle reactivation varies significantly between lines and even within the same line over time. Since this directly impacts the level of antigen available for presentation and therefore $CD8^+$ T cell recognition, it is important to measure the level of lytic cycle in each cell line. Previously this has been assessed by measuring the level of BZLF1 protein, as an indicator of levels of lytic cycle (Croft *et al.,* 2009). In this study, lines which showed the highest proportion of lytic cycle were diluted with a tightly latent ΔBZLF1-LCL from the same donor, until an equal proportion of BZLF1 positive cells was reached, this then allowed for direct comparison of T cell recognition between these LCL lines. However, this method assumes that the level of BZLF1 expression is directly related to the expression of other lytic cycle antigens, which is not always the case (discussed later in this chapter). Perhaps more importantly, it has been found that only a small proportion of cells expressing BZLF1 will progress the full way through lytic cycle, therefore, cells expressing late antigens may well be diluted out. Therefore, equalising different LCL lines on the level of BZLF1 expression may be misleading and lead to incorrect interpretation of T cell recognition data.

As newly translated mRNAs supply the majority of peptides for MHC class I molecule presentation (DRiPs) (as discussed in chapter 1), it was reasoned that a more accurate measure of target antigen expression would be obtained by qRT-PCR analysis of the levels of the relevant mRNAs encoding the lytic antigen specifically recognised by each individual T cell clone. For example, for a $CD8^+$ T cell which recognises the YVL epitope, derived from the

lytic antigen BRLF1, the mRNA level of BRLF1 would be measured and subsequently used to normalise the level of IFN-γ release (T cell recognition) by this T cell.

To confirm that there is a direct correlation between the level of target antigen-mRNA and $CD8^+$ T cell recognition, an initial experiment was performed. This involved diluting aliquots of spontaneously lytic LCL with tightly latent ΔBZLF1-LCLs, to obtain a controlled range of decreasing levels of antigen, for a constant number of cells. These cell mixes were used as targets for a GLC (BRLF1)-specific $CD8^+$ T cell, where the level of recognition was measured using IFN-γ release. Alongside this, the level of BRLF1 mRNA in each diluted mix was also measured. As shown in Fig 4.1 there was a direct correlation between the level of BRLF1 mRNA expression and recognition of the GLC epitope by $CD8^+$ T cells. Thus, by measuring the mRNA-expression level of target antigens we can accurately account for differences in lytic cycle in LCL target cell lines and use this data to normalise $CD8^+$ T cell recognition data (IFN-γ release levels).

Table 4.2 CD8$^+$ T cell clones used in T cell recognition assays

Phase of expression	Protein	Epitope sequence	HLA restriction	Number of clones used
IE	BRLF1	YVLDHLIVV	A2	2
E	BMRF1	TLDYKPLSV	A2	2
	BMLF1	GLCTLVAML	A2	2
L	BALF4	FLDKGTYTL	A2	2

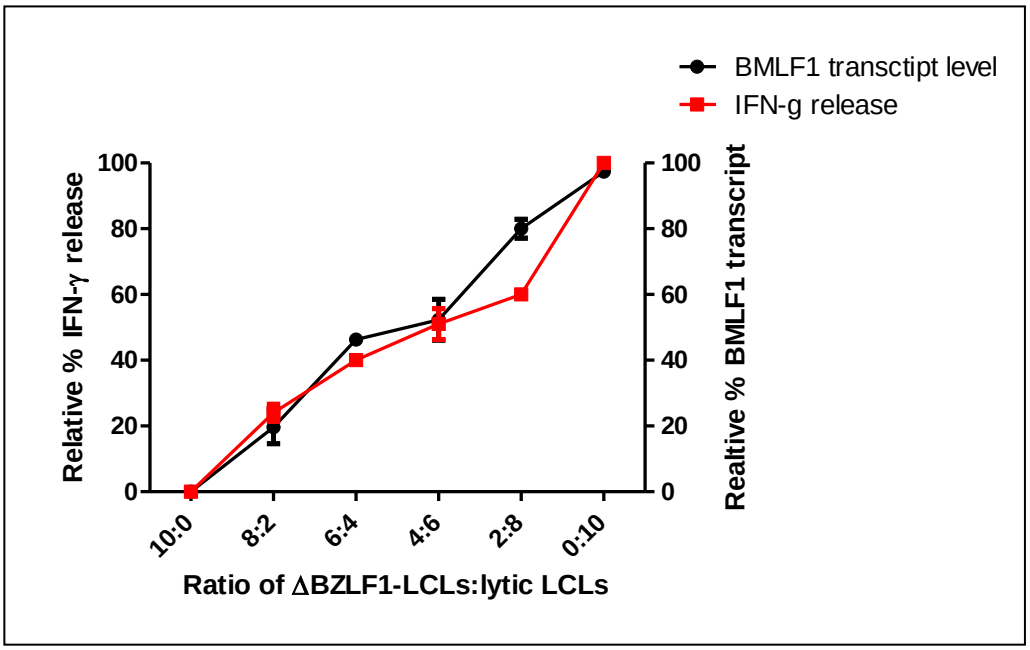


Figure 4.1 Correlation between mRNA antigen expression and CD8$^+$ T cell recognition.

A B95.8-LCL line in which 5% of the line was expressing BZLF1 protein (detected via intracellular staining) was selected. These lytic cells were then serially diluted with tightly latent ΔBZLF1-LCLs, so that the proportion of lytic cell line ranged from 100% to 0%. These cell mixes were then used as targets for a GLC-specific CD8$^+$ T cell clone in a T cell recognition assay. Recognition is shown as percentage IFN-γ release, where 100% release is that seen in undiluted lytic B95.8 LCLs. An aliquot of these cell mixes was also taken to extract RNA and carry out qRT-PCR analysis to detect the level of BMLF1 mRNA. This is shown as % of BMLF1, where 100% is taken as the level of BMLF1 in the lytic B95.8-LCLs before dilution with ΔBZLF1-LCLs cells.

4.3. **Recognition of WT2089-, ΔBNLF2a-, ΔBILF1- and ΔBGLF5-transformed LCLs by immediate early antigen-specific CD8$^+$ T cells**

To determine the effects BNLF2a, BILF1 and BGLF5 have on antigen presentation during the IE phase of EBV lytic cycle, donor 1 (Table 4.2) LCLs were generated using wt-2089**,** ΔBNLF2a-, ΔBILF1-, ΔBGLF5 or ΔBZLF1-virus. These were used as targets for HLA-A2 restricted CD8$^+$ T cell clones which recognise the YVL epitope of the IE protein BRLF1. This was done using two different T cell clones of the same specificity and each experiment was performed twice.

Figure 4.2a shows the raw T cell recognition of HLA-A2 positive Donor 1 LCLs by a HLA-A2 restricted, YVL specific, CD8$^+$ T cell (clone 113). To confirm the specificity and sensitivity of this T cell clone, target ΔBZLF1-LCLs were sensitised with synthetic YVL peptide and as a negative control, the level of IFN-γ release by c113 in the absence of target cells was also measured. As shown, the lack of BNLF2a, BILF1 and BGLF5 expression resulted in increased levels of IFN-γ release by c113, where BNLF2a deletion resulted in the most dramatic increase in recognition. However, as discussed above, these LCL lines spontaneously enter lytic cycle to different frequencies. To account for the fact that a higher frequency of lytic cycle, and therefore more antigen expression may be the reason for this difference in recognition levels, the expression level of BRLF1 mRNA in each cell line was measured (Fig 4.2c), which was found to be similar for each target LCL. IFN-γ release was then normalised against the expression level of BRLF1 mRNA. This normalised data showed the extent of increase in YVL-epitope recognition differed between the different LCLs, where the knockout of BNLF2a shows the greatest increase in YVL-epitope recognition, resulting in an 11-fold increase above WT2089 recognition (Fig4.2e). BILF1 deletion resulted in no

increase in recognition and the lack of BGLF5 expression increased the recognition of YVL-epitope 4-fold above wt2089-LCL

This assay was repeated using the same donor LCLs as targets for a different YVL-specific clone (c36) (Fig4.2b,d,f). In this example the level of IFN-γ release and BRLF1 mRNA expression levels are similar to that seen above (Fig4.2b,c). Thus, following normalisation, the effect of deleting BNLF2a, BILF1 and BGLF5 expression on recognition of an IE-epitope remains similar to that seen in Fig4.2e. Again, the most dramatic effect on increasing $CD8^+$ T cell recognition was seen in cell lines lacking the expression of BNLF2a (Fig4.2f), while the deletion of BILF1and BGLF5 had little or no effect on recognition. These data show that BNLF2a-deletion has the most dramatic effect on recognition of an IE epitope and thus antigen presentation during the IE phase of lytic cycle.

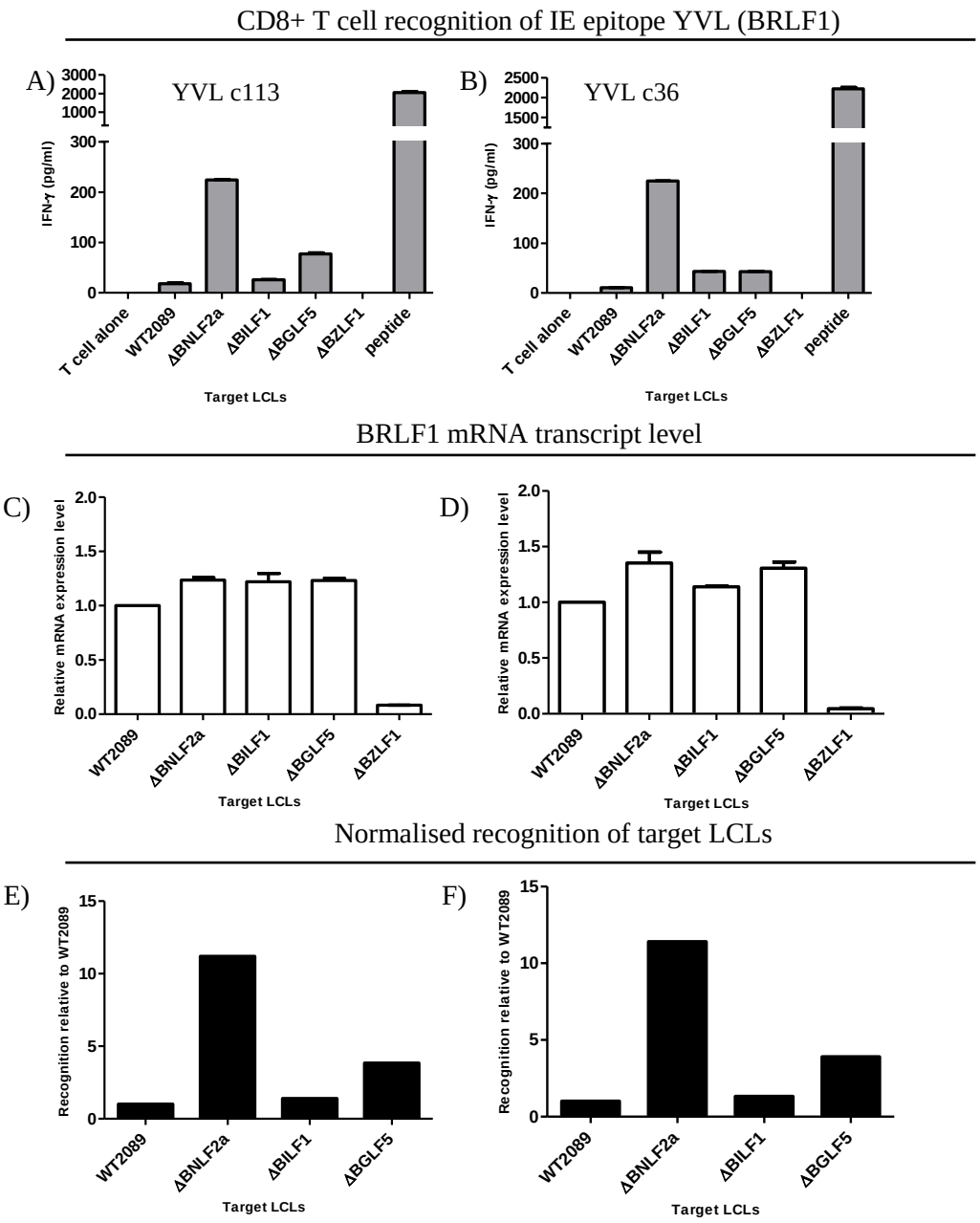


Figure 4.2. Recognition of IE-YVL epitope presented by donor 1 ΔBNLF2a, ΔBILF1 and ΔBGLF5-LCLs

A,B) LCLs were incubated overnight with two different HLA-A2 restricted $CD8^+$ T cell clones specific for the YVL epitope of BRLF1 (c113 and c36). IFN-γ release was measured using an ELISA. ΔBZLF1-LCLs were sensitized with synthetic YVL peptide to confirm T cell activity and specificity. C,D) The mRNA expression of BRLF1 was measured using qRT-PCR. Levels are expressed as mRNA expression relative to wt2089-LCLs. E,F) IFN-γ release was normalized against mRNA expression level. Results expressed as T cell recognition relative to wt2089-LCLs. Representative of two repeats for each T cell.

4.4. Recognition of WT2089-, ΔBNLF2a-, ΔBILF1- and ΔBGLF5-transformed LCLs by early antigen-specific $CD8^+$ T cells

Having established that in donor 1 LCLs the deletion of BNLF2a appears to have the most dramatic effect of T cell recognition of an IE-epitope, the effect that these immune evasion genes have on recognition by E antigen-specific $CD8^+$ T cells was then investigated. In the following representative examples, two different E-epitope specific T cells were used; one specific for the GLC epitope of BMLF1 (c112) and one for the epitope TLD, derived from BMRF1 (c2). Both of these T cells are restricted through HLA-A2 (Table 4.2).

In a similar manner to the experiment in section 4.2, donor 1 wt-2089, ΔBNLF2a-, ΔBILF1- and ΔBGLF5-LCLs were incubated over night with $CD8^+$ T cell clones described above. The level of IFN-γ release by these T cells is shown in Figure 4.3a,b. The increase in raw T cell recognition of LCLs lacking BNLF2a expression compared to wt-2089-LCLs shows ΔBNFL2a-LCLs were more highly recognised. After normalisation of T cell IFN-γ release against the according expression of T cell antigen (Fig 4.3c,d), it is clear that BNLF2a deletion has the most dramatic effect on increasing E epitope recognition. Whereas the deletion of BILF1 increased recognition of these epitopes to a lesser extent and the lack of BGLF5 expression did not appear to increase recognition of these epitopes (Fig 4.3e,f). In this example, noting that this is derived from just one donor, it would appear that the results in Figure 4.3 e and f show essentially the same result as Figure 41.2 e and f, with the strongest effect shown by BNLF2a deletion, a small but measurable increase by BILF1 deletion and in this example no increase in the absence of BGLF5.

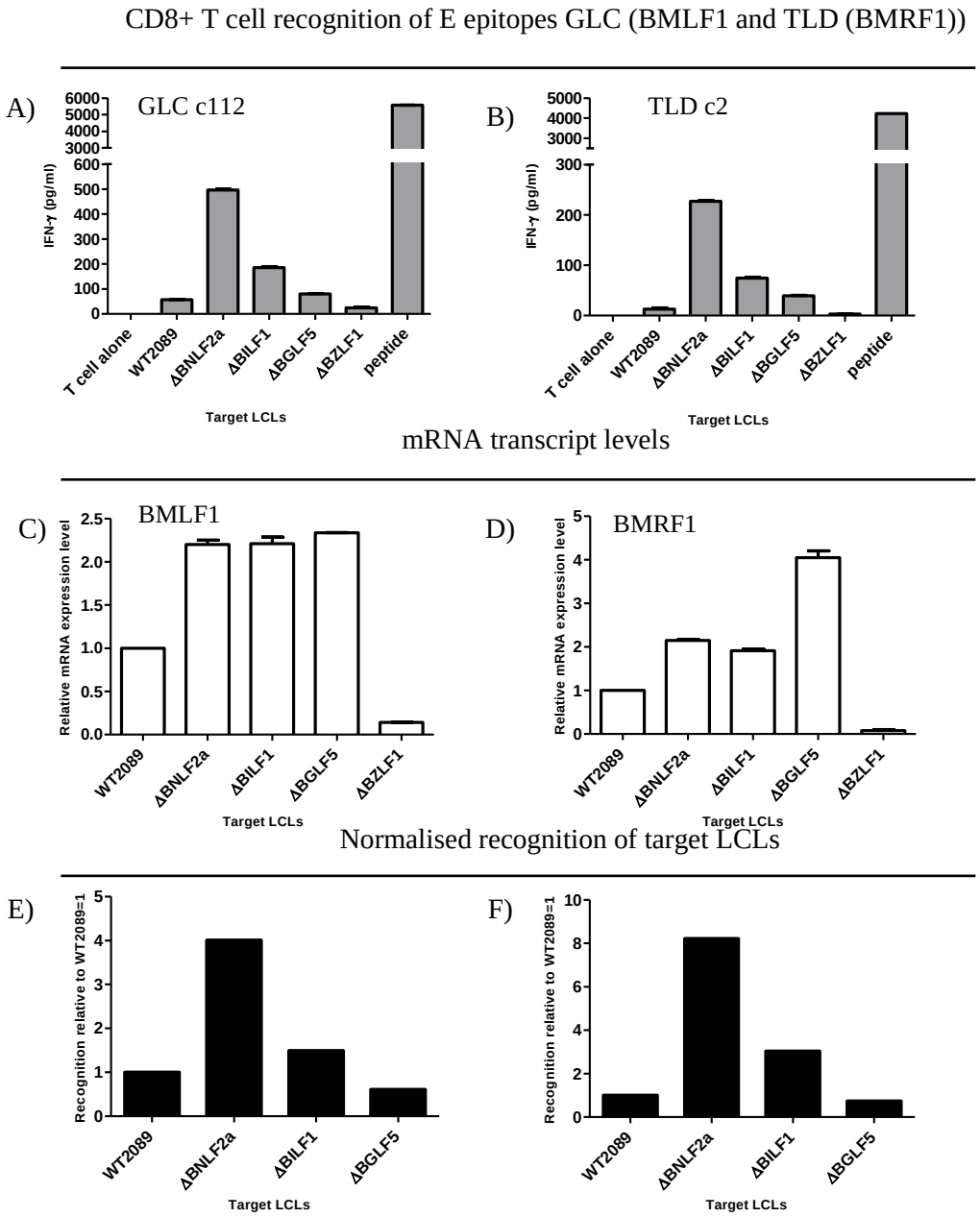


Figure 4.3. Recognition of E epitopes GLC and TLD presented by donor 1 ΔBNLF2a, ΔBILF1 and ΔBGLF5-LCLs

A) LCLs were incubated overnight with a HLA-A2 restricted $CD8^+$ T cell specific for the GLC (BMLF1) epitope (c112) or (B) the TLD (BMRF1) epitope (c2), at a ratio of 10 to 1 target:effector. IFN-γ release was measured using an ELISA. ΔBZLF1-LCLs were sensitized with synthetic YVL peptide to confirm T cell activity and specificity. C) mRNA expression of BMLF1 or (D) BMRF1 was measured using qRT-PCR. Levels are expressed as mRNA expression relative to wt2089. E,F) IFN-γ release was normalized against mRNA expression levels. Results are expressed as T cell recognition relative to wt2089-LCLs. Representative of two repeats for each T cell.

4.5. Recognition of WT2089-, ΔBNLF2a-, ΔBILF1- and ΔBGLF5-transformed LCLs by late antigen-specific CD8$^+$ T cells

Again, using donor 1 LCLs, recognition of wt2089 Vs ΔBNLF2a-, ΔBILF1- and ΔBGLF5-LCLs was measured using two different CD8$^+$ T cell clones (clone 36 and 96) restricted through HLA-A2, both of which recognised the FLD-epitope derived from a L lytic protein BALF4. Recognition of donor 1 LCLs by clones 96 and 36 is shown in Figure 4.4a,b. The highest level of raw recognition in this example is of ΔBILF1-LCLs, followed by ΔBNLF2a and the lowest level of recognition is of ΔBGLF5-LCLs. As shown in Fig4.4c and d, the different LCL lines show different patterns of expression of BALF4 mRNA. Despite this, the pattern of increasing recognition, following normalisation remains consistent between both examples (Fig e and f). In marked contrast to the recognition of IE and E epitopes (Fig 4.2 and 4.3), the data in Figure 4.4e and f shows that T cell recognition of a L epitope is most enhanced by deletion of BILF1, less so by the deletion of BNLF2a and not at all or to a lesser extent by the deletion of BGLF5.

Overall, the data in Figures 4.2-4.4 suggest that BNLF2a has the greatest immune suppressive effect upon recognition of IE and E epitopes, whilst BILF1 has the greatest effect upon recognition of L epitopes. Notably, the effect of BGLF5, if any, was consistently weaker than either BNLF2a or BILF1 for recognition of any target epitope. However, the above data relate to just one HLA restriction element, HLA-A2, and need to be confirmed with other restriction elements. Therefore, it was necessary to extend this work to cover a variety of donor LCLs and a more extensive panel of CD8$^+$ T cells. However, upon repeating this with numerous different donors, a major experimental problem was encountered, whereby some of the target LCL panel were not always undergoing lytic cycle replication to a high enough proportion for T cell recognition. An example of this is shown in Figure 4.5. Here, donor 2 wt2089-,

ΔBILF1-, ΔBNLF2a- and ΔBGLF5-LCLs were used in a similar assay to above, this time using a HLA-B8 restricted T cell specific for the IE-epitope RAK, derived from BZLF1. Firstly, the level of RAK epitope presented by all LCLs was very low, resulting in low recognition (Fig4.5a), despite good recognition of peptide sensitised ΔBLZF1-LCLs. Moreover, ΔBNLF2a LCLs were not recognised due to low levels of lytic cycle and thus BZLF1mRNA expression (Fig4.5b). Since we rely on these cell lines spontaneously entering lytic cycle, such a low level of lytic cycle became a problem. Often, T cell recognition was only slightly above background, which was less than ideal, or more problematic was that some of the panel of LCLs were not lytic at all and thus there was no detectable T cell recognition, meaning that these experiments could not be carried out. To tackle this problem we first sought to design a way in which we could induce lytic cycle in LCLs.

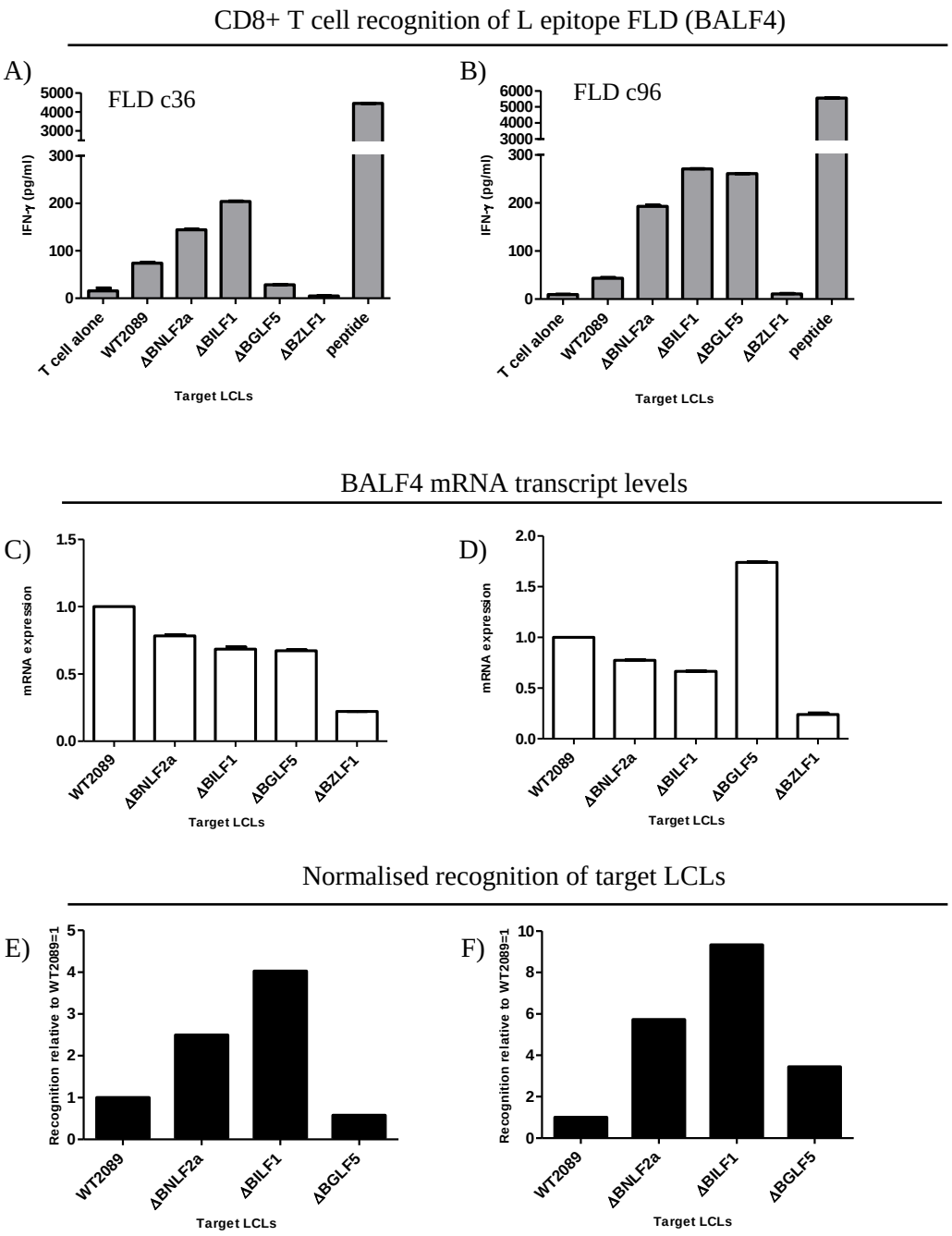


Figure 4.4. Recognition of L FLD epitope presented by donor 1 ΔBNLF2a, ΔBILF1 and ΔBGLF5-LCLs.

A,B) LCLs were incubated overnight with two different HLA-A2 restricted CD8+ T cell specific for the FLD epitope of BALF4 (c36 and c96). IFN-γ release was measured using an ELISA. ΔBZLF1-LCLs were sensitized with FLD peptide to confirm T cell activity and specificity. C,D) The mRNA expression of BALF4 was measured using qRT-PCR. Levels are expressed as mRNA expression relative to WT2089-LCLs. E,F) IFN-γ release was normalized against BALF4 mRNA expression. Results are expressed as the increase in $CD8^{+}$T cell recognition relative to wt2089-LCLs. Representative of two repeats for each T cell.

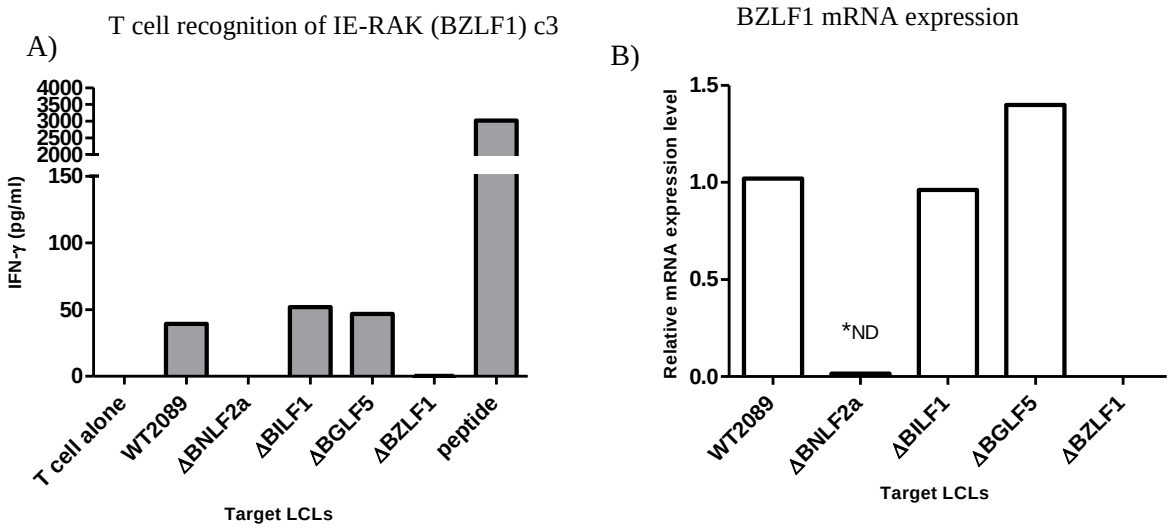


Figure 4.5. Recognition of IE RAK epitope presented by donor 2 ΔBNLF2a, ΔBILF1 and ΔBGLF5-LCLs

A) LCLs were incubated overnight with a HLA-B8 restricted $CD8^+$ T cell specific for the RAK epitope of BZLF1. IFN-γ release was measured using an ELISA. ΔBZLF1-LCLs were sensitized with synthetic RAK peptide to confirm T cell activity and specificity. B) mRNA expression of BZLF1 was measured using qRT-PCR. Gene expression levels are shown as levels of mRNA expression in all cell lines relative to wt2089-LCLs. These data are representative of two repeats.

4.6. **Inducing lytic cycle in LCLs**

Since it was determined during the course of the above work that we absolutely required at least 1% of target LCL lines to be undergoing lytic cycle, it was decided to attempt inducing lytic cycle in LCLs. Although there are methods which are known to induce lytic cycle in LCLs, such as exposure to sodium butyrate (Luka *et al.,* 1979), antibody mediated cross-linking of surface immunoglobulin (Tovey *et al.,* 1978; Takada and Ono 1989) and exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) (zur Hausen *et al.,* 1978), these were not suitable for this work, due to the induction of a plethora of side effects such as changes in MHC class I levels. Since the aim of this work is to investigate the roles of BNLF2a, BILF1 and BGLF5 in the context of whole virus biology, with as little manipulation as possible, these were undesirable methods, as controlling for the side effects would be difficult.

For this reason, inducing lytic cycle by overexpressing EBVs lytic switch protein, BZLF1, was investigated. To do this B95.8-transformed LCLs were electroporated with either BZLF1 expressing plasmid (BZLF1 transfected cells) or a control plasmid (mock transfected cells), as shown in Figure 4.6. After 24 hours, cells were intracellularly stained for an IE expressed protein, BRLF1 (Fig4.6b), and a L expressed protein, BALF4 (Fig4.6c). In order to measure transfection efficiency, BZLF1 protein levels were also measured (Fig4.6a). As shown in Figure 4.6a, BZLF1 staining of mock transfected cells shows that less than 0.5% of cells were spontaneously undergoing lytic cycle replication. Following transfection with a BZLF1-plasmid around, 6% of cells were expressing BZLF1 protein (Fig4.5a). Although this is considered a low transfection efficiency, it would be a high enough proportion of cells undergoing lytic cycle for use in T cell recognition assays involved in this research project. However, only 2% of cells were shown to be expressing the other IE protein BRLF1 (Fig4.5b). Furthermore, only 0.5% were expressing the L lytic protein BALF4 (Fig4.5c).

From these data it would appear that not all cells which were successfully transfected actually progressed through lytic cycle to express BRLF1. In addition of those that did express BRLF1, not all then progressed to express the late lytic protein, BALF4. Therefore, this method of lytic cycle induction was not efficient, although there is clearly induction of BRLF1 expression in some cells. This is perhaps due to poor transfection efficiency of BZLF1-plasmid, which results in a low level of BZLF1 in the first instance and since not all cells that express BZLF1 will progress through lytic cycle, it may be that the transfection efficiency is the limiting factor, thus it was next decided to attempt to increase the transfection efficiency and/or enrich the transfected cell population.

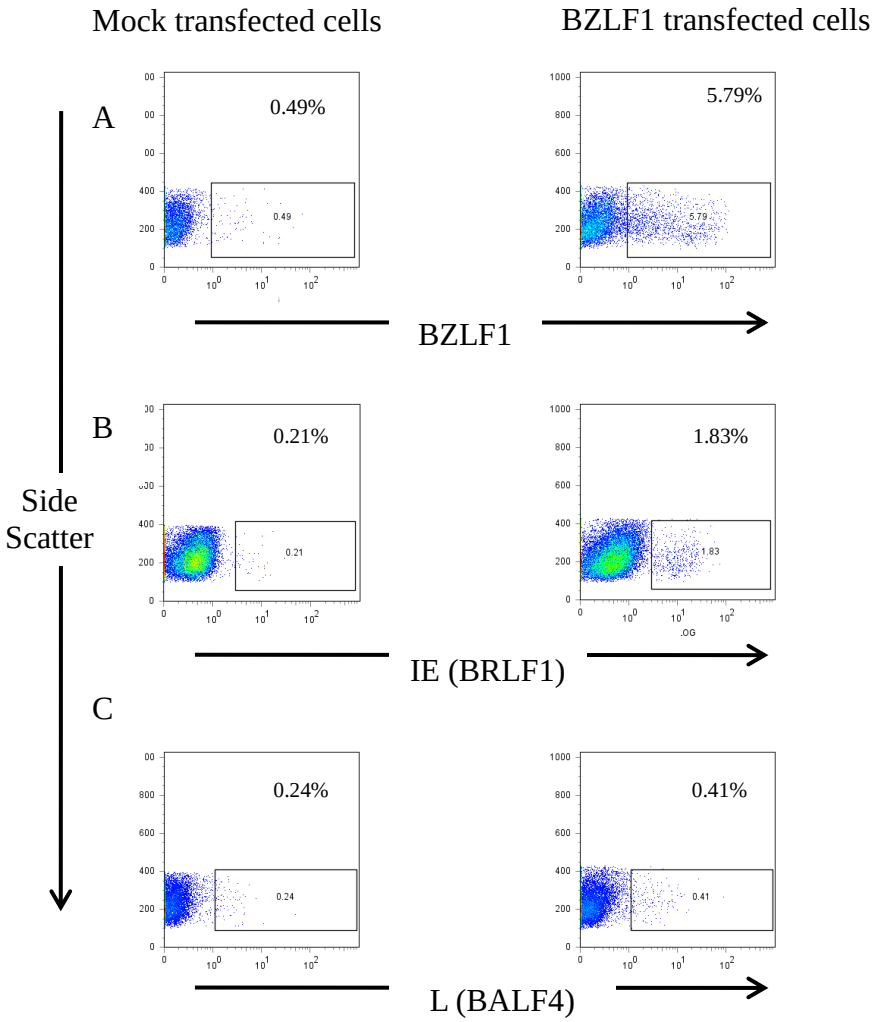


Figure 4.6. Transfection of BZLF1 plasmid into target LCLs does not results in high levels of subsequent lytic cycle.

A) Target LCLs were electroporated with the BZLF1-plasmid or a mock plasmid and 24hr later they were intracellularly stained for lytic proteins. The transfection efficiency was analysed by measuring the level of BZLF1 in transfected cells (A). The same cells were also stained for the expression of an IE protein BRLF1 (B) and a L-protein BALF4 (C).

Therefore, another system of lytic cycle induction was used whereby BZLF1 expression could be induced (Bornkamm *et al.*, 2005). To do this, B95.8-transformed LCLs were transfected with either BZLF1-inducible vector (pRTS-CD2-BZLF1) or control vector (pRTS-CD2-control), after which, successfully transfected cells were enriched. These cells were then induced using doxycycline (dox) and 24 hours later, the expression of IE- (BRLF1), E- (BHRF1) and L- (BALF4) expressed lytic proteins were analysed using intracellular staining and flow cytometry (Fig 4.7). Upon treatment with dox, these cells co-induce both BZLF1 and GFP, which means that the level of induction can be measured by using flow cytometry to detect the proportion of cells expressing GFP. As an additional control, the level of BZLF1 expression was also measured. As shown in Figure 4.7a, 0.6% of control-induced cells were expressing GFP, while only 0.1% of BZLF1-induced cells expressed GFP. However, 1.3% of BZLF1-induced cells were expressing BZLF1. This was likely due to the process of intracellular staining which often quenches the fluorescence level of GFP. It should be noted that this is not due to the presence of dox, as the same effect was not seen in control-induced cells. Thus from here on the levels of GFP expression will be ignored, instead the expression of lytic proteins will be used as the measure of lytic cycle progression. To this end, the expression of BRLF1 in BZLF1-induced cells, was seen to be similar to the level of BZLF1 expression (1.2%) (Fig. 4.7b). However, none of these cells showed detectable levels of the early EBV protein BHRF1 (Fig 4.7c) or the late protein BALF4 (Fig 4.7d). This would suggest that the expression of BZLF1 and BRLF1 is not suffice to induce full lytic cycle in LCLs, meaning this system of lytic induction is also not appropriate for $CD8^+$ T cell recognition assays. Thus, whilst the Bornkamm vector has the potential to sort on the basis of rat-CD2 or GFP, this approach was not used as the above data were not promising with regards to E and L lytic cycle progression.

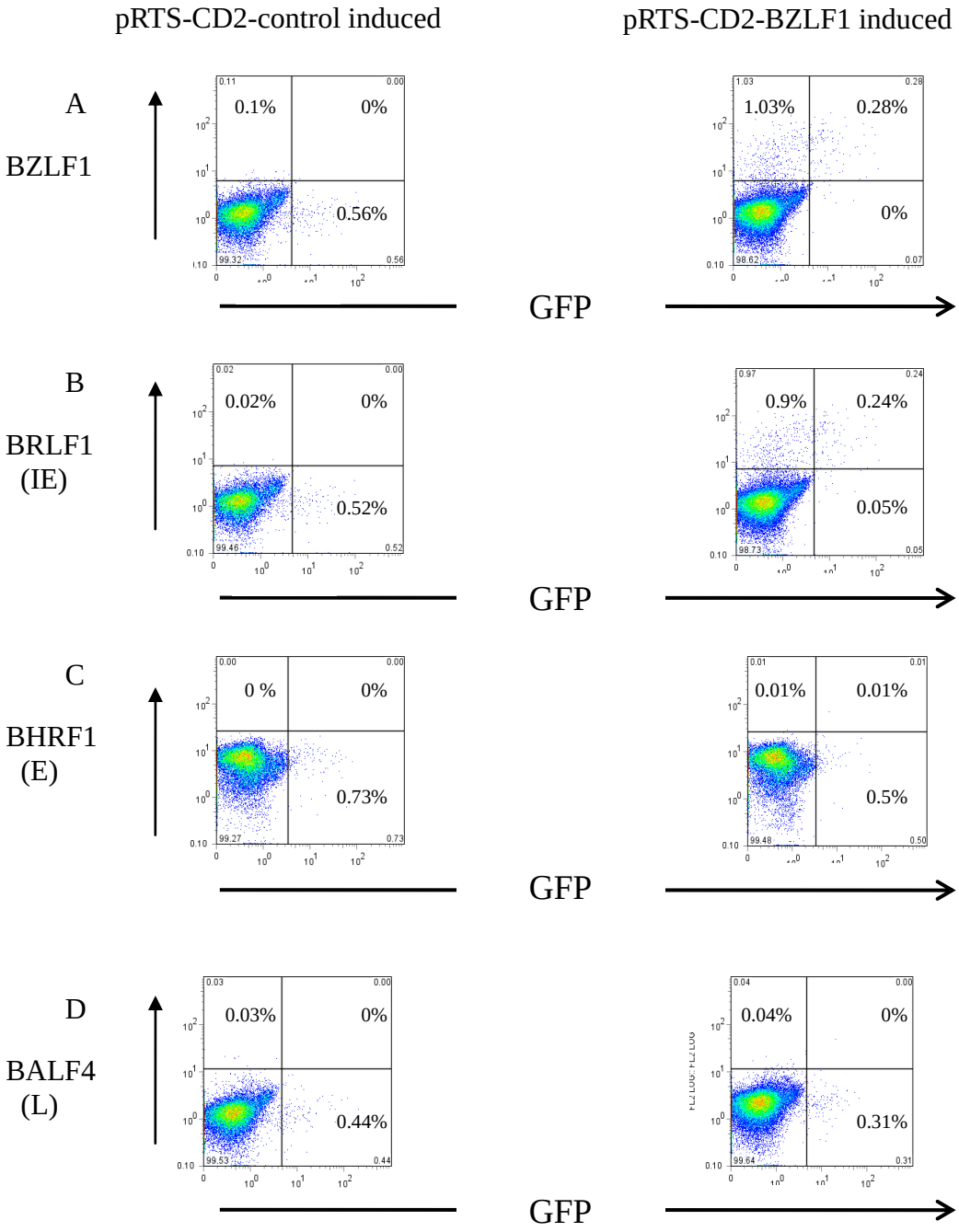


Figure 4.7. Lytic cycle does not progress to late lytic protein expression in LCLs after dox-induced expression of BZLF1 using a Bornkamm vector.

Cells were transduced with a control- or BZLF1- vector followed by selection of positive cells. Cells were then dox-induced to express BZLF1 and 24hrs post-induction assayed for the expression of IE, E and L-lytic proteins alongside GFP, which is expressed by cells successfully induced, using intracellular protein staining and flow cytometry. A) Induced cells were stained for the expression of BZLF1 and GFP. Cells were also stained for the IE protein BRLF1 (B), the E protein BHRF1 (C) and L protein BALF4 (D). The reason not all cells which express lytic proteins also express GFP, as would be expected, is that the process of intracellular staining quenches the signal of GFP.

4.7. **Discussion**

Overall, the data shown in Figures 4.2-4.4 suggest that BNLF2a plays the strongest role in interfering with the presentation of viral epitopes during the IE and E phases of lytic cycle, whereby the deletion of this gene results in enhanced recognition of 10-fold above wild type LCLs for IE-YVL, a 4-fold increase in E-GLC recognition and a 8-fold recognition of E-TLD. BILF1, on the other hand, appears to have the greatest effect upon recognition of L epitopes, increasing recognition of L-FLD epitope 4- and 8-fold above wild type, for two different T cell clones. While at late stages BNLF2a appears to play a less dominant but still a notable role. In contrast, the deletion of BGLF5 showed little or no effect on increasing T cell recognition of IE- E- or L-epitopes, suggesting that BGLF5 may play a little or no role in interfering with antigen presentation at all stages of lytic cycle. The BNLF2a data generally confirm the previously published data of Croft *et al.* except that they observed no enhanced recognition of L epitope in ΔBNLF2a-LCL targets, whereas the present data show clear enhanced of recognition, albeit less marked than shown by the deletion of BILF1. This discrepancy may be attributed to two factors: firstly, the T cell clones generated in this thesis are more potent than those used by Croft *et al*, and secondly, the method of normalisation of the T cell recognition has been improved in the present study.

However, upon repeating the experiments presented in this chapter target LCLs were not always undergoing lytic cycle reactivation to a high enough proportion, or more importantly, some of the panel were not lytic at all. This made the confirmation of these findings using other donor LCLs and T cell specificities impossible. Since it was not feasible to wait for these target cells to become lytic, with no way of controlling this, it was attempted to induce lytic cycle. An obvious alternative would be to use the BAC-293 virus recombinant virus producer cell line. In these cells, the production of virus can be induced using BZLF1, thus

would make ideal targets. However, when this was attempted, although lytic cycle was induced and this progressed to late stages, there was no detectable T cell recognition (data not shown); therefore this of lytic induction was not pursued further. Likewise, chemical induction was not ideal, due to the pleptrophic effects these inducers have on cells. The failed attempts at inducing lytic cycle using induced expression of BZLF1 meant that an alternative strategy was required to confirm and extend the T cell recognition data shown in Figures 4.2-4.4. During the course of this work it became clear that LCLs transformed using the B95.8 strain of EBV showed consistently higher levels of spontaneous lytic cycle when compared to LCLs transformed with 2089-EBV strain (data not shown). This led us to consider whether we could select from appropriate HLA-typed donor B95.8-transformed LCLs that showed consistently high subpopulations (more than 2%) of LCLs in lytic cycle, and use a small hairpin RNA (shRNA) approach to selectively knockdown the expression of BNLF2a, BILF1 or BGLF5. Such experiments are documented in the following chapter.

CHAPTER 5

DEVELOPING ALTERNATIVE BNLF2A, BILF1 AND BGLF5 KNOCKDOWN MODELS IN SPONTANEOUSLY LYTIC LCLS

The method of gene knockdown, involving the expression of a shRNA, which binds to complementary mRNA and suppresses its translation, is widely used in other experimental models. However, there appears to be no reports in the literature of attempts to use shRNA-mediated knockdown of EBV lytic gene expression in LCLs. This chapter outlines the results obtained from designing and generating shRNAs to knockdown the expression of BILF1, BNLF2a and BGLF5 in spontaneously lytic B95.8-LCLs.

To order design successful shRNAs, standard practice is to identify a small interfering RNA (siRNA) which can transiently silence the expression a target gene, and use the sequence of this siRNA to design shRNAs. siRNAs are double stranded RNA molecules around 20-25 base pairs in length, with a sequence complementary to the mRNA of the gene which is to be silenced. For this work, either two or three siRNAs were designed and generated using a service offered by sigma, to target the mRNA of either BILF1, BNLF2a or BGLF5 (sequences shown in Table 5.1). To test these siRNAs, LCLs were not used, as siRNA transfection of LCLs is generally inefficient, therefore, would require high concentrations of siRNA in order to achieve knockdown. Instead, it was decided instead to use 293 cells to screen the panel of siRNAs. The advantage of using 293 cells are that they are readily transfectable with gene expression plasmids and siRNAs, thus they provide a level of sensitivity that permits replicate experiments requiring minimal amounts of siRNA.

Table 5.1 siRNA sequences generated and tested.

Target gene and siRNA sequence (Sigma)

Gene Target	siRNA	Sequence 5'-3'
BNLF2a	-036	CACAGAGUACCACCAGGAG
	-123	CGGGCAGGCCGCAGGCAGA
	-140	GAGGACUGCUGCUCUAGCA
BILF1	-251	GUGAAGGUGACGUUGCAUA
	-456	CCAUGGUAAUGAGGAGGAU
	-664	CGAGAACUCCUGAAUCAUU
BGLF5	-363	GUGGAUUGAUGAAGAUGUU
	-541	GCGCUUACGGACAUCUUUA
	-881	CAGAUGAGCUUACAGACAA
	-096	CACGUACGAGCAGAGAACA

5.1. **Screening BNLF2a-, BILF1- and BGLF5-siRNAs**

The experimental approach to screening the panel of candidate siRNAs listed in Table 5.1 is shown schematically in Figure 5.1. Briefly, aliquots of 293 cells were transfected with plasmids expressing BNLF2a, BILF1 or BGLF5. After 8 hours these transfected cells were divided into four wells of a 12-well plate. This avoided any differences in the transfection efficiency since each well would theoretically express the same level of target mRNA. The next day cells were transfected with individual siRNAs, or a control siRNA. After 24 hours, cells were harvested and the level of protein knockdown was examined using western blot analysis. Each candidate siRNA screen was repeated three times, using a range of siRNA concentrations.

Figure 5.2a shows an example of the results for one such experiment, in which three candidate BNLF2a-siRNAs (-036, -123 and -140) were tested at a dose of 100nM. As shown, siRNA-036 and siRNA-140 showed substantial BNLF2a protein knockdown, compared to control siRNA transfected cells (lane 1). This is representative of numerous repeats using siNRA concentrations ranging from 50nM to 500nM. In each case siRNA-036 showed the highest level of BNLF2a protein knockdown. Therefore, the siRNA sequence 5' CACAGAGUACCACCAGGAG 3' was selected for subsequent generation of shRNA-lentivirus targeting BNLF2a.

Screening of candidate BILF1 siRNAs (-664, -251, and -456) was carried out in a similar way, the only difference being that 293 cells were transfected with a HA tagged BILF1 expression plasmid, since an antibody against BILF1 protein is not available. A representative example of the level of BILF1-HA protein knockdown in these cells is shown in Figure 5.2b.

siRNA-251 and siRNA-664 both caused an obvious reduction in BILF1-HA protein, whilst the siRNA-456 reproducibly upregulated BILF1-HA expression. Other experiments with lower concentrations of siRNA (data not shown) indicated that siRNA-664 gave more efficient knockdown than siRNA-251. Therefore, the siRNA-664 sequence 5'-CGAGAACUCCUGAAUCAUU-3' was taken forward to generate shRNA constructs. It should be noted, that the increase in BILF1-HA expression in the presence of siRNA-456 was unexpected, and is likely due to this particular siRNA activating the expression of BILF1.

Finally, similar experiments were performed to screen four BGLF5 targeting siRNAs (siRNA-363, -541, -881, -096). A representative example of these results is shown in Figure 5.2c which shows knockdown of BGLF5 protein by three of the candidate siRNAs (siRNA-363, -541 and -881). siRNA-363 reproducibly resulted in the most efficient knockdown in all experiments performed, over a range of concentrations. Therefore, shRNA was subsequently designed around the sequence of the siRNA-363 (5' GUGGAUUGAUGAAGAUGUU 3').

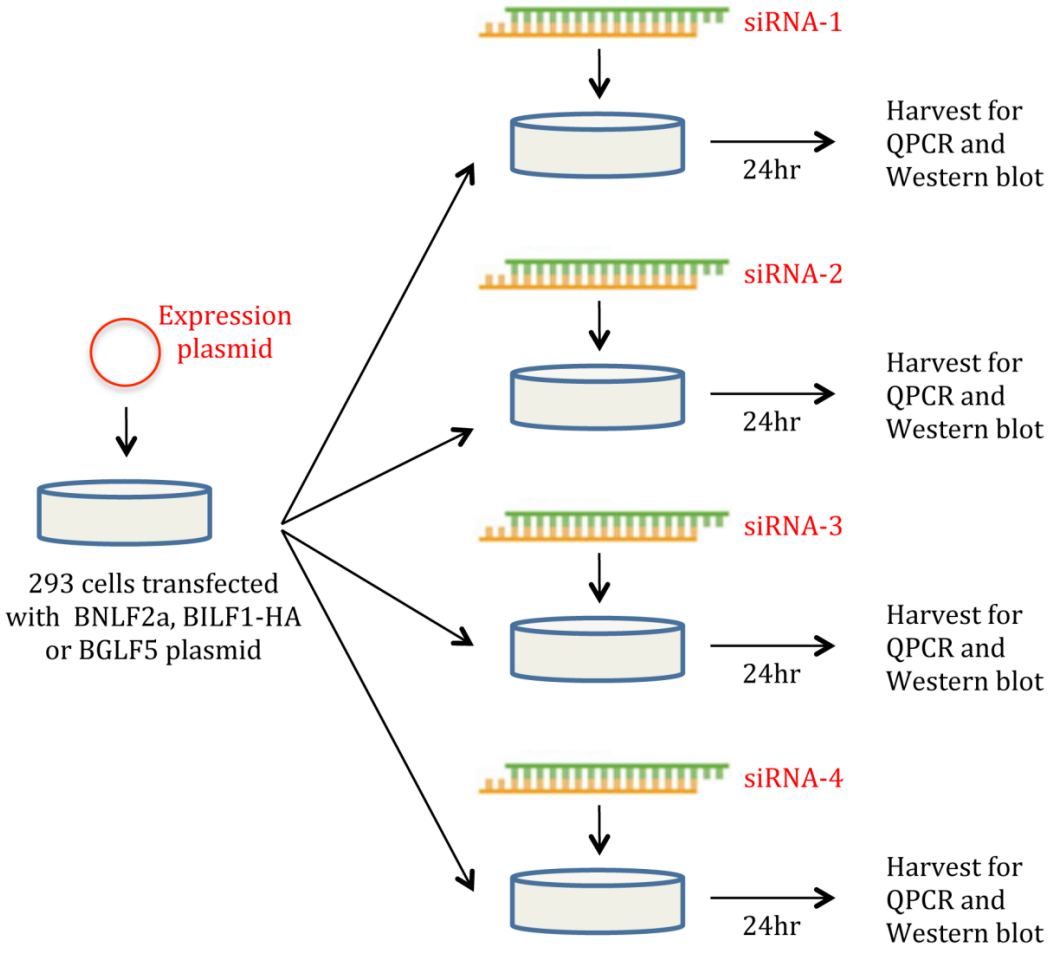


Figure 5.1 Experimental method used to screen candidate siRNAs.

293 cells were plated into a 25cm^2 flask on day 0. The following day, cells were transfected with either BNLF2a, BILF1-HA or BGLF5 expressing plasmid. After 8 hours, these cells were transfered to a 12-well plate and left overnight. The following moring, cells were trasnfected with appropriate siRNAs and a control siRNA. After 24 hours cells were harvested and the level of protein knockdown was assessed using western blot analysis.

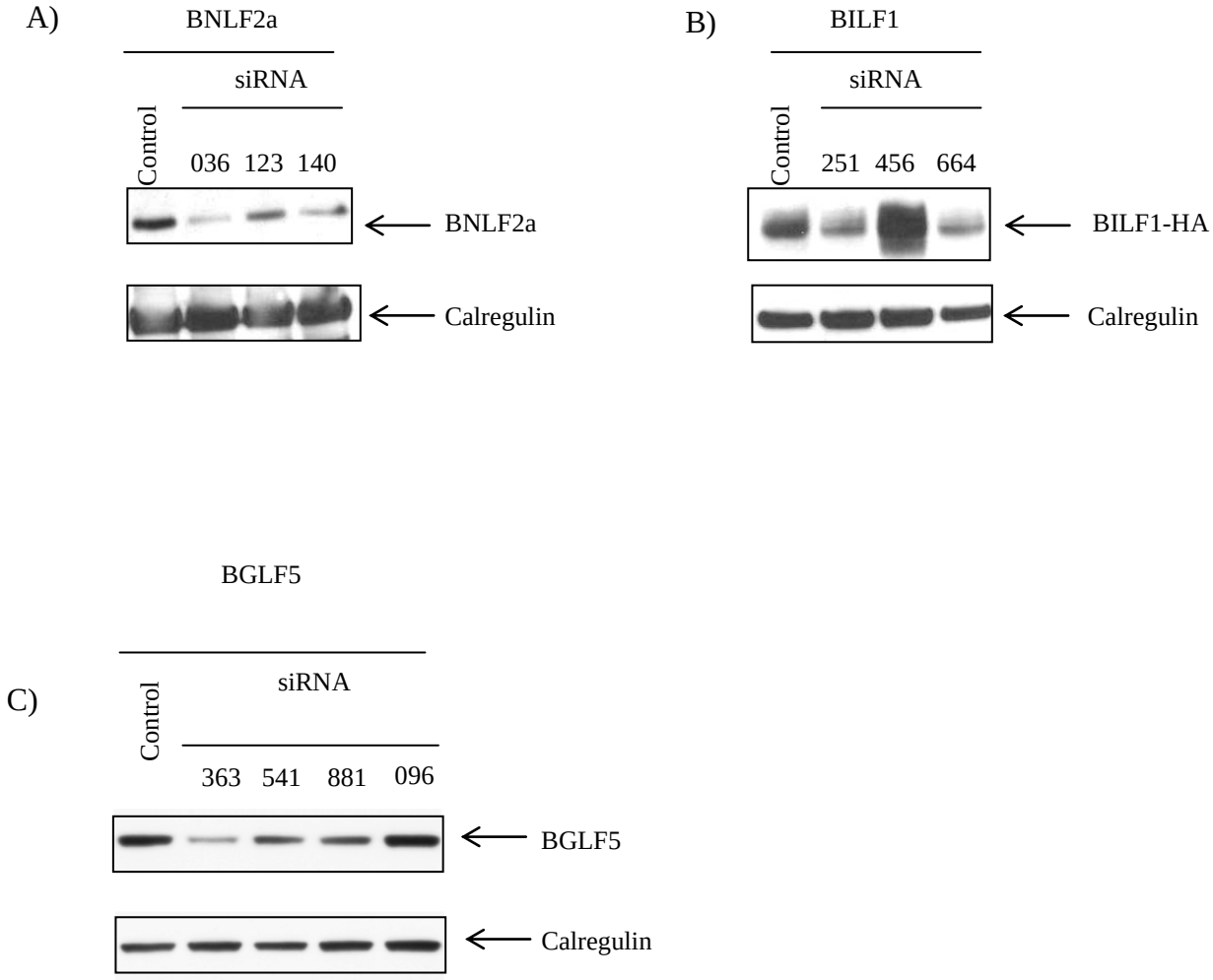


Figure 5.2 Knockdown of BNLF2a, BILF1 and BGLF5 using siRNAs

293 cells were transiently transfected with BNLF2a (A), BILF1-HA (B) or BGLF5 (C) expressing plasmids. 8 hours later these cells were split into 4 wells. 16 hours later, each well was transfected with either specific siRNA or a control siRNA (100uM). 24 hours later these cells were harvested and screened for the level of BNLF2a (A), BILF1-HA (B) or BGLF5 (C) protein knockdown using western blot analysis. Each result is a representative example of numerous repeats using a range of different siRNA concentrations.

5.2. **Generating shRNA-lentiviruses**

The selected siRNA sequences (Table 5.2) were then used to generate four shRNA-lentiviruses (Fig 5.3a) (Service offered by sigma). The advantage of using shRNA-lentiviral delivery rather than transient transfection of siRNA is that shRNAs are integrated into the genome of cells so that the knockdown of target genes is constitutive and stable. In order to detect, and subsequently sort transduced cells, fluorescent tags were also expressed from the lentiviral vectors; different coloured tags were used for each shRNA vector, as shown in Table 5.2. These constructs also expressed a puromycin resistance gene, which allowed for the enrichment of transduced cells. Therefore, this overall approach allowed for the selection and maintenance of successfully transduced cells by sorting on the expression of a specific tag and by adding puromycin to cell culture, if necessary.

Table5.2 shRNA-lentivirus constructs used.

Including information on the vector which encoded the shRNA, the gene targeted the fluorescent tag which was encoded for each shRNA and the DNA sequence of the shRNA. All vectors also encoded a puromycin resistance.

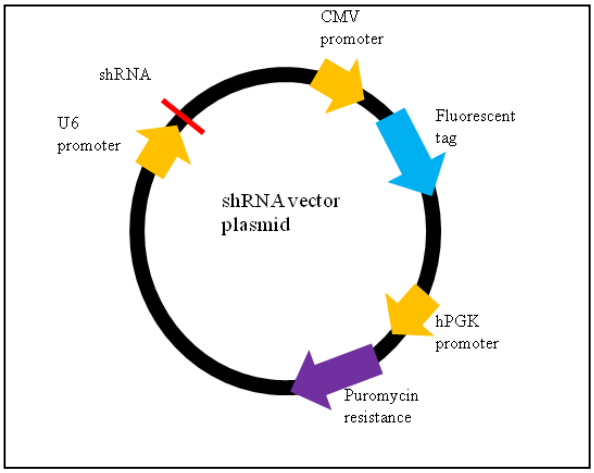
Vector	**Gene targeted**	**Fluorescent tag**	**DNA sequence 5'-3'**
pLKO.1-puro-CMV-TagCFP	BNLF2a	CFP	CACAGAGTACCACCAGGAG
pLKO.1-puro-CMV-TagFP635	BGLF5	FP635	GTGGATTGATGAAGATGTT
pLKO.1-puro-CMV-TagYFP	BILF1	YFP	CGAGAACTCCTGAATCATT
pLKO.1-puro-CMV-tCFP	None	CFP	TCCTAAGGTTAAGTCGCCCTC

5.3. **shRNA-lentivirus transduction of lytic B95.8-LCLs**

Donor B95.8 LCLs for use in T cell assays were initially selected on the basis of their HLA type and subsequently, the proportion of cells undergoing lytic cycle was assessed using intracellular BZLF1 staining. While carrying out the work outlined in chapter 4, it became clear that if target LCLs were less than 1% BZLF1 positive, $CD8^+$ T cell recognition was too low for accurate quantitation. For this reason, only B95.8 LCLs that were more than 1% BZLF1 positive were selected for transduction.

As depicted in Figure 5.2b, selected lytic B95.8 LCLs were transduced with lentiviruses expressing shRNA-Non-target-CFP, shRNA-BNLF2a-CFP, shRNA-BGLF5-FP635 or shRNA-BILF1-YFP. Cultures were then expanded, and successfully transduced cells were selected on their expression of CFP, FP635 or YFP using a MoFlo fluorescence activated cell sorter. There cells were then re-cultured, with drug selection if required, until sufficient numbers were reached to validate the knockdown of the selected EBV lytic genes This allowed for the generation of populations of successfully transduced B95.8-LCLs, in which the lytic subpopulation were single knockdowns for either BNLF2a, BILF1 or BGLF5. These were then used as targets in $CD8^+$ T cell recognition assays.

A


CMV
promoter
shRNA
U6
promoter
Fluorescent
tag
shRNA vector
plasmid
hPGK
promoter
Puromycin
resistance

B

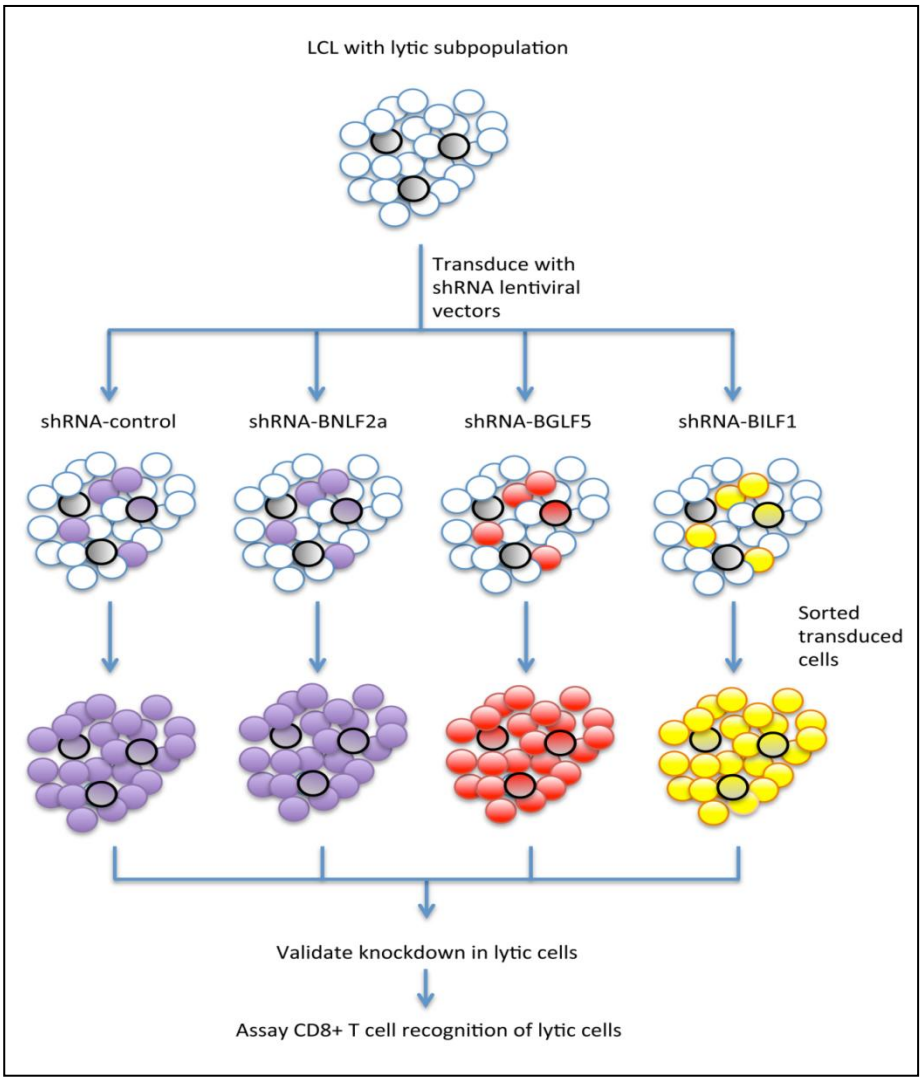

LCL with lytic subpopulation
Transduce with
shRNA lentiviral
vectors
shRNA-control
shRNA-BNLF2a
shRNA-BGLF5
shRNA-BILF1
Sorted
transduced
cells
Validate knockdown in lytic cells
Assay CD8+ T cell recognition of lytic cells

Figure 5.3 Schematic of shRNA-lentiviruses transduction of B95.8 LCLs.

A) Diagram of shRNA vector plasmid used. B) The experimental approach used to generate shRNA transduced LCLS. B95.8-LCLs are selected on the basis of their HLA type and the frequency of cells undergoing lyric cycle. Cells are transduced with each different shRNA-lentivirus. Cell lines are then expanded and transduced cells are selected on the expression of the appropriate fluorescent tag. Knockdown is then confirmed and cells are used in downstream T cell assays.

5.4. **shRNA knockdown of BNLF2a in lytic B95.8-LCLs**

Following transduction and expansion of parallel B95.8-LCL cultures with shRNA-BNLF2a lentivirus, transduced LCLs expressing high levels of CFP were observed (Fig 5.4a). Following selection by fluorescence activated cell sorting, and further expansion, expression of BNLF2a transcripts was assayed and found to be of similar levels in both shBNLF2a-LCLs and shNon-target-LCLs (Fig 5.4b), despite the fact that shBNLF2a-LCLs were around three times as lytic as control shNon-target-LCLs (Fig 5.4c). Therefore, when the expression of BNLF2a was normalised against the level of BZLF1 transcript (Fig 5.4d), the knockdown of BNLF2a mRNA was around 70%, compared to BNLF2a expression in shNon-target-LCLs. It should be noted, that although in this example the level of BZLF1 expression was used to measure lytic cycle and normalise BNLF2a expression, in other experiments, normalisation against BRLF1, BMLF1 or BZLF1 all confirmed the efficiency of BNLF2a knockdown in shBNLF2a-LCLs.

The level of BNLF2a protein knockdown in these cells was also investigated using western blot analysis. This was complicated by the fact that shBNLF2a-LCLs in this example were more lytic than their shNon-target-LCLs counterparts. In order to address this difference in lytic cycle, the shBNLF2a-LCL sample was diluted 1 in 2 and 1 in 4, so that there was decreasing levels of BZLF1 protein in each lane. As shown in Figure 5.4e, the level of BZLF1 expression was most comparable between shNon-target- (lane 1) and shBNLF2a-LCLs which have been diluted 1 in 4 (lane 3). When comparing these lanes, the expression of BNLF2a in shBNLF2a-LCLs is dramatically reduced compared to that in shNon-target LCLs. This effect can also be seen in undiluted shBNLF2a-LCLs, where when there is more BZLF1 protein, there are still lower levels of BNLF2a protein, compared to shNon-target-LCLs. Thus the

knockdown of BNLF2a in B95.8-LCLs using shBNLF2a-lentivirus was successful. It was also noted that there appeared to be unexpected expression of BNLF2a transcript and protein in ΔBZLF1 LCLs (negative control). This will be addressed at the end of this chapter.

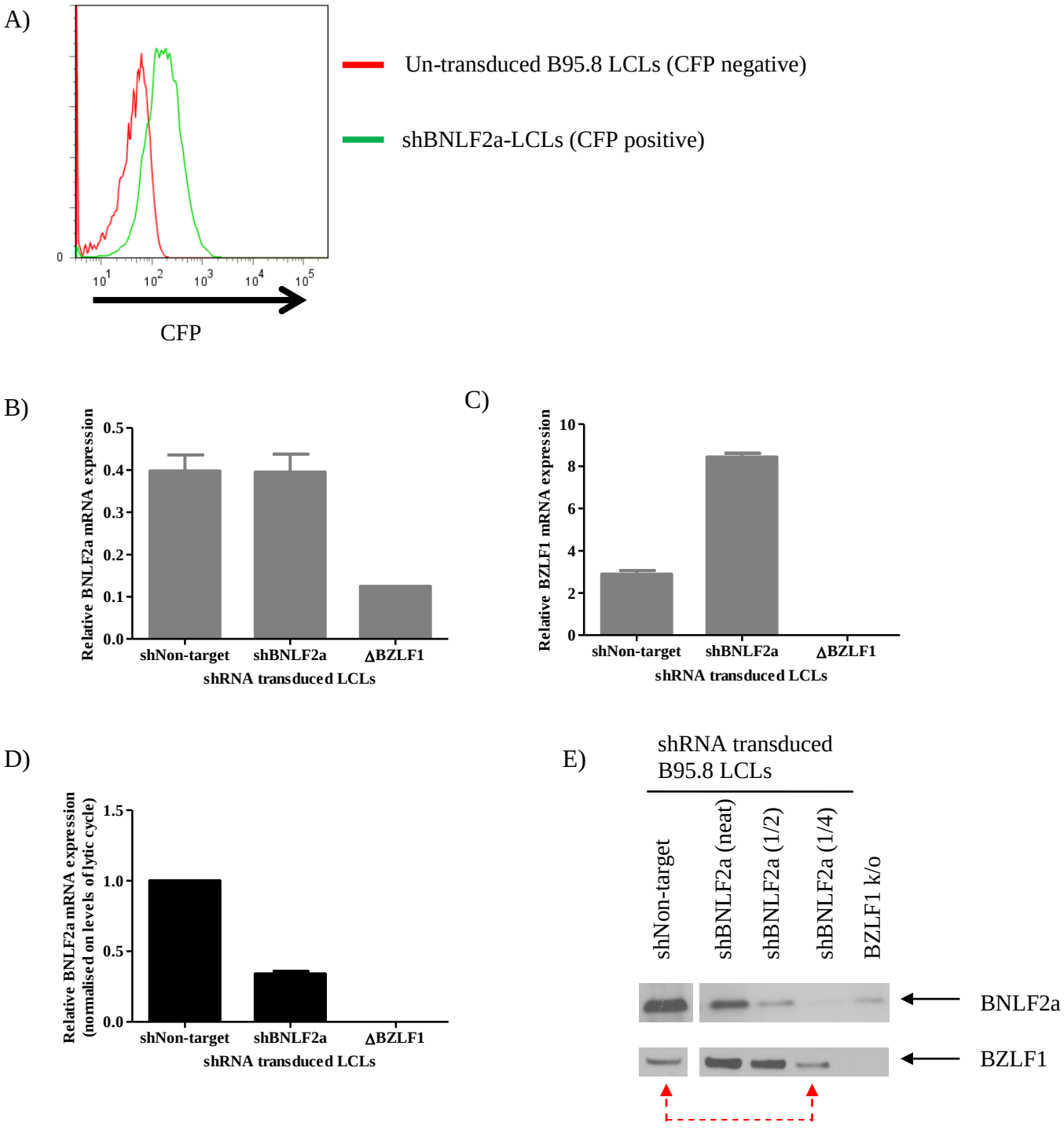


Figure 5.4 BNLF2a knockdown in B95.8-LCLs

A) shBNLF2a-LCLs were expanded, and the CFP positive cells were selected. (B,C) qRT-PCR was used to measure the level of BNLF2a and BZLF1 mRNA. Data is shown as relative transcript level. D) BNLF2a mRNA expression was normalized against BZLF1. Data is shown as BNLF2a mRNA in shBNLF2a-LCLs relative to shNon-target. E) BNLF2a protein knockdown was assessed using western blot analysis. Protein levels of BNLF2a and BZLF1 was measured in shNon-target-, shBNLF2a- and ΔBZLF1-LCLs. Since shBNLF2a-LCLs were more lytic, a 1 in 2 and 1 in 4 dilution of shBNLF2a-LCLs was carried out, so that a similar level of BZLF1 expression was achieved (Red line).

5.5. **Knockdown of BILF1 in lytic B95.8-LCLs using shRNA lentiviruses**

Similarly to the above, parallel cultures of B95.8 LCLs were transduced with shRNA-BILF1-YFP lentivirus and expanded. Firstly, the level of YFP expression in transduced cultures was measured (Fig 5.5a), and those cells expressing high levels of YFP were selected using cell sorting and subsequently expanded. The level of BILF1 mRNA knockdown in these cells was then measured using qRT-PCR analysis. As was the case with shBNLF2a-LCLs, shBILF1 LCLs expressed slightly more BZLF1 mRNA than their shNon-target-LCL counterparts (Fig 5.5c). Despite this, the level of BILF1 mRNA in shBILF1-LCLs was lower than in shNon-target-LCLs (Fig5.5b). Therefore, after normalisation of BILF1 expression against BZLF1 (Fig 5.5d), the knockdown of BILF1 in shBILF1-LCLs amounted to around 75%. This was repeated using other lytic genes to normalise for lytic cycle, and the level of BILF1 knockdown was always 70-90%. Unfortunately, since there is no available anti-BILF1 antibody, protein knockdown could not be confirmed.

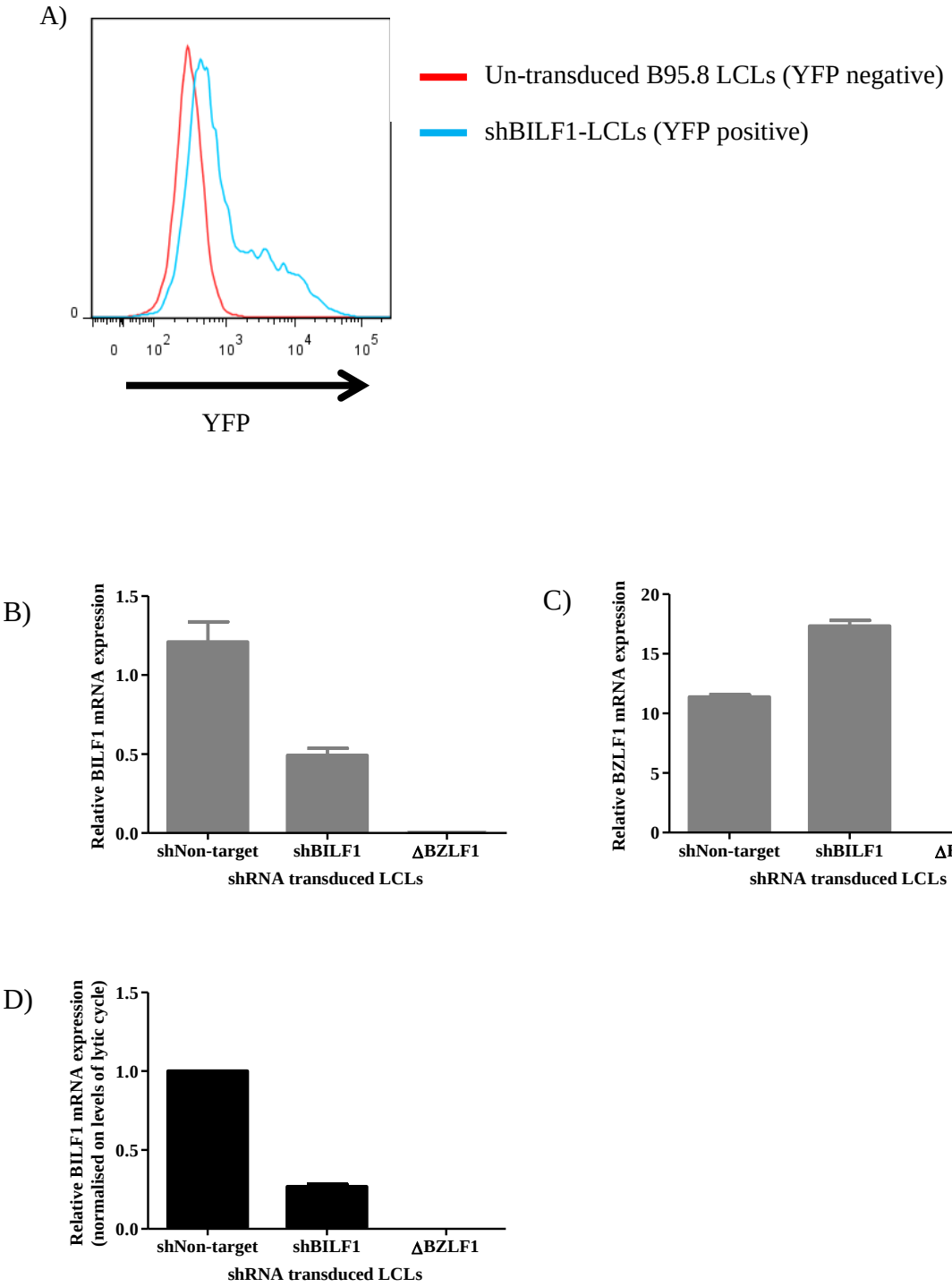


Figure 5.5 BILF1 knockdown in B95.8-LCLs

A) shRNA-BILF1-LCLs expressing YFP were selected, using cell sorting. B,C) qRT-PCR analysis was performed to measure the level of BILF1 (B) and BZLF1 (C) transcripts in shNon-target and shBILF1-LCLs. As a negative control ΔBZLF1-LCLs were included. Data is shown as transcript level relative to induced AKBM cells. D) BILF1 expression was normalized against the level of BZLF1 transcript. Data is shown as BILF1 expression in shBILF1-LCLs relative to that in shNon-target cells.

5.6. **Knockdown of BGLF5 in lytic B95.8-LCLs using shRNA lentiviruses**

Following sorting of shBGLF5-LCLs on the expression of FP635 (Fig5.6a) and subsequent expansion of these cells, the successful knockdown of BGLF5 was measured using qRT-PCR analysis to quantitative the levels of BGLF5 mRNA in shBGLF5-LCLs compared to shNon-target LCLs. As shown in Figure 5.6b, the level of BGLF5 transcript in shBGLF5-LCLs was less than that in shNon-target LCLs. As shown in Figure 5.6c, shBGLF5-LCLs were in fact more lytic than the shNon-target LCLs. Therefore, when the expression of BGLF5 was normalised against the level of BZLF1 transcript, the knockdown of BGLF5 expression was seen to be 70%, compared to BGLF5 expression in shNon-target LCLs (Fig5.6d)

Next, the level of BGLF5 protein knockdown was investigated. As shown in Figure 5.6e, the level of BZLF1 protein expression in shBGLF5 LCLs was slightly higher than in shNon-target LCLs, which reflected the mRNA data in 5.6c. Despite these comparable levels of BZLF1 protein, there was a dramatic decrease in BGLF5 protein expression in shBGLF5-LCLs compared to their shNon-target-LCL counterparts. Thus the knockdown of BGLF5, BNLF2a and BILF1 in lytic B95.8-LCLs using this method was successful.

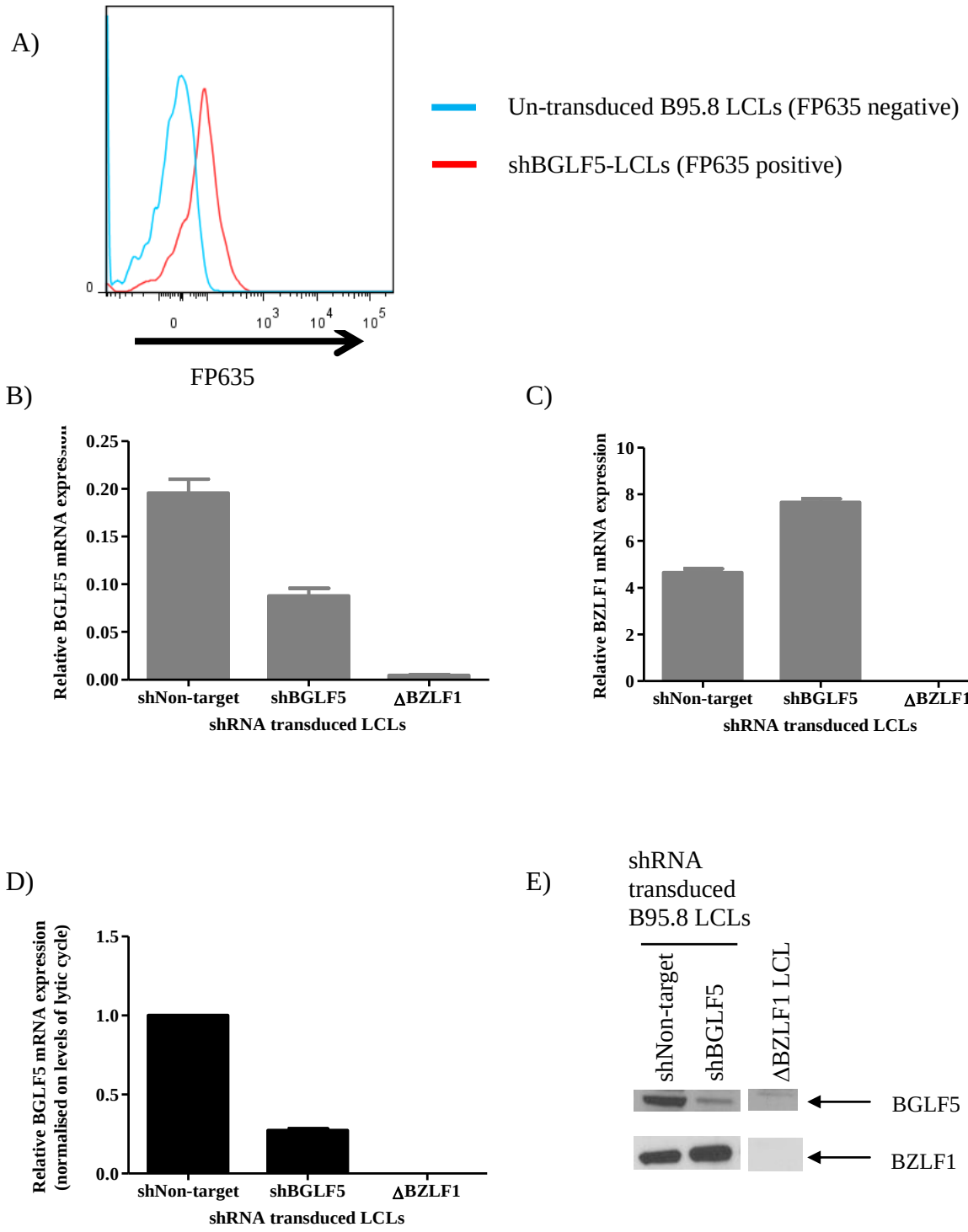


Figure 5.6 BGLF5 knockdown in B95.8-LCLs

A) shRNA-BGLF5-LCls were sorted on the expression of FP635 (B,C) qRT-PCR analysis was used to measure the level of BGLF5 (B) and BZLF1 (C) transcripts. Data is shown as transcript level relative to a standard of lytic EBV cells. D) BGLF5 expression was then normalized on the level of BZLF1 transcript. Data is shown as BGLF5 expression in shBGLF5-LCLs relative to that in shNon-target cells. E) BGLF5 knockdown was then confirmed at the protein level using western blot analysis. The expression of BGLF5 and BZLF1 protein was measured in shNon-target-LCLs (no knockdown), shBGLF5-LCLs (knockdown) and as a negative control, BZLF1 ko-LCLs were included.

5.7. **Expression of BNLF2a in latent EBV infected cells**

As shown in Figure 5.4, it was apparent that replication deficient ΔBZLF1-LCLs expressed detectable levels of BNLF2a transcript and protein. This finding was unexpected and warranted further investigation.

The ability of HLA-A2 restricted $CD8^+$ T cells specific for the BNLF2a epitope, VLFGLLCLL, (Bell *et al.*, 2009) to recognise ΔBZLF1-LCLs was investigated. Firstly, the expression of BNLF2a in ΔBZLF1-LCLs was confirmed using qRT-PCR (Fig5.7a). As a positive control, lytic 2089-EBV (wild-type) transformed LCLs and negative control of ΔBNLF2a-LCLs were included. As shown in Figure 5.7a, in this particular example, both wild-type 2089-LCLs and ΔBZLF1 LCLs expressed BNLF2a mRNA to similar levels, whereas there was no detectable BNLF2a expression in ΔBNLF2a-LCLs. As shown in Figure 5.7b BNLF2a specific $CD8^+$ T cells were able to recognise 2089- and ΔBZLF1-LCLs, whilst there was no recognition of their ΔBNLF2a-LCL counterparts. This confirms that BNLF2a is indeed expressed in ΔBZLF1-LCLs, that are considered to display only latent infection and, moreover, it is processed and presented.

To confirm that ΔBZLF1-LCLs were not expressing significant levels of other lytic transcripts, the level of the lytic antigen BRLF1, was assayed, and its recognition by a BRLF1-specific $CD8^+$ T cell measured. As shown in Figure 5.7c, BRLF1 transcript was present in 2089- and ΔBNLF2a -LCLs, while there was no detectable expression by ΔBZLF1-LCLs. Furthermore, there was no recognition of ΔBZLF1 LCLs by BRLF1 specific T cells (Fig 5.7d), and 2089- and ΔBNLF2a-LCLs were recognised by this T cell. Thus, ΔBZLF1-

LCLs are not undergoing lytic cycle replication, however they do express BNLF2a. Next, the expression of BNLF2a in other tightly latent EBV infected cells was investigated.

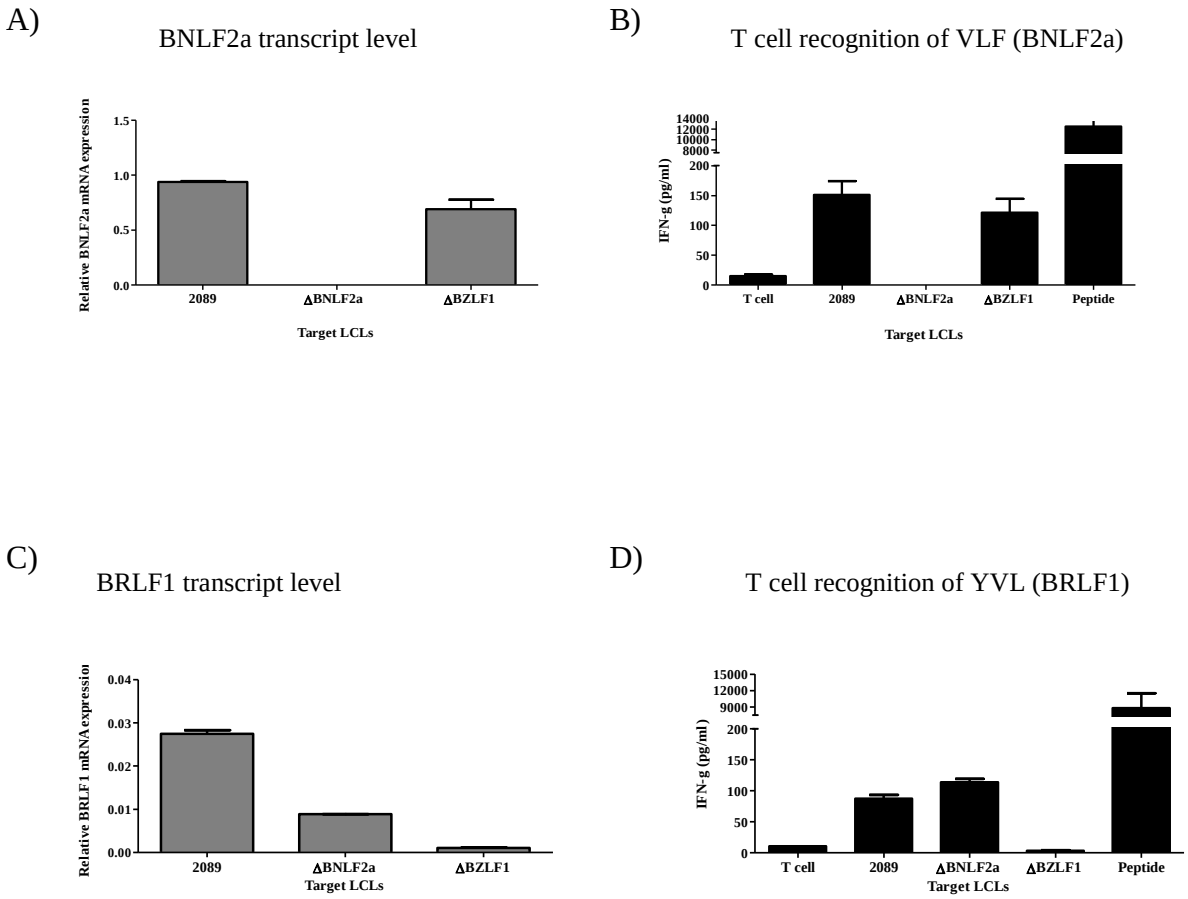


Figure 5.7 Recognition of BZLF1 ko LCLs by BNLF2a specific T cells.

qRT-PCR analysis was used to measure the level of BNLF2a (A) and BRLF1 transcripts in 2089-, ΔBNLF2a - and ΔBZLF1 -LCLs. Results are shown as expression relative to induced AKBM cells. (B,D,) $CD8^+$ T cell recognition of BNLF2a and BRLF1 epitopes in 2089-, ΔBNLF2a - and ΔBZLF1 - LCLs was measured using IFN-γ ELISA. Background IFN-γ release by T cell alone was measured and as a positive control, T cell recognition of peptide loaded cells was measured. Results are shown as IFN-γ release (pg/ml).

5.8. **Expression of BNLF2a in latent EBV infected SNK/T cell lines**

In order to confirm that the expression of BNLF2a during latency was not exclusive to ΔBZLF1-LCLs, the expression of BNLF2a in other latent EBV infected cell lines was investigated. To this end, the expression of BNLF2a in three NK/T cell lines derived from patients with EBV-associated NK/T cell lymphoma (ENKTL) or chronic active EBV (CAEBV): SBK6, SNK10 and SNT16, was assayed. As shown in Figure 5.8a, these cell lines do not express BZLF1 or other lytic transcripts (data not shown), thus they are considered tightly latent. However, they do express varying levels of BNLF2a (Fig 5.8b), whereby SNK6 shows the highest level of transcript expression. This raises the question as to whether BNLF2a is functionally active in theses latent cells, and if it could be playing a functional role in latent $CD8^+$ T cell evasion. However, due to time constraints, this work could not be pursued further during this research project.

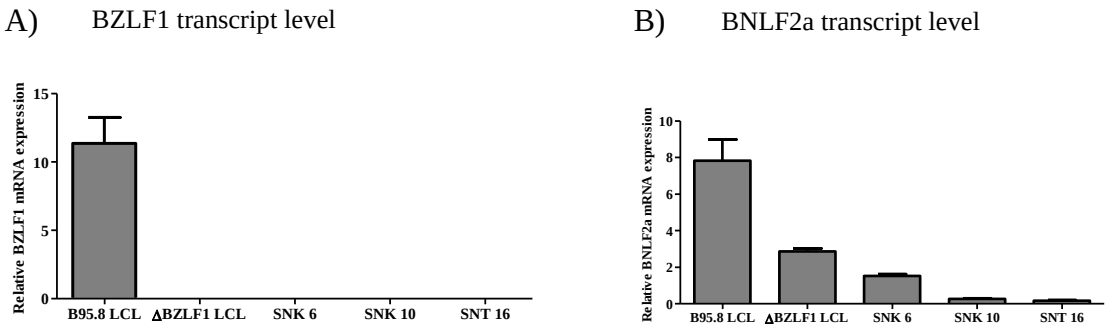


Figure 5.8 Expression of BNLF2a in SNK and T cell lines.

Using qRT-PCR analysis, the level of BZLF1 (A) and BNLF2a (B) transcripts in SNK-6,-10 and SNT 16 cell lines was measured. As a control, B95.8 and ΔBZLF1-LCLs were also included. Results are shown as transcription level relative to induced AKBM cells.

5.9. **Discussion**

Although the experimental approach outlined in this chapter did enable the generation of single knockdown LCLs, there were some issues which complicated matters. Firstly, transduced LCLs did not survive for long periods of time. This was likely due to untransduced LCLs outgrowing transduced LCLs; however, it cannot be ruled out that transduced cells died-off faster, as a result of the transduction process. Moreover, sorted cells did not expand at the same rate as untransduced B95.8-LCLs and thus, the ability to use knockdown LCLs in T cell recognition assays was not always possible (discussed in chapter 6). This meant that all T cell assays, described in chapter 6, involved new transductions of B95.8-LCLs in parallel, for each experiment, which was not ideal and limited the amount of effector T cells which could be assayed. In addition, sorting of shBGLF5-LCLs was hampered by the fact that the sorting facility available did not possess a laser which could effectively excite the FP635 tag and thus cell sorting was less efficient. However, this method of gene knockdown was ultimately a success and allowed for the further investigation of the roles BNLF2a, BILF1 and BGLF5 play in interfering with antigen presentation at the three different stages of lytic cycle (IE, E and L).

The novel finding of BNLF2a expression in latent EBV infected cells raises some interesting issues. It suggests that BNLF2a may play a role in interfering with antigen presentation in latent infections, implicating it as an important immune evasion mechanism in both latent and lytic forms of EBV infection. Interestingly, Jochum *et al* have recently shown that BNLF2a plays an important role in protecting newly infected B cells from T cell recognition (Jochum *et al.,* 2012; Jochum *et al.,* 2012). In this study (as discussed in chapter 1), the presence of BNLF2a transcript was detected in the EBV virion and it was found to be expressed and active early in infection, before the initiation of lytic cycle replication.

Regardless of whether BNLF2a expression in latency is sufficient to protect cells from $CD8^+$ T cell recognition, the data presented in this chapter (Fig 5.7) suggest BNLF2a could be a potential T cell target and thus could possibly be used in treatment of EBV infections and associated disease.

CHAPTER 6

RECOGNITION OF EBV LYTIC CYCLE EPITOPES IN shRNA-LENTIVIRUS TRANSDUCED LCLS LACKING THE EXPRESSION OF BNLF2A, BILF1 OR BGLF5

In order to assess the roles BNLF2a, BILF1 and BGLF5 play in interfering with presentation of IE, E and L lytic epitopes, we selected lytic B95.8 LCLs and silenced the expression of BNLF2a, BILF1 or BGLF5 using shRNA-lentiviruses, as described in chapter 5. These LCLs, alongside their shNon-target- and ΔBZLF1 counterparts, were then used as targets for $CD8^+$ T cells specific for IE, E or L lytic epitopes. In order to minimise any experimental variation, we aimed to simultaneously measure the recognition of these transduced LCLs, by $CD8^+$ T cells restricted through the same HLA-molecule, showing specificity towards epitopes derived from each phase of lytic cycle (IE, E and L).

6.1. **Experimental approach**

The approach adopted for this chapter was to select a range of lab donor B95.8-LCLs (Table 6.1) which possessed the relevant HLA alleles and were no less than 1% lytic (according to intracellular BZLF1 staining). These were transduced with shNon-target-, shBNLF2a-, shBILF1- or shBGLF5-lentiviruses, in order to knockdown the expression of the appropriate EBV immune evasion genes. Following expansion of these transduced LCLs, the level of transduction was measured using flow cytometry to detect the level of CFP- (shBNLF2a and shNon-target), YFP- (shBILF1), or FP635- (shBGLF5) tag expression, as described in chapter 5. Transformed LCLs which were more than 80% transduced were used immediately in T cell

recognition assays or; those which were less than 80% positive were enriched using MoFlo fluorescence activated cell sorting, and subsequently used in T cell recognition assays. To account for any differences in lytic cycle between target LCLs, the level of mRNA expression of each T cell target antigen, was also measured using qRT-PCR, as described in chapter 4. T cell recognition data was then normalised against the mRNA expression level of the target antigen. This allowed us to investigate the increase in T cell recognition of IE, E and L epitopes in the absence of BNLF2a, BILF1 or BGLF5 expression, when compared to shNon-target-LCLs. The T cell cloning experiments described in chapter 3 provided the reagents to enable simultaneous assessment of recognition of both HLA-A2 and HLA-B7 restricted epitopes derived from IE, E and L phases of lytic cycle (Table 6.2).

Table 6.1 HLA types of donors used to generate shNon-target-, shBNLF2a-, shBILF1-, shBGLF5- and ΔBZLF1-LCLs

Donor	HLA-A		HLA-B		HLA-C	
1	2	24	27	35	2	4
3	2	3	7	27	1	7
4	1	2	39	40	3	12
5	2	ND	58	55	3	7
6	2	ND	47	60	ND	ND
7	2	68	35	49	4	7
8	2	29	7	44	7	16

*ND HLA allele not determined

Table 6.2 Peptide epitope specificities of CD8+ T cell clones used in recognition assays

Phase of expression	EBV protein	Peptide epitope	HLA restriction	No. of clones
Immediate early	BZLF1	DPYQVPFVQAF	B7	1
	BRLF1	YVLDHLIVV	A2	3
Early	BMLF1	GLCTLVAML	A2	3
	BMRF1	TLDYKPLSV	A2	1
	BNLF2b	RPGRPLAGFYA	B7	1
Late	BALF4	FLDKGTYTL	A2	2
	BNRF1	WQWEHIPPA	A2	1
		YPRNPTWQGNI	B7	1

6.2. Recognition of HLA-A2 presented IE-, E- and L- lytic epitopes on LCLs lacking BNLF2a expression

In this section a selection of HLA-A2 positive lab donor shBNLF2a-LCLs (Table 6.1) were used to assess the ability of a panel of HLA-A2 restricted T cells to recognise IE (BRLF1), E (BMLF1, BMRF1) and L (BALF4, BNRF1) lytic epitopes, in the absence of BNLF2a.

HLA-A2 positive donor 3 shBNLF2a-LCLs were first used to study the recognition of the HLA-A2 restricted IE-YVL (BRLF1), E-GLC (BMLF1) and L-FLD (BALF4) epitopes. Figure 6.1 shows a full set of representative data for one $CD8^+$ T cell clone per epitope. Figure 6.1a shows the raw level of recognition (IFN-γ release) by each T cell clone, including peptide pulsed ΔBZLF1-LCLs to show maximal recognition, and ΔBZLF1-LCL recognition as a negative control. In this example, shBNLF2a-LCLs were more highly recognised than shNon-target-LCLs, by YVL-, GLC- and FLD- specific T cells. These shBNLF2a-LCLs were expressing marginally higher levels of BRLF1-, BMLF1- and BALF4-transcripts, compared to that of shNon-target LCLs, as shown in Figure 6.1b. After normalising the level of T cell recognition on the level of antigen expression (Fig 6.1c), there was a clear increase in recognition of LCLs lacking BNLF2a by each T cell. Notably, the level of increased recognition differed between each epitope; recognition of the IE-YVL epitope was increased 13-fold, E- GLC epitope recognition was increased 9-fold, whereas the increase in recognition of L-FLD epitope was less dramatic (2-fold increase).

To confirm that this finding was not donor specific, the same experiment was carried out using different donor target LCLs (donor 4) and a different set of $CD8^+$ T cell clones with the same specificities as those used in Figure 6.1. As shown in Figure 6.2a, shBNLF2a-LCLs were again better recognised by YVL-, GLC-, and FLD-epitope specific T cells (Fig 6.2a). In

this example however, shBNLF2a-LCLs were expressing substantially less lytic cycle antigen than the shNon-target control LCLs (Fig 6.2b). Although such differences were potentially detrimental to the interpretation of data, after normalisation of T cell recognition against measured transcript levels for the respective target antigens, the overall pattern of recognition of shBNLF2a-LCLs (Fig 6.2c) was remarkably similar to that seen in Figure 6.1c. Again, the recognition of YVL- and GLC-epitopes was dramatically increased in the absence of BNLF2a (30- and 24-fold respectively), whereas the increase in recognition of the FLD-epitope was less dramatic.

In order to confirm this pattern remained consistent between different donors and other HLA-A2 restricted T cell specificities, this work then was performed using three further sets of donor LCLs. Using donor 5 shBNLF2a-LCLs as targets for the same panel of HLA-A2 restricted clones used in Figure 6.1, the same pattern of increased recognition following normalisation, was seen (Fig 6.3c). This was then extended to use donor 5 and 6 LCLs as targets for a slightly different panel of T cells which recognised IE-YVL (BRLF1), E-TLD (BMRF1) and L-WQW (BNRF1). As shown in Figure 6.4c and 6.5c, the pattern of increased recognition of epitopes derived from antigens which are expressed at the three different stages of lytic cycle remains consistent (IE>E>>L). It should be noted that due to BNRF1 expression in ΔBZLF1-LCLs, as described in chapter 3, it was not possible to use these LCLs as negative controls for WQW specific T cells, for this reason, HLA mismatched LCLs were instead used. Finally, a more extensive panel was used to test the recognition of IE-YVL (BRLF1), E-GLC (BMLF1), E-TLD (BMRF1) and L-FLD (BALF4) when presented by donor 7 shBNLF2a-LCLs (Fig 6.6). As shown in Figure 6.6c, the pattern described above remained consistent. Indeed, the most dramatic increase in recognition was of IE-YVL and E-GLC/E-TLD epitopes with a less dramatic increase in L-FLD epitope recognition. Therefore, despite

varying levels of lytic antigen expression, the pattern of increased recognition of different lytic cycle antigens (IE>E>>L) was consistent. All presented data, and that data to follow was repeated one or two further times, using different clones of the same specificity, if possible. Although this could not always be carried out simultaneously, due to limited cell numbers, the patterns shown in Figures 6.1-6.6 remained consistent, where the most dramatic increase in recognition was of IE- and especially E-epitopes, while the increase in L-epitope recognition was marginal, all data obtained during the course of this work is summarised Table 6.3. The difference in the magnitude of increase in recognition between donors is likely due to experimental variation of donor LCLs and T cells. However, the fact that the observed pattern remained consisted confirmed that the above experimental approach was valid and reliable for this work. For this reason, only normalised data will be shown for the rest of this chapter.

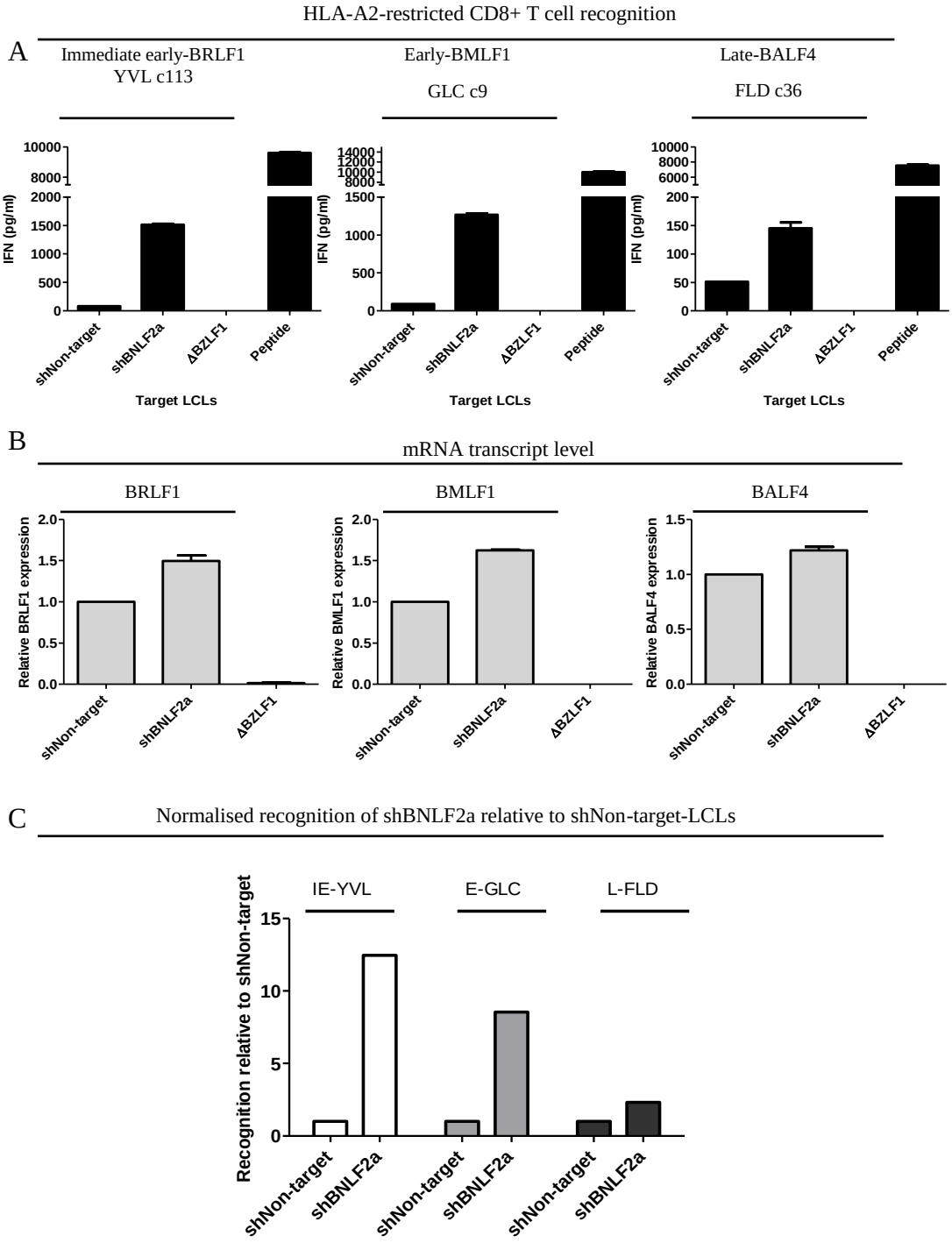


Figure 6.1 Recognition of donor 3 shBNLF2a-LCLs.

A) Recognition of donor 3 LCLs by a IE-YVL, E-GLC and L-FLD specific $CD8^+$ T cell clones. Recognition is shown as IFN-γ (pg/ml) release by T cell. Maximal recognition is shown as recognition of peptide sensitised ΔBZLF1 LCLS. (B) Level of IE-BRLF1 (YVL), E-BMLF1 (GLC) and L-BALF4 (FLD) mRNA transcript in target LCLs used in A. (C) Relative recognition of shBNLF2a-LCLs relative to shNon-target-LCLs, after normalisation of IFN-γ release against transcript level.

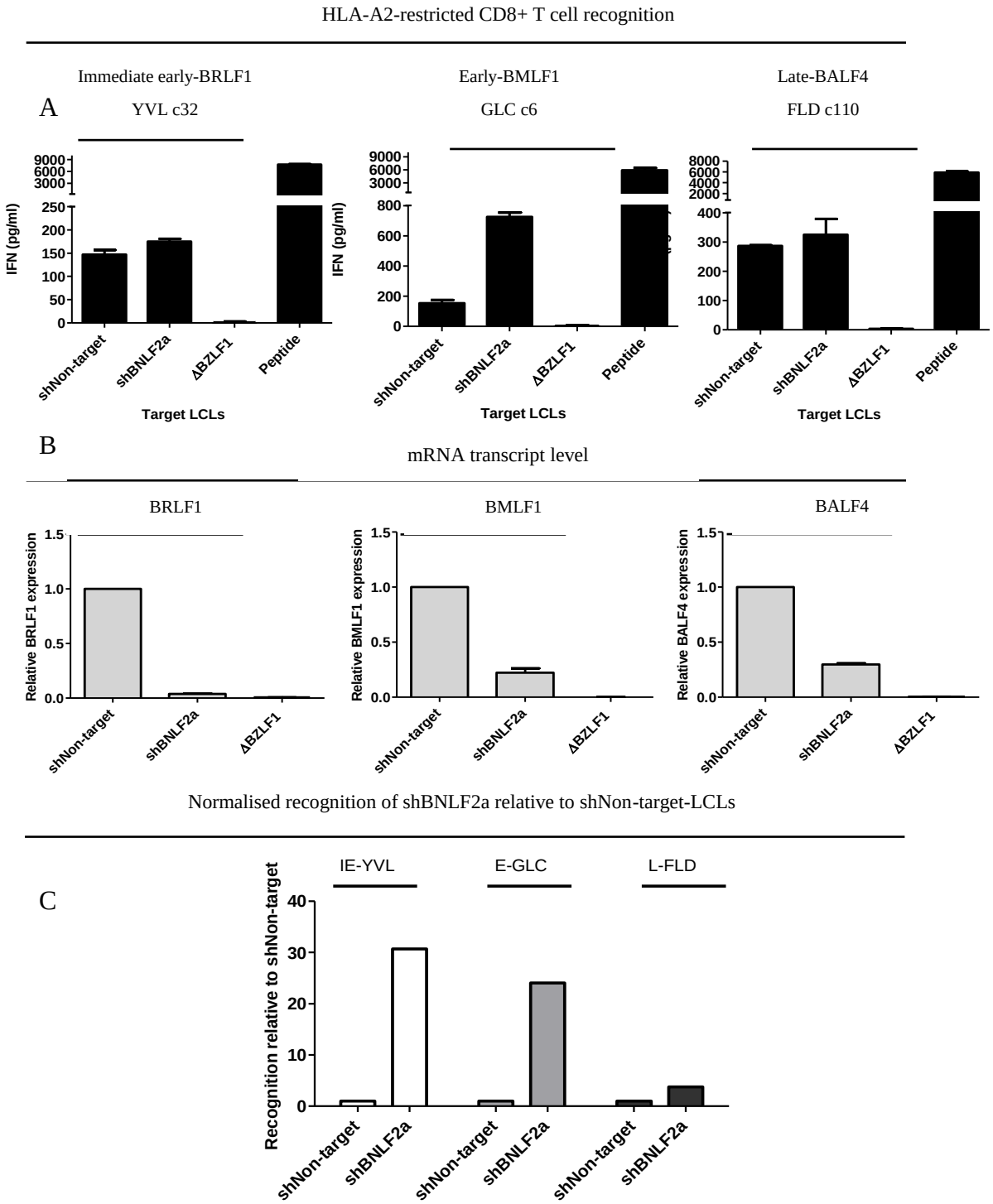


Figure 6.2 Recognition of donor 4 shBNLF2a-LCLs by HLA-A2 restricted T cell clones.

A) Recognition of donor 4 LCLs by IE-YVL, E-GLC and L-FLD specific $CD8^+$ T cell clones. Recognition is shown as IFN-γ (pg/ml) release. Maximal recognition is measured using peptide pulsed ΔBZLF1-LCLs. (B) Level IE-BRLF1 (YVL), E-BMLF1 (GLC) and L-BALF4 (FLD) mRNA transcript in target LCLs used in A. C) Relative recognition of shBNLF2a-LCLs relative to shNon-target-LCLs, after normalisation of IFN-γ release using transcript level.

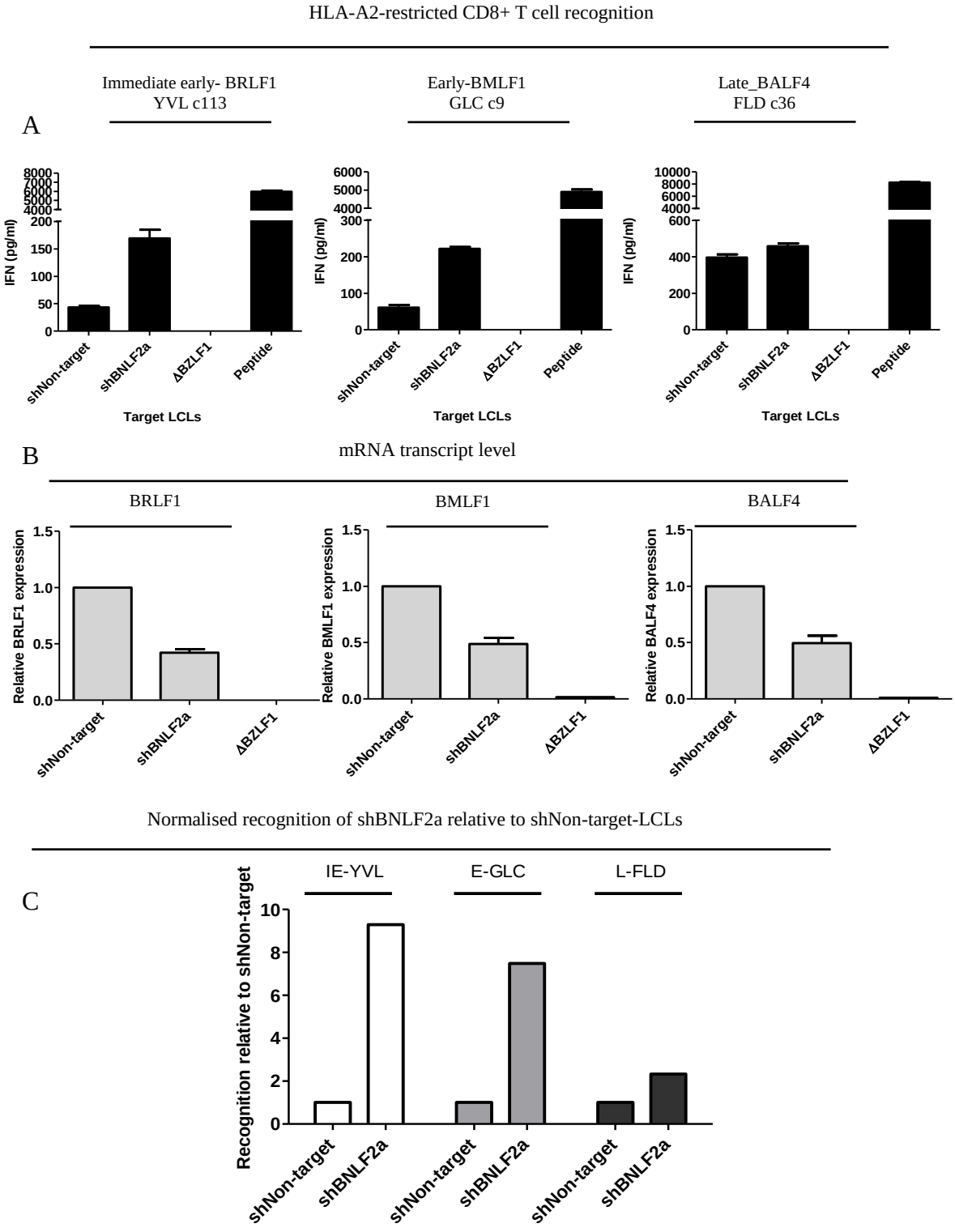


Figure 6.3. Recognition of donor 5 shBNLF2a-LCLs by HLA-A2 restricted T cell clones.

A) Recognition of donor 3 LCLs by an IE-YVL, E-GLC and L-FLD specific CD8+ T cell clones. Recognition is shown as IFN-g (pg/ml). B) Level of mRNA transcript of IE-BRLF1 (YVL), E-BMLF1 (GLC) and L-BALF4 (FLD) in target LCLs used in A. C) Relative recognition of shBNLF2a-LCLs relative to shNon-target-LCLs, after normalisation of IFN-g release using transcript level.

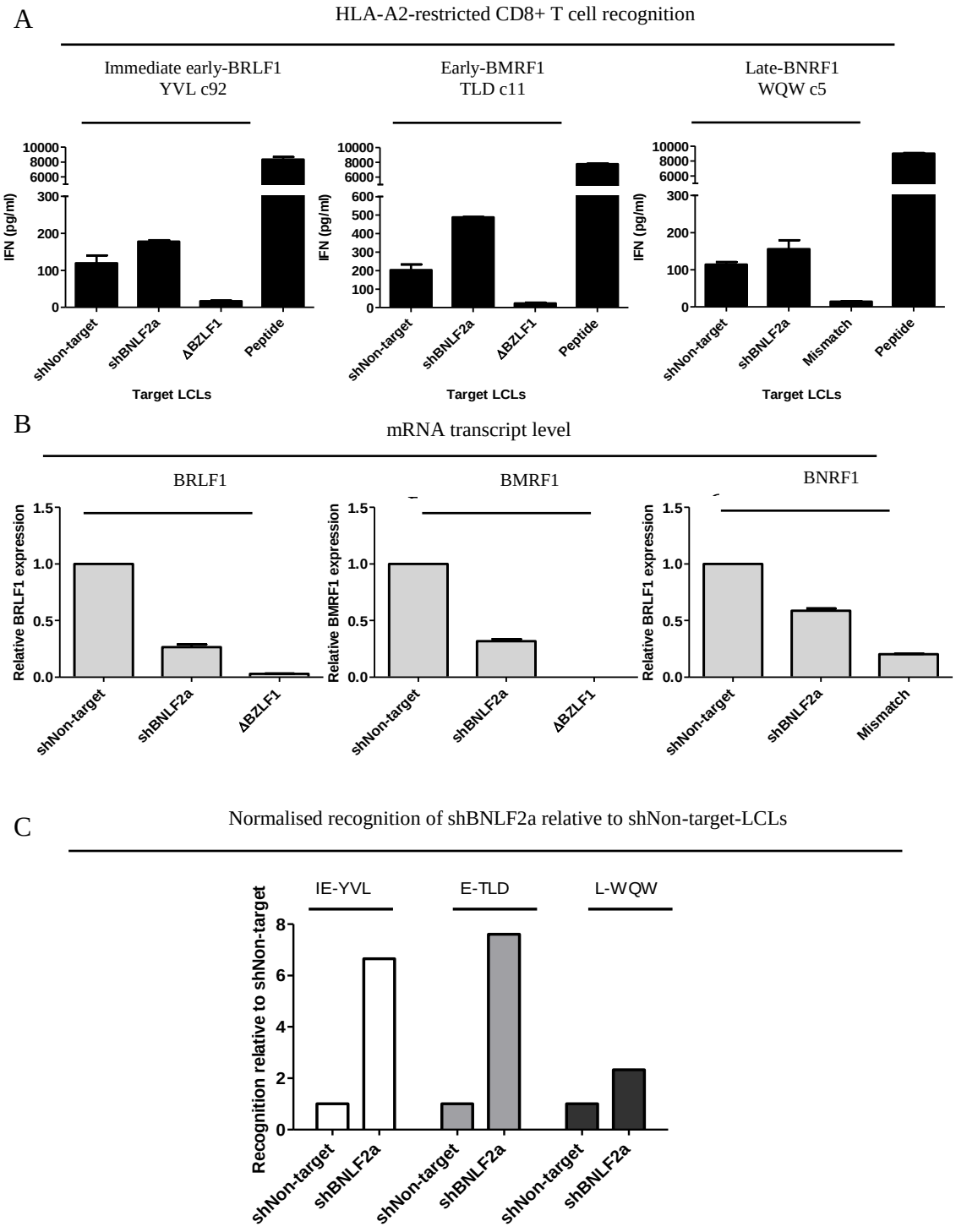


Figure 6.4. Recognition of donor 5 shBNLF2a-LCLs by HLA-A2 restricted T cell clones.

A) Recognition of donor 3 LCLs by an IE-YVL, E-TLD and L-WQW specific CD8+ T cell clones. Recognition is shown as IFN-g (pg/ml) release by T cell. B) Level of mRNA transcript of IE-BRLF1 (YVL), E-BMRF1 (TLD) and L-BNRF1 (WQW) in target LCLs used in A. C) Relative recognition of shBNLF2a-LCLs relative to shNon-target-LCLs, after normalisation of IFN-g release using transcript level.

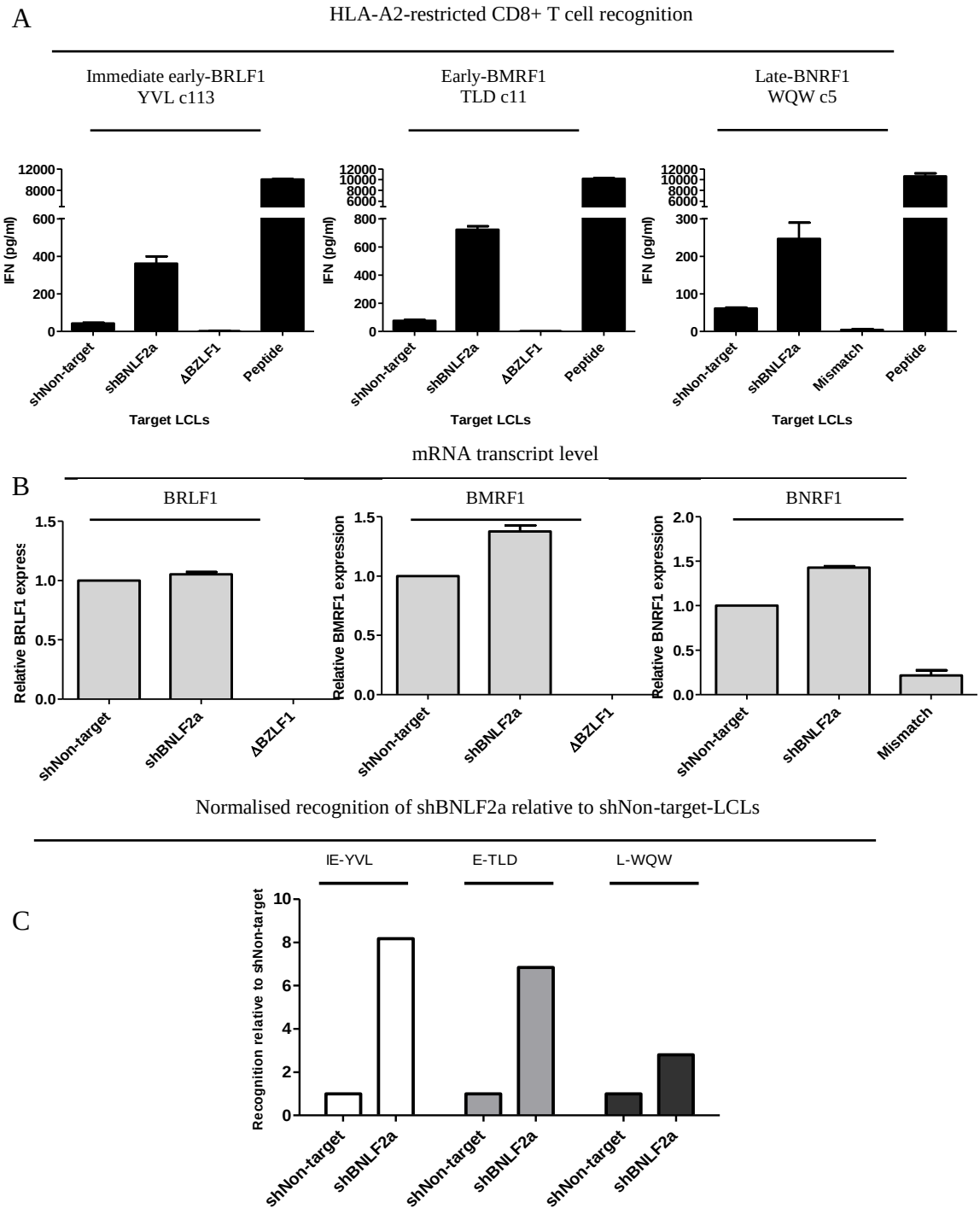


Figure 6.5. Recognition of donor 6 shBNLF2a-LCLs by HLA-A2 restricted CD8+ T cells

A) Recognition of donor 3 LCLs by an IE-YVL, E-TLD and L-WQW specific CD8+T cell clones. Recognition is shown as IFN-g (pg/ml) release by T cell. (B) Level of mRNA transcript of IE-BRLF1 (YVL), E-BMRF1 (TLD) and L-BNRF1 (WQW) in target LCLs used in A. (C) Relative recognition of shBNLF2a-LCLs relative to shNon-target-LCLs, after normalisation of IFN-g release using transcript level.

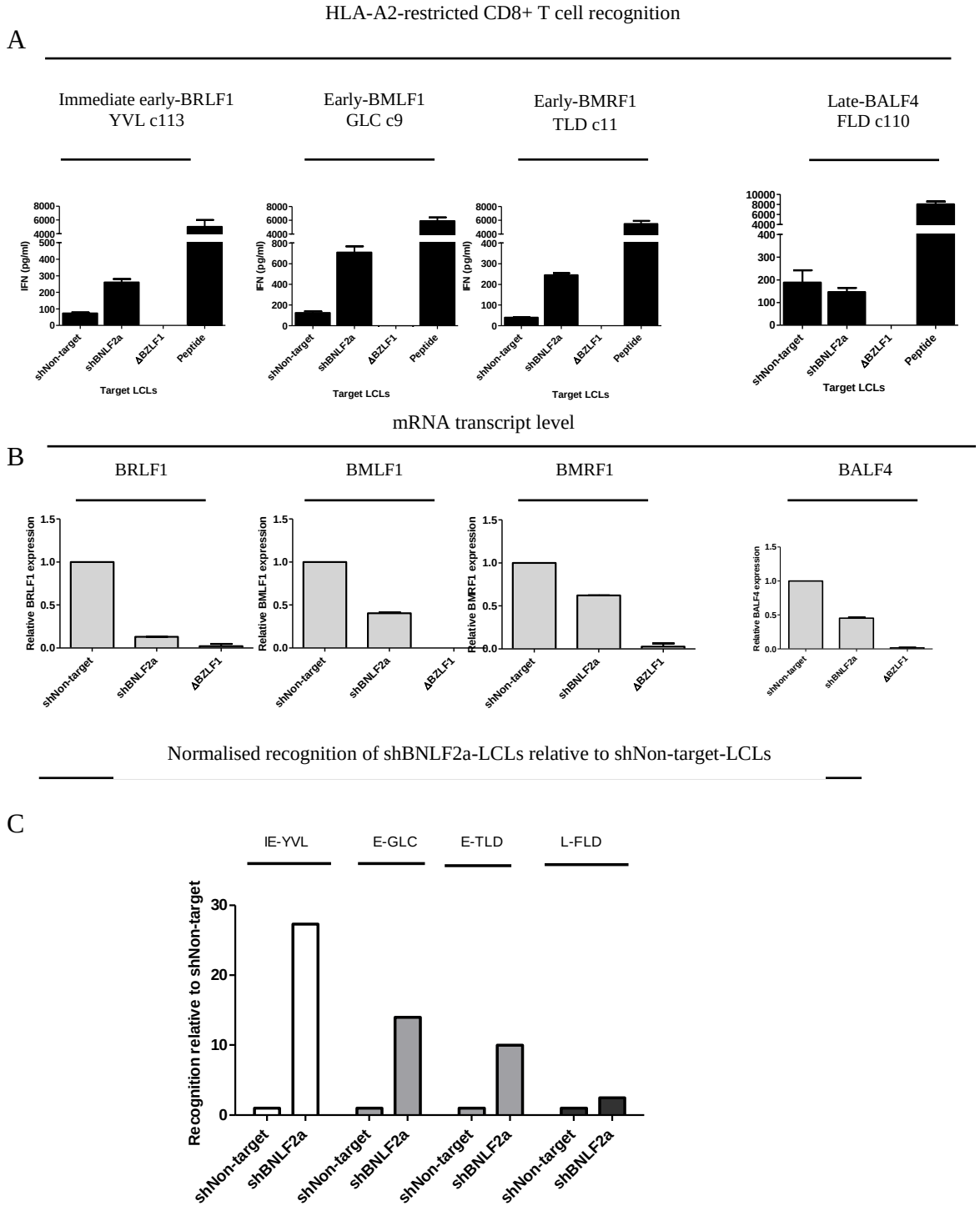


Figure 6.6. Recognition of donor 7 shBNLF2a-LCLs by HLA-A2 restricted CD8+ T cell clones.

A) Recognition of donor 7 LCLs by an IE-YVL, E-GLC, E-TLD and L-FLD specific CD8+T cell clones. Recognition is shown as IFN-g (pg/ml) release by T cell. (B) Level of mRNA transcript of IE-BRLF1 (YVL), E-BMRF1 (TLD) and L-BNRF1 (WQW) in target LCLs used in A. (C) Relative recognition of shBNLF2a-LCLs relative to shNon-target-LCLs, after normalisation of IFN-g release using transcript level.

6.2.1. Recognition of HLA-B7 presented immediate early, early and late lytic antigens in the absence of BNLF2a expression

Next, the possibility that the inhibition of antigen processing by BNLF2a may be confined to HLA-A2 molecules was addressed. Although this was unlikely as BNLF2a acts upon the TAP transporter, and thus is unlikely to target certain HLA molecules, it was still interesting to determine if the above pattern remained consistent when investigating the effects of BNLF2a knockdown on the recognition of epitopes presented by HLA-B7 molecules. To do this, a panel of HLA-B7 restricted $CD8^+$ T cell clones (Table 6.2) which recognise the IE-DPY (BZLF1), the E-RPG (BNLF2b) and the L-YPR (BNRF1) epitopes were used as effector cells against a selection of HLA-B7 positive shBILF1-LCLs.

Using donor 3 and 8 shBNLF2a-LCLs, as shown in Figure 6.7, the pattern of increased recognition in the absence of BNLF2a was similar to that seen when using HLA-A2 restricted effector T cells. Again, recognition of the IE (DPY) and E (RGP) epitopes was increased more dramatically than that of a L (YPR) epitope.

In summary, using a panel of HLA-A2 restricted T cells against 5 different sets of donor LCLs and a panel of HLA-B7 restricted T cells against 2 different sets of donor LCLs, as well as experiments carried out using partial panels of T cells, antigen presentation was found to be increased in the absence of BNLF2a for IE, E and L antigens. The extent of this increase differed, whereby more dramatic increases were seen for IE epitopes, less dramatic for E antigens and less again for L epitopes, as summarised in Table 6.3 is in agreement with data presented in chapter 4, and together these data suggest that at the IE and E phase of lytic cycle, BNLF2a plays a strong role in interfering with antigen presentation, and at the L phase, BNLF2a is likely playing a lesser role. This finding implies that at the L stages of lytic cycle,

when EBV has been found to be highly protected against $CD8^+$ T cell recognition, other immune evasion mechanisms must be active and functional.

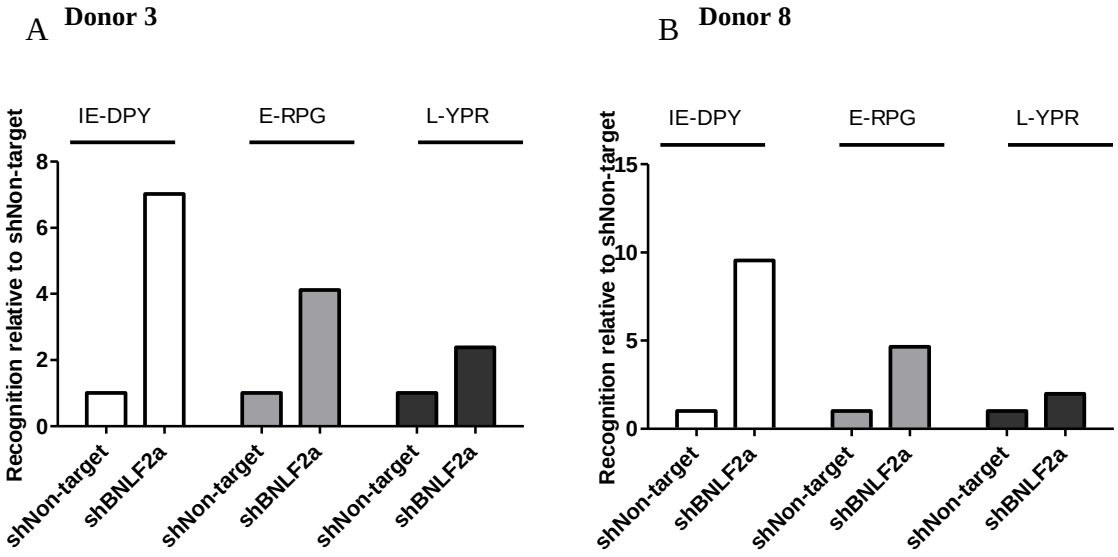


Figure 6.7 Normalised relative recognition of donor 3 and 8 shBNLF2a-LCLs by a panel of HLA-B7 restricted CD8+ T cell clones specific for the IE-, E- and L-lytic epitopes.

(A) Relative recognition of donor 3 HLA-B7 positive shBNLF2a-LCLs, compared to shNon-target LCLs, by HLA-B7 restricted T cells specific for the IE-DPY (BZLF1), E- RPG (BNLF2b) and L-YPR (BNRF1) epitopes**.** (B) Relative recognition of donor 8 HLA-B7 positive shBNLF2a-LCLs compared to shNon-target LCLs, by HLA-B7 restricted T cells specific for the IE-DPY (BZLF1), E- RPG (BNLF2b) and L-YPR (BNRF1) epitopes.

Table 6.3 Summary of fold increase in CD8$^+$ T cell recognition of IE-, E-, and L-antigens when presented by shBNLF2a-LCLs compared to shNon-target-LCLs

					Fold increase	
Phase of expression	**EBV protein**	**Epitope**	**HLA restriction**	**No. of expts***	**Range**	**Median**
Immediate early	BRLF1	YVL	A2	11	6.5-30.7	17
	BZLF1	DPY	B7	4	7-14	8.3
Early	BMLF1	GLC	A2	10	7-24	11.5
	BMRF1	TLD	A2	5	7.5-12	9
	BNLF2b	RPG	B7	4	4.1-7	5.5
Late	BALF4	FLD	A2	11	2-5	2.3
	BNRF1	WQW	A2	4	2.3-3	2.5
		YPR	B7	4	2-3.5	2.5

*More than one effector clone was used where possible (see Table 6.2). In total 7 different donor LCLs were used.

6.3. **Recognition of HLA-A2 presented IE, E and L lytic epitopes presented by LCLs lacking BILF1 expression**

Next, the effect of BILF1 on antigen presentation during lytic cycle was investigated using similar experiments to above. To this end, a selection of lab donor shBILF1-LCLs (Table 6.1) were used as targets for the panels of HLA-A2 restricted $CD8^+$ T cell clones outlined in Table 6.2.

This was first carried using donor 1 and 3 HLA-A2 positive shBILF1-LCLs as targets for two different sets of HLA-A2 restricted T cells specific for IE-YVL (BRLF1), E-GLC (BMLF1) and L-FLD (BALF4) epitopes. A representative example of these results, for one clone per epitope, is shown in Figure 6.7a and b. In these particular examples, the recognition of E-GLC and L-FLD epitopes was clearly increased in the absence of BILF1, compared to recognition of shNon-target counterparts, where clearly the increase of L-FLD recognition was more dramatic. In contrast, the increase in recognition of the IE-YVL epitope was minimal.

This was then repeated using donor 5, 6 and 7 shBILF1-LCLs as targets for a more extensive panel of HLA-A2 restricted T cells which recognised IE-YVL, E-GLC or E-TLD, and L-FLD and/or L-WQW epitopes. In each representative example, the lack of BILF1 expression resulted in an increase in recognition of the E-epitopes TLD (Fig 6.8 d,f) and GLC (Fig 6.8c,e), and a more striking increase in recognition of L-FLD (Fig 6.8c,e,f) and L-WQW (Fig 6.8d,e) epitopes. In all examples, there was a minimal increase in recognition of the IE-YVL epitope. Clearly, the pattern of increased recognition of different lytic cycle epitopes in the absence of BILF1 is in contrast to that of BNLF2a. The pattern of increased recognition of epitopes when BILF1 was not expressed shows increased recognition of E epitopes, a more exaggerated increase in recognition of L epitopes, and minimal increases in recognition of IE

epitopes (IE<E<<L). This pattern remained consistent upon repeating the examples shown and when using shBILF1-LCLs as targets for partial panels of HLA-A2 restricted T cells, as summarised in Table 6.7.

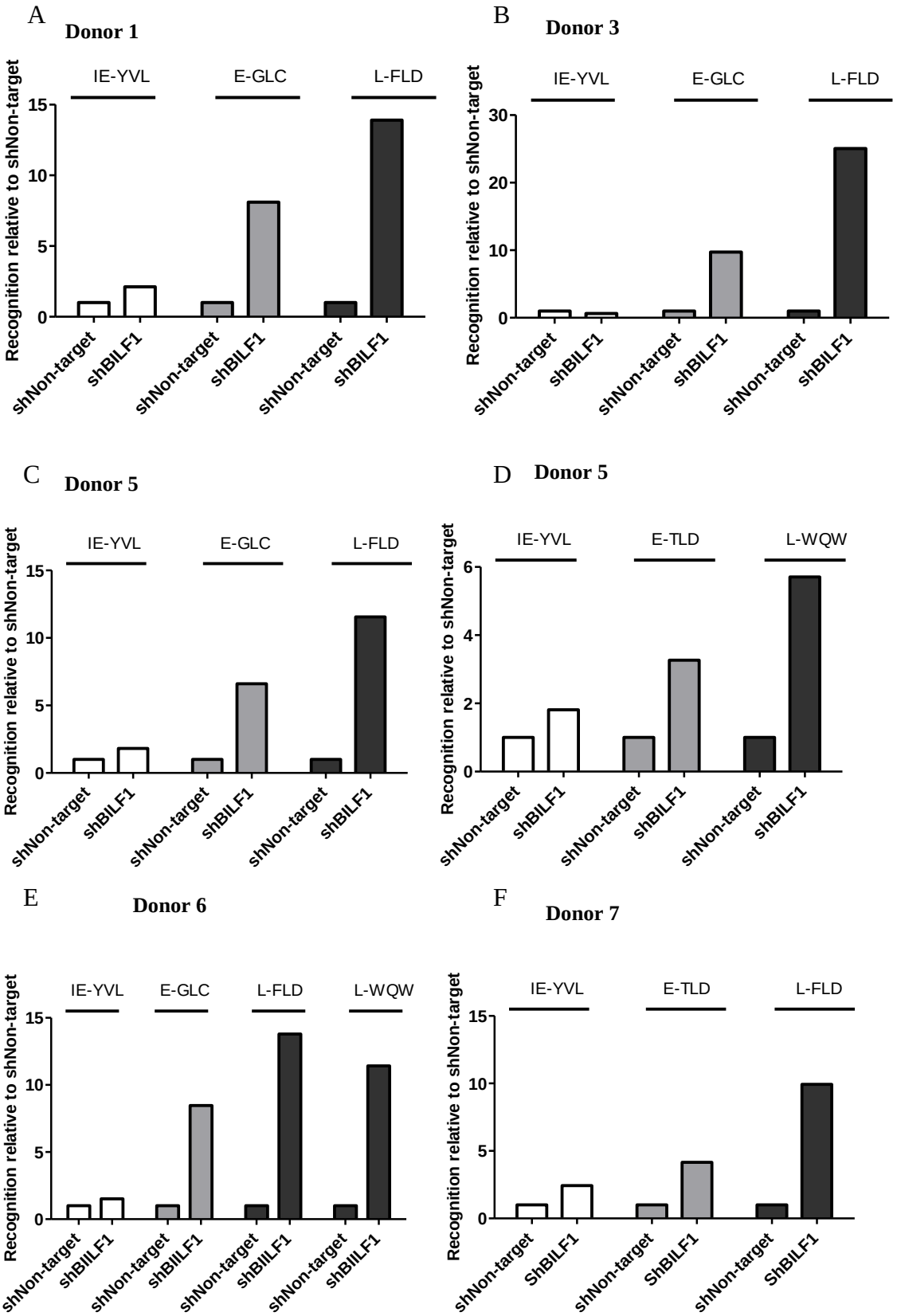

A
Donor 1
IE-YVL
E-GLC
L-FLD
Recognition relative to shNon-target
15
10
5
0
shNon-target
shBILF1
B
Donor 3
30
20
C
Donor 5
D
Donor 5
IE-TLD
L-WQW
6
4
2
E
Donor 6
L-WQW
F
Donor 7

Figure 6.8. Normalised relative recognition of shBILF1-LCLs, by a panel of HLA-A2 restricted CD8+ T cells.

(A,B) Relative recognition of donor 3 and 2 shBILF1-LCLs by IE-YVL (BRLF1), E-GLC (BMLF1) and L-FLD (BALF4) specific T cells. (C) Relative recognition of donor 7 shBILF1-LCLs by IE-YVL (BRLF1), E-TLD (BMRF1) and L-FLD (BALF4) specific T cells. (D) Relative recognition of donor 6 shBILF1-LCLs by a panel IE-YVL (BRLF1), E-GLC (BMLF1), L-FLD (BALF4) and the L-WQW (BNRF1) specific T cells. (E,F) Relative recognition of donor 5 shBILF1 LCLs IE-YVL (BRLF1), E-GLC (BMLF1) and L-FLD (BALF4) (C) and, in a separate experiment, IE-YVL (BRLF1), E-TLD (BMRF1) and LWQW (BNRF1) epitopes.

6.3.1. Recognition of HLA-B7 presented IE, E and L lytic epitopes in shBILF1-LCLs

As it is perhaps more likely that BILF1 could target specific HLA molecules, given that it interacts directly with MHC class I molecules, recognition of IE, E and L epitopes presented by HLA-B7 molecules was investigated. To do this, HLA-B7 positive shBILF1-LCLs were used as targets for a panel of HLA-B7 restricted T cells. These T cells recognised the IE-DPY (BZLF1), the E-RPG (BNLF2b) and the L-YPR (BNRF1) epitopes. Representative examples of these results, from two donors, for one T cell clone per epitope, are shown in Figure 6.9. Using donor 3 shBILF1 LCLs (Fig 6.9a), that was also used in Figure 6.8b, and donor 8 shBILF1-LCLs (Fig 6.9b); there was an increase in recognition of E-RPG epitope, and a more exaggerated increase in recognition of L-YPR epitope. In a similar pattern to that described for the effect of BILF1 knockdown on recognition by HLA-A2 restricted T cells, the increase in recognition of IE-DPY epitope was minimal. This pattern is similar to that observed for HLA-A2 epitope recognition. These data show that the effect of BILF1 on antigen presentation is not confined to HLA-A2 restricted epitopes.

As described previously, although the individual examples of results shown in Figures 6.8 and 6.9 were carried out using one set of donor shBILF1-LCLs as targets for the panel of T cells indicated, this was not always possible when trying to repeat these experiments. This was due to limiting cell numbers and as a result of this, partial panels of HLA-A2 or HLA-B7 restricted T cells had to be used when using complete T cell panels was not feasible. A summary Table of all completed experiments and the fold increase in recognition seen for all HLA-A2 and HLA-B7 T cells is shown in Table 6.4. Taken together, these data suggest that BILF1 plays a strong role in interfering with the presentation of antigens during the E and L

phases of lytic cycle, with its strongest effect being at the L-phase, while its effect at the IE-phase is relatively weak.

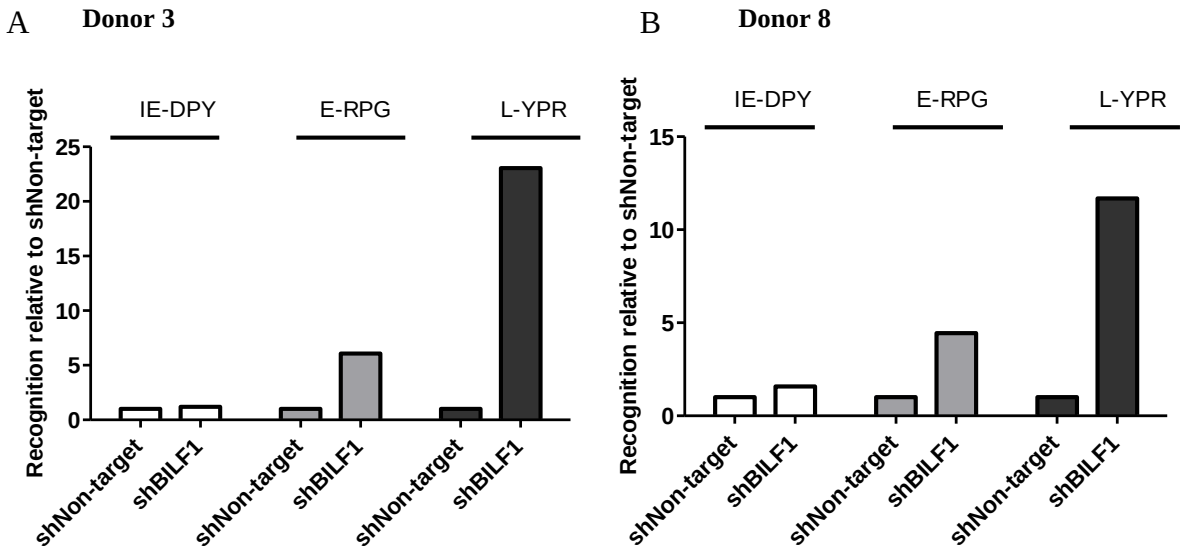


Figure 6.9 Relative recognition of donor 3 and 8 shBILF1-LCLs by a panel of HLA-B7 restricted CD8+ T cell clones.

(A) Relative recognition of donor 3 shBILF1-LCLs, compared to shNon-target LCLs, by HLA-B7 restricted T cells specific for the IE-DPY (BZLF1), E- RPG (BNLF2b) and L-YPR (BNRF1) epitopes. (B) Relative recognition of donor 8 HLA-B7 positive shBILF1-LCLs compared to shNon-target LCLs, by HLA-B7 restricted T cells specific for the IE-DPY (BZLF1), E- RPG (BNLF2b) and L-YPR (BNRF1) epitopes.

Table 6.4 Summary of fold increase in CD8+ T cell recognition of IE-, E-, and L-epitopes when presented by shBILF1-LCLs compared to shNon-target-LCLs

Phase of expression	EBV protein	Epitope	HLA restriction	No. of expts*	Fold increase Range	Median
Immediate early	BRLF1	YVL	A2	10	No increase-2.5	1.9
	BZLF1	DPY	B7	4	1.2-1.8	1.7
Early	BMLF1	GLC	A2	8	7-11	9.1
	BMRF1	TLD	A2	5	3.2-10	6
	BNLF2b	RPG	B7	4	4.5-7	6
Late	BALF4	FLD	A2	8	10-25	13.5
	BNRF1	WQW	A2	4	5.7-16	12.2
		YPR	B7	4	9-23	11.3

*More than one effector clone was used where possible (see table 6.2). In total 6 different donor LCLs were used.

6.4. Recognition of shBGLF5 LCLs by HLA-A2 and HLA-B7 restricted T cells specific for IE, E and L lytic epitopes

Next the role that BGLF5 plays in interfering with antigen presentation during the three different phases of lytic cycle was investigated. These sets of experiments were more problematic due to the nature of shBGLF5-LCLs. These LCLs did not survive for longer than three weeks following transduction, thus the ability to simultaneously examine the recognition of shBGLF5-LCLs by our panels of HLA-A2 or HLA-B7 T cells was rarely possible. This meant that most experiments were carried using a partial panel of HLA-A2 or B7 restricted T cells (summarised in Table 6.5). Despite this, it was possible to assess the recognition of two sets of donor shBGLF5-LCLs using a panel of HLA-A2 T cells specific for; IE-YVL; E-GLC; and L-FLD or L-WQW epitopes. As shown in Figure 6.10a and b, there was minimal increased recognition of IE-, E- and L-epitopes in LCLs lacking BGLF5 expression, compared to their shNon-target LCL counterparts. Although there appeared to be a more dramatic increase in recognition of the L-WQW epitope (Fig 6.10b), this pattern was not consistent when repeated. Although, as mentioned, using complete panels of HLA-A2 restricted T cells was not always possible, numerous experiments were carried out using partial panels of HLA-A2 restricted T cells and, as shown in Table 6.5, this pattern of minimal effect of increased recognition in the absence of BGLF5 expression was reproducible.

To determine if the observed minimal effect of BGLF5 knockdown on T cell recognition was a HLA-A2 phenomenon, the effect that knocking down BGLF5 had on recognition by HLA-B7 T cells was investigated. Unfortunately, due to the technical difficulties described above, only one complete set was generated using donor 3 shBGLF5 LCLs (Fig 6.10c). In this example, the increase in recognition of IE-DPY, E-RPG and L-YPR epitopes was not more

than 2.5-fold above shNon-target LCLs. This pattern is almost identical to that seen for the HLA-A2 restricted T cell recognition of shBGLF5-LCLs (Fig 10a,b). Following repeats of these experiments, it was clear that the lack of BGLF5 expression consistently showed minimal effects on increasing recognition of IE- E- and L- epitopes, as summarised in Table 6.5

Overall, these findings suggest that BGLF5 plays a minimal role in interfering with antigen presentation at all three stages of EBVs lytic cycle. In some cases there was no effect of BGLF5 knockdown on increasing epitope recognition and in other others, there was a minimal increase in recognition. However, this should be taken with the caveat that these assays were complicated by the fact that shBGLF5-LCLs were more difficult to work with than shBNLF2a- and shBILF1-LCLs. It is also important to note that the lack of phenotypic effect of BGLF5 knockdown could be due to the activity of any residual BGLF5 expression in the low number of LCLs entering lytic cycle. However, the above data is consistent with data accumulated using BGLF5 knockout LCLs, as described in chapter 4, thus the above phenotypic effects of BGLF5 knockdown on T cell recognition are likely a true reflection of the effect of BGLF5 knockdown.

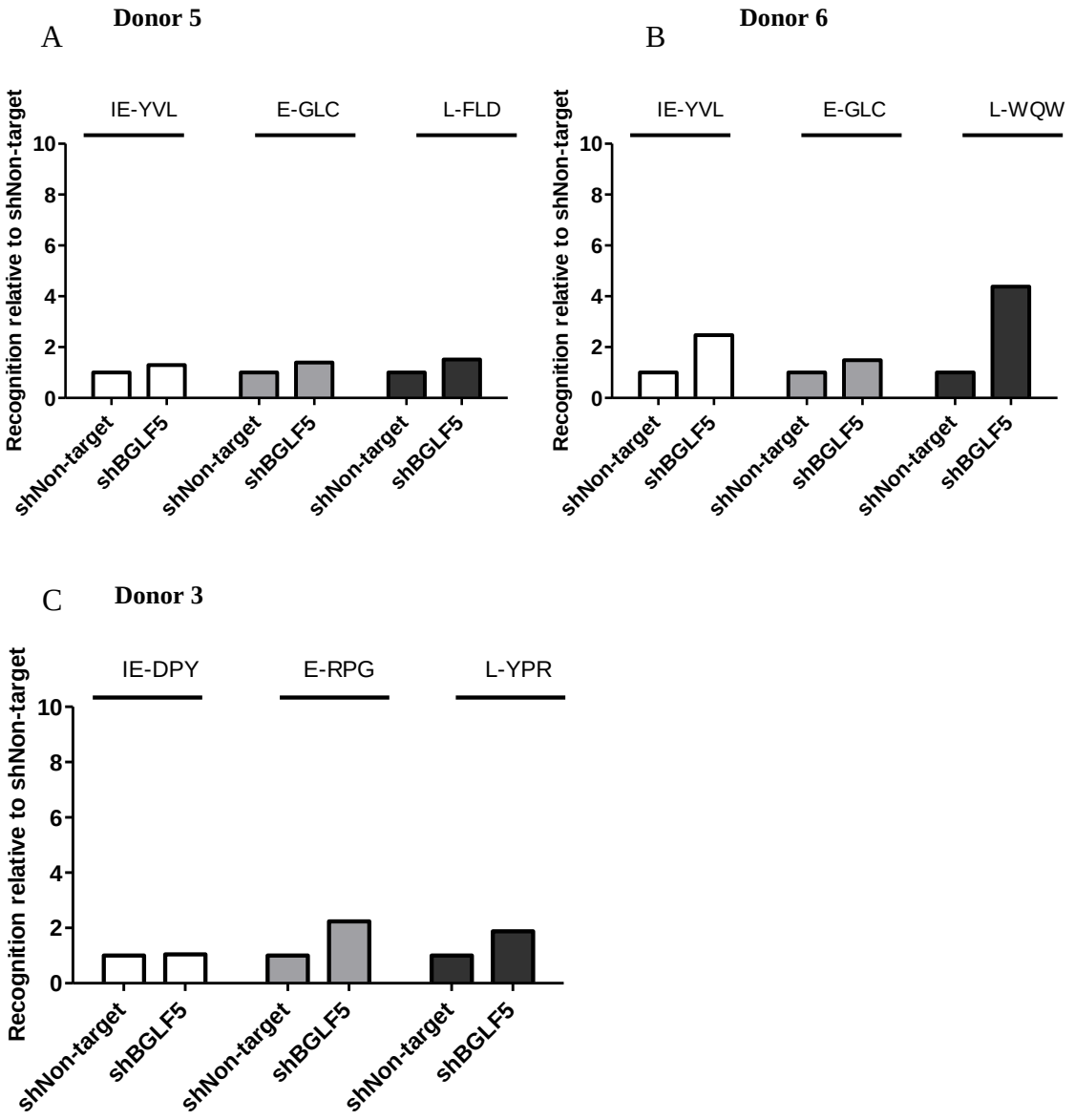


Figure 6.10. Normalised relative recognition of donor 5, 6 and 3 shBGLF5-LCLs by HLA-A2 and HLA-B7 restricted CD8+ T cell clones.

(A) Relative recognition of donor 5 shBGLF5-LCLs, compared to shNon-target-LCLs, by a panel of HLA-A2 restricted CD8+ T cells specific for IE-YVL (BRLF1), E-GLC (BMLF1) and L-FLD (BALF4) epitopes. (B) Relative recognition of donor 6 shBGLF5-LCLs, compared to shNon-target-LCLs, by a panel of HLA-A2 restricted $CD8^+$ T cells specific for IE-YVL (BRLF1), E-GLC (BMLF1) and L-WQW (BNRF1) epitopes. (C) Relative recognition of donor 3 HLA-B7 positive shBGLF5-LCLs, compared to shNon-target LCLs, by HLA-B7 restricted T cells specific for the IE-DPY (BZLF1), E- RPG (BNLF2b) and L-YPR (BNRF1) epitopes**.**

Table 6.5 Summary of fold increase in CD8+ T cell recognition of IE-, E-, and L-epitopes when presented by shBGLF5-LCLs compared to shNon-target-LCLs

Phase of expression	EBV protein	Epitope	HLA restriction	No. of expts*	Fold increase Range	Fold increase Median
Immediate early	BRLF1	YVL	A2	10	No Increase-2.5	1.3
	BZLF1	DPY	B7	2	1.1-1.4	1.2
Early	BMLF1	GLC	A2	8	No Increase-4	1.6
	BMRF1	TLD	A2	3	1.4-1.7	1.5
	BNLF2b	RPG	B7	2	1.9-2.2	2
Late	BALF4	FLD	A2	4	1.5-3	1.8
	BNRF1	WQW	A2	3	1.9-4.3	2.6
		YPR	B7	3	1.5-1.9	1.9

*More than one effector clone was used where possible (see Table 6.2). In total 6 different donor LCLs were used.

6.5. Assessing the effect of BNLF2a, BILF1 and BGLF5, in parallel, on recognition of HLA-A2 presented IE- E- and L-epitopes

The data shown in the preceding sections (6.2-6.4) provide a clear indication of the relative effects of each candidate immune evasion gene on recognition of epitopes at different stages of lytic cycle. However, as the effects of BNLF2a, BILF1 and BGLF5 were examined in separate experiments, the magnitude of the effects relative to one other cannot be ascertained with confidence. To this end, whilst technically challenging, the following set of experiments were performed in order to investigate in a single experiment the relative effects of BNLF2a, BILF1 and BGLF5 on recognition of IE, E and L lytic epitopes.

This was done by generating donor 5 shBNLF2a-, shBILF1- and shBGLF5- and shNon-target- LCLs, and measuring the relative recognition of these LCLs, in parallel, by a panel of HLA-A2 restricted $CD8^+$ T cells. In the representative example shown in Figure 6.11, those clones used recognised the IE-YVL (BRLF1), E-GLC (BMLF1) and L-FLD (BALF4) epitopes. After normalisation on the expression of BRLF1, BMLF1 and BALF4, the patterns of increased recognition remained similar to the previous findings. LCLs lacking BNLF2a were better recognised by IE- and E-specific T cells (15- and 10-fold respectively), whereas the increased recognition in L-epitopes was minimal. In contrast, those LCLs lacking BILF1 expression were better recognised by E- and L-epitope specific T cells (6- and 12-fold respectively), where the most dramatic effect is seen in the recognition of L-FLD and finally, LCLs lacking in BGLF5 expression showed a small level of increased recognition of E- and L-epitopes. Unfortunately, the level of BRLF1 expression by shBGLF5-LCLs was insufficient for T cell recognition, thus, a result could not be determined here.

Relatively speaking, this result suggests that at the IE phase of lytic cycle, BNLF2a plays the most dominant role in interfering with antigen presentation, while BILF1 plays a small role. At the E phase of lytic cycle, both BILF1 and BNLF2a appear to play strong roles, where BNLF2a is possibly more dominant and finally at L phase lytic cycle; BILF1 plays a more dominant role, while BNLF2a contributes a small effect. In contrast, BGLF5 appears to contribute little or no effect at any phase of lytic cycle. However, it should be noted that this could not be repeated in other donors, due to the unreliability of transduced shBGLF5 transduced LCLS, where some were no longer lytic, or did not expand to a high enough number for use. Nevertheless, these data are consistent with the conclusion that BNLF2a and BILF1 are the predominant effectors of immune evasion and that BNLF2a plays a decreasing role as lytic cycle progresses, while BILF1 plays an increasing role.

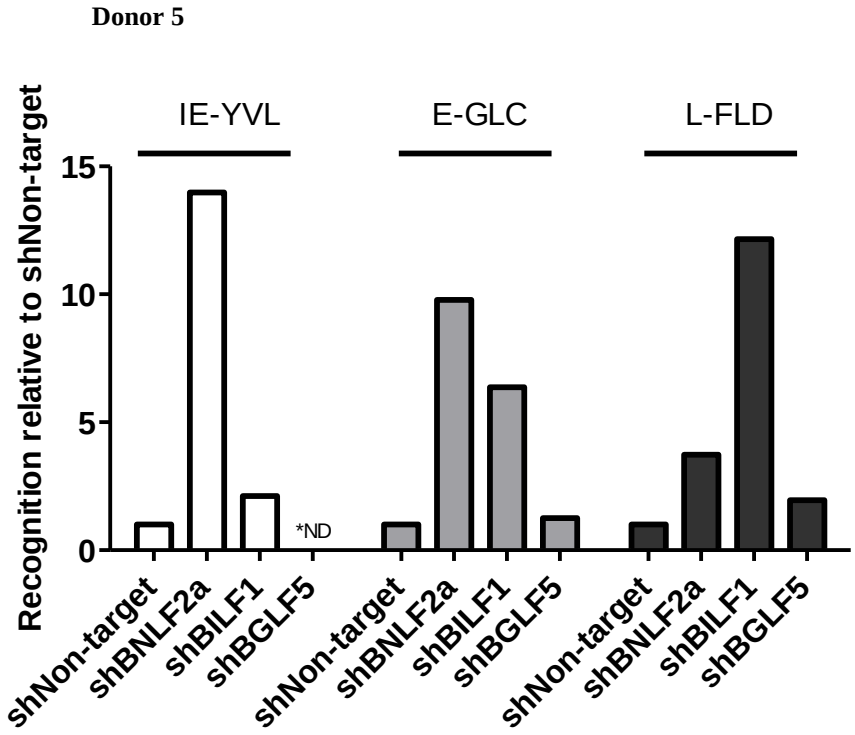


Figure 6.11 Direct comparisons of the relative effects of BILF1, BNLF2a and BGLF5 on recognition of IE-, E-, and L-epitopes by HLA-A2 restricted T cells specific for IE-YVL (BRLF1), E-GLC (BMLF1) and L-FLD (BALF4).

Recognition of epitopes presented by each LCL was measured simultaneously. T cell recognition (IFN-g release) was then normalised on the expression of each appropriate mRNA transcript. Data is shown as recognition of knockdown LCLs relative to shNon-target LCLs.

*ND-Expression levels of target antigen were not sufficient in shBGLF5-LCLs. No result was seen.

6.6. **Correlation of epitope recognition with lytic cycle expression**

The above results are perhaps surprising since it has previously been reported that BNLF2a is expressed as an E lytic gene, therefore the strong effect it has on IE-epitope presentation is unexpected. In order to determine if we could explain the patterns of effect that BNLF2a, BILF1 and BGLF5 have on antigen presentation during lytic cycle, we next measured the kinetics of expression of these genes in cells undergoing synchronous lytic cycle. To do this, an EBV-infected cell line known as Akata was used. Akata cells can be efficiently and synchronously induced into lytic cycle by ligation of surface IgG (Takada and Ono 1989). Following induction, gene expression was measured at successive time points.

The expression kinetics of the IE gene BZLF1, E gene BMRF1 and L gene BALF4 were measured, alongside that of BNLF2a, BILF1 and BGLF5. RNA samples were harvested at time points 0-48 hours post induction. cDNA was then generated and qRT-PCR analysis was used to assess the relative levels of transcription of each gene at each time point. Figure 6.12 shows a representative example of hours post induction plotted against the level of transcript expressed as a percentage of their maximum, the timing of each phase of lytic cycle is also indicated.

As shown in Figure 6.12b, the expression of BNLF2a was first detected at 2 hours post-induction. This is almost the same time at which BZLF1 is first detected (Fig 6.12a). BNLF2a expression then steadily increases and peaks at the E time point of 12 hours, decreasing thereafter. Thus, although BNLF2a is considered an E expressed lytic gene, it is clearly expressed, to up to 73% of its maximum level of expression, during IE-phase lytic cycle. It is therefore unsurprising that it is most effective at interfering with antigen presentation during IE and E phase lytic cycle, when its expression is at its highest. Notably, the expression of

BNLF2a transcript remains high, at 73% during L-stage lytic cycle, yet does not have a strong effect on antigen presentation at this phase. This is due to a rapid decrease in protein expression after 12 hours post transduction. In fact, BNLF2a protein expression is almost undetectable by 24-48 hours post induction (data not shown), indicating that this level of transcript is not directly proportional to the level of protein expression, explaining why despite high levels of transcript, there is little phenotypic effect of BNLF2a.

The pattern of BILF1 transcript expression is slightly different, in that the initial expression of BILF1 is detected later than BNLF2a, at around 4-hours post-induction (Fig 6.12b), which is in the E phase of lytic cycle, as indicated by the expression of BMRF1 (Fig 6.12b). BILF1 expression then rapidly increases and peaks at 8 hours, declining thereafter. This would explain why BILF1 has a subtle effect on the presentation of IE-lytic epitopes and a stronger affect on E-lytic epitope presentation. Interestingly, the expression of BILF1 transcript declines to 77% by 24 hours, and continues to decrease beyond this time. Given that lytic cycle can last for up to 4 days, this is perhaps surprising, since it is at the L-phase of lytic cycle when BILF1 appears to exert its strongest effect. Presumably, this level of transcript translates into relatively high levels of protein, which is active and functional during the late phase of lytic cycle. Unfortunately, the lack of available anti-BILF1 antibody means this cannot be confirmed. However, the obvious phenotypic effect that knocking down BILF1 has on presentation of L-lytic epitopes means that it can be confidently assumed that this is the case.

The kinetics of BGLF5 expression is perhaps more surprising. BGLF5 transcript can be detected at the same time as BILF1, after which it increases, peaking during L phase lytic cycle, at 24 hours. Thus, it would be expected that its immune evasion function would be active during the E-phase of lytic cycle and more so at the L phase. However, the data

presented in the above sections suggest this is not the case. This could perhaps be because the effect of BGLF5 on antigen presentation, in the context of whole virus infection is minimal throughout lytic cycle.

Taken together these data suggest that the roles that BNLF2a and BILF1 play in interfering with antigen presentation are perhaps a consequence of their timings of expression. However, the minimal effect of BGLF5 throughout lytic cycle cannot be explained in this way, this is perhaps a consequence of a weak immune evasion function of BGLF5, compared to BILF1 and BNLF2a. It would be an advantage to assess the kinetics of BNLF2a, BILF1 and BGLF5 protein expression; however, due to the lack of suitable BILF1 antibody this is not possible.

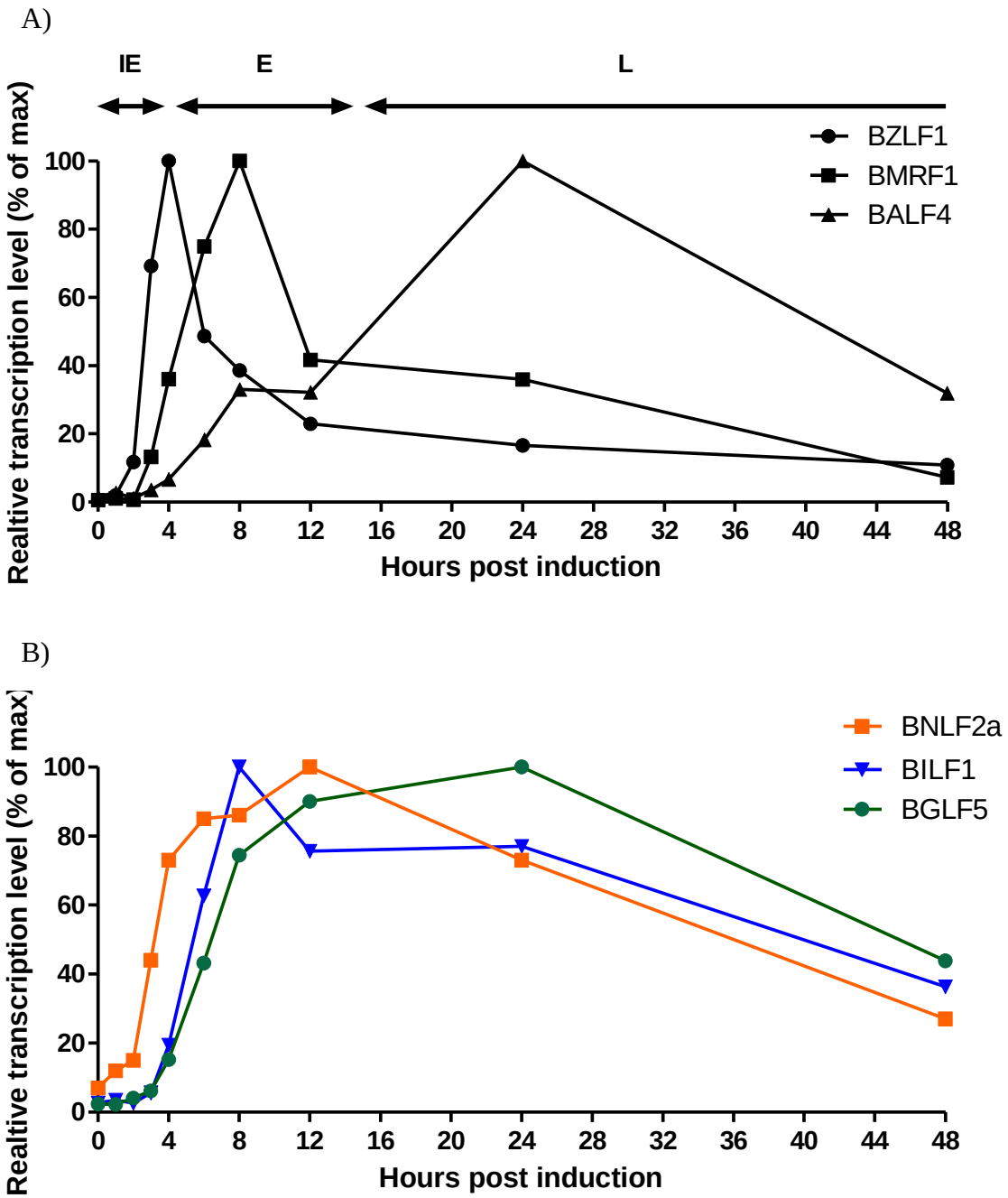


Figure 6. 12 Expression kinetics of EBV lytic cycle.

EBV infected cells (AKATA) were induced into lytic cycle. RNA was harvested at the indicated time points and cDNA was then synthesised followed by qRT-PCR analysis to detect the expression of (A) IE-BZLF1, E-BMRF1 and L-BALF4 genes and (B) BNLF2a, BILF1 and BGLF5 genes. Samples were tested in duplicate and normalised to cellular GAPDH. Data is expressed as the relative number of transcripts as percentage of the maximum for each gene.

6.7. Assessment of co-operation or redundancy of BNLF2a and BILF1 using dual knockdown of BILF1 and BNLF2a-LCLs

Finally, to ascertain if there was any co-operation or redundancy between BNLF2a and BILF1 at the IE- and L- phases of lytic cycle, double knockdown-LCLs were used as targets for IE- and L-antigen specific T cells. To do this, donor LCLs in which both BNLF2a and BILF1 had been silenced using shRNA-lentiviruses, were generated. The recognition of these double knockdown LCLs by IE- or L-epitope specific HLA-A2 restricted T cells was then assessed, alongside the recognition of their shBNLF2a-, shBILF1- and shNon-target-LCL counterparts.

In order to treat all cells in the same way, LCLs were transduced with two lentiviruses; shBNLF2a and shNon-target; shBILF1 and shNon-target; shNon-target and shNon-target or; shBNLF2a and shBILF1. It was determined that the best way to generate these double knockdown LCLs was to transduce with one virus, sort the cells on the basis of their expressed fluorescence tags, and then transduce with the second appropriate virus. This strategy was technically challenging and was only successful using two sets of donor LCLs.

Using donor 7 and 8 LCLs, we first investigated the recognition of the HLA-A2 restricted IE-YVL (BRLF1) epitope. As shown in Figure 6.13, using two different IE-YVL epitope specific T cells, the recognition of shBILF1- and shBNLF2a-LCLs fits the pattern discussed previously, where there was slight increase in recognition of shBILF1-LCLs, by both clones, and a more substantial increase in the recognition of YVL presented by shBNLF2a-LCLs (Fig 6.13a,b). Interestingly, there was a more dramatic increase in recognition of YVL presented by LCLs lacking in both BILF1 and BNLF2a. This suggests that BNLF2a and BILF1 synergise in their interfering with antigen presentation during the IE phase of lytic cycle, since

the increased recognition of the double knockdown is more dramatic than the sum of the two single knockdowns.

These same donor LCLs were then used in a separate experiment in order to assess the level of co-operation or redundancy between BILF1 and BNLF2a during L phase lytic cycle. In the representative examples shown in Figure 6.13c and d, the pattern of recognition of single knockdown shBILF1- and shBNLF2a-LCLs was again as expected, where L-FLD is better recognised when presented by shBILF1-LCLs, compared to shBNLF2a-LCLs. As noted previously, as with the recognition by IE- specific effectors, the simultaneous knockdown of both BILF1 and BNLF2a resulted in a more dramatic increase in recognition of the L-FLD (Fig 6.13c and d) epitope. This suggests that at L-phase lytic cycle BILF1 and BNLF2a may co-operate or indeed synergise, albeit to a lesser extent than at the IE phase.

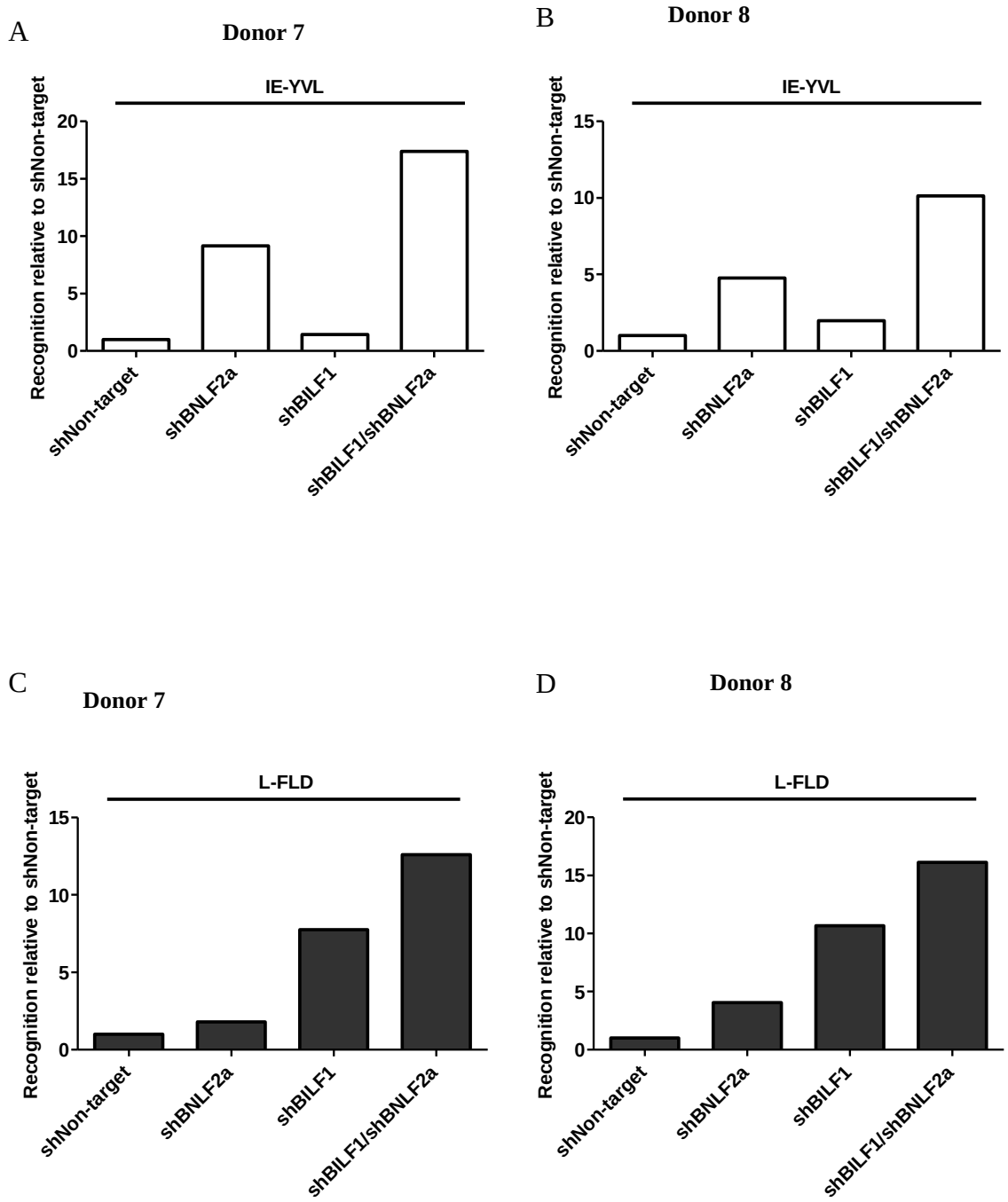


Figure 6.13 Relative recognition of LCLs lacking in both BNLF2a and BILF1 expression by an IE-YVL (BRLF1) and a L-FLD (BALF4) specific, HLA-A2 restricted CD8+ T cell clones.

A,B) Recognition of IE-YVL presented by donor 8 and 7 LCLs was measured simultaneously. T cell recognition (IFN-g release) was then normalised on the expression of BRLF1 mRNA transcript. Data is shown as recognition of knockdown LCLs relative to shNon-target LCLs.(C,D) Recognition of L-FLD presented by each donor 8 and 7 LCLs was measured simultaneously. T cell recognition (IFN-g release) was then normalised on the expression of BRLF1 mRNA transcript. Data is shown as recognition of knockdown LCLs relative to shNon-target LCLs.

6.8. **Discussion**

Experimental approach

This chapter utilised shBNLF2a-, shBILF1 and shBGLF5-LCLs, generated in chapter 5, to assess the roles that the immune evasion proteins BNLF2a, BILF1 and BGLF5 play in interfering with antigen presentation to $CD8^+$ T cells during EBV lytic cycle. These LCLs, which were spontaneously reactivating in to lytic cycle, were used as target populations, alongside their shNon-target-LCLs counterparts, in $CD8^+$ T cell recognition experiments. In the first instance this was carried out in separate experiments using single knockdown LCLs, to compare the effect that knockdown had on epitope recognition. Following this, single knockdown LCLs were used in parallel, in the same experiment, to allow for a direct comparison of the effects of these immune evasion mechanisms on lytic epitope presentation. Finally, to investigate the possible co-operation or redundancy between BILF1 and BNLF2a, dual knockdown LCLs were generated. In order to minimise any experimental variation, the ability of IE-, E- and L-epitope specific T cells to recognise target LCLs was done in parallel, where possible.

Although the above experimental approach was valid, since results were consistent and any differences in lytic antigen expression by LCLs did not affect the observed pattern, it did pose technical challenges that limited the number of complete experiments that could be performed. Most often this was due to the fact that transduced LCLs could not be cultured for long periods of time, meaning that T cell assays had to be performed shortly after transduction, which meant that cell numbers became the limiting factor.

Immediate early and early epitopes are better presented by LCLs which lack BNLF2a expression

The data accumulated within the first section of this chapter (section 6.2) show that there is a clear increase in $CD8^+$ T cell recognition of IE and E lytic epitopes when presented by LCLs lacking BNLF2a (shBNLF2a-LCLs), compared to their shNon-target-LCL counterparts. This implies that BNLF2a functions to protect these epitopes from presentation to $CD8^+$ T cells. This increase in recognition remained consistent across a range of epitopes and HLA class I alleles, although the magnitude of increase for each epitope differed between donors.

The increased recognition of the IE epitopes YVL (BRLF1) and DPY (BZLF1) was consistently more dramatic than the increase seen for E- and L-epitopes, in the same experiment (with the exception of one donor). However, IE-YVL recognition was increased 6.5-30.7 fold, in the absence of BNLF2a (Table 6.3), while the increased recognition in DPY ranged from 7-14 fold. This difference in magnitude may be due to the fact that BZLF1, from which the DPY epitope originates, is expressed earlier than BRLF1 and thus even earlier than BNLF2a, therefore the ability of BNLF2a to interfere with DPY epitope presentation may be diminished. However, this cannot be drawn as a firm conclusion due to variations in the magnitude of increasing recognition between donors (discussed below).

Recognition of E epitopes in the absence of BNLF2a was also substantially increased across a range of donors, using both HLA-A2 and HLA-B7 restricted T cells. The epitopes investigated here included GLC (BMLF1) and TLD (BMRF1), both restricted through HLA-A2 and RPG (BNLF2b), restricted through HLA-B7. In these experiments, the recognition of GLC and TLD was seen to be highly increased (7-24 fold and 7.5-12 fold respectively) in the absence of BNLF2a. Although these magnitudes vary, the increase in E-epitope recognition,

within the same donor, was consistently less than the increase seen in IE-epitope recognition, with the exception of one example (Fig 6.3B). However, the range of increase in recognition of RPG (BNLF2) was smaller (4.1-7 fold). This is likely due to a problem with the experimental approach taken, BNLF2b expression is driven from the same transcript as BNLF2a, and although the shRNA used in this work was specific for BNLF2a transcript, it became apparent that the expression of BNLF2b was also decreased in shBNLF2a-LCLs, albeit to a lesser extent than BNLF2a. Therefore, it is likely that we are underestimating the effect of BNLF2a on interfering with the presentation of BNLF2b-RPG epitope. Ideally a different method of gene silencing or deletion would be used, however, due to time constraints, this was not feasible.

Recognition of late epitopes, which included the HLA-A2 presented FLD (BALF4) and WQW (BNRF1) epitopes and the HLA-B7 presented YRP (BNRF1) epitope, were consistently marginally increased in the absence of BNLF2a (2-5 fold increases).

The differences in magnitude of recognition between donors is likely due to differences in epitope display levels by different donor LCLs and differences between T cell clone avidity on different days. Although it should be noted, that the avidity of the different T cell clones used tended to be similar and since in each case, increased recognition was compared to control transduced cell, any differences in T cell affinity was somewhat controlled for. The apparent differences in donor LCL epitope presentation and T cell avidity is the reason we attempted to carry out T cell recognition of epitopes expressed at the different phases simultaneously, although this was not always possible.

Finally, the peptide sequence may play a role in the sensitivity of epitopes to BNLF2a action. A panel of EBV specific epitopes have been identified as TAP-dependant or TAP-

independent, according to their level of hydrophobicity (Lautscham *et al.,* 2001; Lautscham *et al.,* 2003; Lautscham *et al.,* 2003). This was assessed using a TAP negative cell line (T2-cells) and thus it cannot be ruled out that the TAP-independent epitopes may preferentially use TAP under normal conditions and are only TAP-independent in the absence of TAP. YVL and GLC are hydrophobic and have been identified as TAP-independent and therefore would be expected to be less affected by the action of BNLF2a (Table 6.6). However, given those results outlined in the present study, these epitopes were dramatically affected by the knockdown of BNLF2a. Also, TLD has been identified as a TAP-dependant epitope, thus we would expect this to be highly affected by the knockdown of BNLF2a, however the range in fold increase in recognition of TLD was similar to that observed for GLC. Therefore it is not possible to predict the effect BNLF2a will have on epitopes according to their TAP dependence.

Table 6.6. TAP dependency and hydrophobicity of peptide-epitopes used.

Antigen	Peptide	HLA restriction	Hydrophobicity (%)*	TAP dependency	Reference
BRLF1	YVLDHLIVV	A2	78	Independent	Lautscham *et al.* 2001
BMLF1	GLCTLVAML	A2	78	Independent	Lautscham *et al.* 2001
BMRF1	TLDYKPLSV	A2	44	Dependent	Lautscham *et al.* 2001
BALF4	FLDKGTYTL	A2	44	Unknown	N/A
BNRF1	WQWEHIPPA	A2	44	Unknown	N/A
BZLF1	DPYQVPFVQAF	B7	55	Unknown	N/A
BNLF2b	RPGRPLAGFYA	B7	45	Unknown	N/A
BNRF1	YPRNPTWQGNI	B7	27	Unknown	N/A

*Hydrophobicity was calculated using software available at http://lifetein.com/peptide-analysis-tool.html.

Although the magnitude of increased recognition varied between donors, the pattern of BNLF2a knockdown effect on recognition remained consistent. In all cases, with the exception of one, the recognition of IE-epitopes was most dramatically increased; E-epitope recognition was substantially increased, albeit to a lesser extent than IE and; increases in L-epitope recognition was minimal in all cases.

This finding is perhaps surprising since BNLF2a is designated as an early expressed lytic gene. To address this, the kinetics of lytic gene expression was assessed using EBV infected cells which could be induced into synchronous lytic cycle. This revealed that although BNLF2a is classified as an E lytic cycle gene is initially expressed during the IE phase of lytic cycle, which is perhaps the reason it has a strong effect on antigen presentation at the IE phase of lytic cycle. In addition, although the transcript levels of BNLF2a remain relatively high during late phase lytic cycle, the protein expression of BNLF2a after 24-hours post induction is dramatically decreased, which explains why it has only a marginal effect on L epitope presentation.

Early and late epitopes are better presented by LCLs lacking in BILF1 expression

In the next section we set out to assess the role BILF1 plays in interfering with antigen presentation during lytic cycle. Using the same experimental approach as above, the pattern in increased recognition in the absence of BILF1 was different to that of BNLF2a. In all examples shown, there was a minimal increase in recognition of IE-epitopes presented by LCLs lacking BILF1 compared to their shNon-target-LCL counterparts (0-2.5 fold increase), a more notable increase in recognition of E-epitopes (3.2-11 fold) and a more dramatic

increase in recognition of L-epitopes (5.7-25). This pattern remained consistent using both HLA-A2 and HLA-B7 panels of T cells and across a selection of donor LCLs.

Again, there were differences in the magnitudes of increases between different donor LCLs, for those reasons described earlier. Within the HLA-B7 assays which were performed, the increase in recognition of the E-RPG (BNRF1) epitope, compared to that of the L-YPR (BNRF1) was a more dramatic difference, perhaps due to the fact that BNLF2b is expressed very early in E lytic cycle and thus before BILF1.

More recently it was reported that BILF1 does not target HLA-C molecules for degradation. It is now known that the specific residues which enable BILF1 to bind to HLA molecules are not expressed in the tail of HLA-C molecules. Unfortunately, during this study the assessment of HLA-C restricted T cell recognition was not included.

The above findings suggest that BILF1 plays a more dominant role in interfering with antigen presentation during L phase lytic cycle and contributes a strong effect during the E phase, having little or no effect at the immediate early stage. Since BILF1 is expressed later than BNLF2a and presumably remains relatively high during L lytic cycle, is perhaps the fact that BILF1 has a stronger effect later in lytic cycle. It would be an advantage to measure the level of BILF1 protein at L stage lytic cycle in order to confirm this observation.

BGLF5 appears to have little or no effect on interfering with antigen presentation during lytic cycle

In the present study the effect of BGLF5 on interfering with antigen presentation during IE-, E- and L-phase lytic cycle appeared to be minimal. Using numerous donors and both HLA-A2 and HLA-B7 restricted T cells the increase in recognition ranged from no increase to 2.5 for

IE-epitopes, no increase-4 for E-epitopes and 1.5-4.3 for L-epitopes. This would suggest that BGLF5 plays a minimal role towards interfering with antigen presentation throughout lytic cycle.

However, it should be noted that the number of donors in which complete panels of T cells which recognised IE-, E- and L- epitopes could be performed at the same time was minimal. This was due to the shBGLF5-LCLs showing little or no cell expansion following transduction, or cells were no longer lytic and thus could not be used. Despite this, in all cases there was minimal increased recognition, across a range of donors and T cells and no pattern of increased recognition was observed. It should be noted that it cannot be ruled out that any residual BGLF5 expression may account for the lack of effect seen following BGLF5 knockdown, although, this is unlikely considering the consistent results seen in Table 6.5. This lack of BGLF5 effect is surprising in one respect since BGLF5 is expressed at the same time as BILF1 and to a high level; however, when considering the function of BGLF5 this finding is perhaps unsurprising. BGLF5 has a global host protein shut off function and has been observed to down regulate other host proteins, including TLR-9. This implies that BGLF5 has a less targeted approach to interfering with antigen presentation, and thus it is perhaps unsurprising that its effect is less dramatic and consistent.

It is also important to consider the possibility that the knockdown of BGLF5 may also result in the knockdown of BGLF4 expression, since these two genes are expressed from the same transcript. BGLF4 is a protein kinase which has been shown to antagonise the host shutoff function of BGLF5 (Feederle *et al.*, 2009). These two enzymes have been shown to act together to modulate viral gene expression and virus protein production. If only BGLF5 was knocked down in our shBGLF5-LCLs, then we may expect to see an increase in the expression of some viral genes including BFLF2 and BFRF1, the expression of which is

stimulated or upregulated by BGLF4. However, studies performed using ΔBGLF5-LCLs (discussed previously) show that a lack of BGLF5 expression does not prevent LCLs progressing through lytic cycle, although there is a lower level of viral production. Alternatively, if both BGLF4 and BGLF5 were knocked down in our experimental system, there would be a more exaggerated decrease in the level of viral production and perhaps a different pattern of gene expression during lytic cycle. In addition, BGLF4 has recently been shown to downregulate MHC class I surface expression (personal communication, Dr J. Zuo), therefore a more exaggerated level of T cell evasion may be expected. Although this is an interesting consideration, this project utilises both BGLF5-knockdown and -knockout LCLs as targets for T cells and the data obtained using these different targets LCLs are similar, the issue of BGLF4 knockdown is perhaps not overly important.

Simultaneous assessment of the role of BILF1, BNLF2a and BGLF5 at all phases of lytic cycle

To allow for the direct comparison of the relative contribution that BILF1, BNLF2a and BGLF5 make towards interfering with antigen presentation during lytic cycle, simultaneous assessment of the recognition of LCLs lacking in the expression of these individual genes, by a panel of HLA-A2 restricted T cells was performed. This confirmed the above conclusions, where LCLs lacking in BNLF2a expression showed a dramatic increase in recognition of IE-YVL epitope, compared to LCLs lacking BILF1 and BGLF5. LCLs lacking in BILF1 and those lacking BNLF2a resulted in an increase in recognition of E-GLC epitope, with shBNLF2a-LCLs showing a higher increase and finally LCLs lacking in BILF1 expression resulted in a dramatic increase in recognition of L-FLD epitope compared to cells lacking in

BNLF2a and BGLf5 expression. Although this was only assessed using one set of donor LCLs, due to the experimental problems which were encountered when attempting to generate a panel of knockdown LCLs which remained lytic and expanded to a an appropriate number, the above mentioned results confirmed what was expected.

Taken together, the above results suggest that at the IE-phase of lytic cycle BNLF2a plays the more dominant role in interfering with antigen presentation, at the E-phase of lytic cycle, BNLF2a and BILF1 both contribute, with BNLF2a perhaps playing more of a role, and at the late stage it is the function of BILF1 which is more dominant. This can be explained by considering the kinetics of expression of these genes. BNLF2a is expressed very early in lytic cycle, peaks at 12 hours and is decreasing during late phase lytic cycle. Conversely, the expression of BILF1 is minimal in the immediate early phase of lytic cycle, peaks during the early phase and remains relatively high at the late phase.

Dual knockdown of BNLF2a and BILF1indicate a co-operative function during the IE- and L- phases of lytic cycle

The data generated in the final section of this chapter suggests that there is a level of synergy between BILF1 and BNLF2a almost certainly at the IE- phase of lytic cycle. This is evident when considering the level of increase when both BILF1 and BNLF2a are not expressed; the level of increase seen is more dramatic than that of shBNLF2a-LCLs. At the L stage of lytic cycle this level of synergy is perhaps less dramatic and could be considered as cooperation of the two, rather than synergy. Regardless, there is most certainly a more dramatic increase in recognition of the L-FLD epitope when both BILF1 and BNLF2a are not expressed. Although this was only completed using two donors and two epitopes, it suggests that there is a level of

synergy between these genes during the IE- and L-stages of lytic cycle, and most certainly no implied redundancy.

To confirm this finding, it would be necessary to assess the increase in recognition of IE-, E- and L-epitopes across a selection of donors and HLA-restrictions and extend this further to confirm the minimal role of BGLF5 by generating a triple knockdown target LCL. However, due to time constraints, this was not possible. Since it is the transduction of two lentiviruses which appeared to be the problem, it could be an advantage to generate one lentivirus which expresses shRNAs to target both BNLF2a and BILF1 or all three genes. However, this has not been attempted using LCLs and there are reported problems with this approach due to the use of two identical promoters to drive the expression of the separate shRNAs.

CHAPTER 7

FINAL DISCUSSION

Like other persistent viruses, EBV has evolved to encode a plethora of immune evasion mechanisms. The work outlined in this thesis focuses on three of these; BNLF2a, BILF1 and BGLF5, that when ectopically expressed in model systems are able to interfere with different stages of the MHC class I antigen presentation pathway. The overall aim of this research project was to investigate the functional effect of these individual immune evasion proteins on peptide presentation to $CD8^+$ T cells during virus lytic cycle and, ultimately to determine the relative contribution each makes towards interfering with antigen presentation at the three different phases of lytic cycle (IE, E and L).

In 2007, BNLF2a was identified as able to interfere with the function of the TAP transporter and reduce peptide presentation to $CD8^+$ T cells (Hislop *et al.*, 2007; Croft *et al.*, 2009). Shortly after this BILF1 and BGLF5 were identified as able to decrease the levels of cell surface expression of MHC class I molecules, upon ectopic expression. BILF1 was subsequently shown to target newly assembled MHC class I molecules for lysosomal degradation and also target MHC class I molecules which make it to the cell surface, for degradation (Zuo *et al.*, 2009; Zuo *et al.*, 2011). The mechanism by which BGLF5 was able to exert this effect was via its host-shutoff function which increases the turnover of cellular mRNA and thus non-specifically reduces the level of newly synthesised MHC class I molecules available for peptide loading and presentation of $CD8^+$ T cells (Rowe *et al.*, 2007).

Whilst at the start of this thesis some details were known of the molecular mechanisms of these potential modulators of antigen presentation, there was a largely unresolved question of

their relative contributions to immune evasion during lytic cycle. In 2009 Croft *et al* carried out a study to examine the effects of BNLF2a on antigen presentation during IE- E- and L-phases of lytic cycle. This study utilised recombinant EBV viruses and successfully showed that in the absence of BNLF2a expression, recognition of IE- epitopes was increased and, more strikingly, recognition of E- epitopes was dramatically increased. This same study indicated that BNLF2a did not play a role in interfering with antigen pretention during L-stage lytic cycle, since cells transformed with EBV lacking BNLF2a were no better recognised by $CD8^+$ T cells specific for L-epitopes than their wild-type counterparts (Croft *et al.,* 2009). This then posed the question as to what exactly is protecting EBV infected cells from recognition by L- epitope specific $CD8^+$ T cells, since it is known that these T cells, despite being avid and functional, show much reduced recognition of target cells. This phenomenon is not explained by level of L antigen expression but is thought to be a direct result of a decrease in efficiency of antigen-epitope presentation in wild-type LCLs as lytic cycle progresses (Pudney *et al.,* 2005). Perhaps this diminished efficiency in epitope presentation is due to the action of BILF1 and BGLF5? T cell studies investigating the functions of these immune evasion proteins side by side, in the context of whole virus life cycle are lacking. Therefore the work outlined in this research project was carried out to study the function of BNLF2a, BILF1 and BGLF5 using lytic LCLs; the most informative results were obtained in which the expression of BNLF2a, BILF1 and BGLF5 had been silenced using shRNAs. These target LCLs served a means of testing the relative contribution of BNLF2a, BILF1 and BGLF5 to T cell recognition of IE-, E- and L- derived lytic cycle epitopes.

Data presented in this thesis show that the relative contribution of BNLF2a, BILF1 and BGLF5 towards interfering with antigen presentation differs between the three different

phases of lytic cycle. In LCLs lacking the expression of BNLF2a there was a dramatic increase in presentation of IE-epitopes and a less dramatic increase in presentation of E-epitopes presented to CD8+ T cells. In contrast to the study by Croft *et al.*, this study also found a marked increase in L- epitope presentation, albeit less dramatic than the increase in IE- and E-epitope presentation (Croft *et al.,* 2009). This minor discrepancy is most likely due to the wider range of potent L epitope-specific $CD8^+$ T cell clones and target cell lines used in this thesis compared to the rather limited T cell/target combination used in the original study. However, the general take-home message is essentially unaltered in that both sets of data suggest that the role BNLF2a plays in interfering with antigen presentation diminishes as lytic cycle progresses (IE>E>>L). Indeed, by studying the kinetics of BNLF2a expression, in relation to the expression of other lytic genes, is was revealed that BNLF2a transcript was detectable and increased dramatically during the IE stage of lytic cycle, peaking at the E stages, which offers an explanation as to why BNLF2a appears to confer such a dramatic effect on the presentation of epitopes at these phases of lytic cycle. Although BNLF2a transcripts show only a modest decrease at the L stage of lytic cycle, we know that this does not correlate with the levels of protein expression, which is actually dramatically decreased by L phase lytic cycle, possibly accounting for why there appears to be less of a dramatic effect of BNLF2a on epitope presentation at this stage of lytic cycle.

A major novel finding of the work outlined in this thesis is the effect of BILF1 on antigen presentation during lytic cycle. This protein appears to exert its strongest effects when BNLF2a is beginning to show weaker effects. Indeed, in the absence of BILF1 there is a dramatic enhancement of presentation of L epitopes to $CD8^+$ T cells with a less dramatic effect seen at the E and an even weaker effect seen on IE (IE<<E<L). Thus, it appears that BILF1 plays the most important role at interfering with antigen presentation during the L

stage of lytic cycle. Again, when studying the kinetics of BILF1 expression it is clear that BILF1 is expressed later than BNLF2a and, given the phenotypic effects shown in these data; it would be reasonable to assume that the level of transcript of BILF1 is also correlated to relatively high levels of protein expression. Unfortunately, without an anti-BILF1 antibody, this is hard to confirm. It could be that BILF1 protein is expressed at higher levels at the L stages, perhaps accumulating in order to have the dramatic effect it does at the this stage. The small effect BILF1 has on IE epitope presentation compared to L stages is perhaps unsurprising considering that IE gene expression peaks before the expression of BILF1 is seen and these genes are expressed to a much lower level when BILF1 is highly expressed, thus the window in which BILF1 is available to interfere with the presentation of these epitopes is minimal. It should be noted that although BZLF1 protein remains expressed throughout lytic cycle, transcription declines, and since newly translated products (DRiPs) provide the peptides for antigen presentation, stable BZLF1 protein probably serves as a minimal source of peptides for presentation.

Quite surprisingly, this thesis revealed that the effect of BGLF5 on antigen presentation is weak throughout lytic cycle, despite its expression and host shut-off function throughout the E and especially L stages. One interesting possibility is that the non-specific down regulation of MHC class I by BGLF5 actually confers little protection from $CD8^+$ T cell recognition. It is known that human cells express a huge excess of MHC class I at the cell surface, thus it may be that the small effect that BGLF5 has on the decrease of these molecules actually makes little difference to T cell recognition of EBV epitopes. It may be that the seemingly minimal role played by BGLF5 is actually more of a fine tuning role.

Most importantly, this thesis has shown a direct comparison of these immune evasion effects. Studying the simultaneous recognition of cells lacking in BNLF2a, BILF1 or BGLF5 expression revealed the stage-specific, relative contribution of these proteins during lytic cycle. At the IE stage, BNLF2a is most important; at the E it is the role of BNLF2a and BILF1 and at the late stage BILF1 has the dominant effect, with a small contribution by BNLF2a. In addition to this, these data revealed, for the first time, the level of synergy or co-operation between the two most potent immune evasion proteins, BNLF2a and BILF1 using double knockdown LCLs. Although experiments were limited due to technical difficulties, this work indicated that at the IE stages of lytic cycle, BNLF2a and BILF1 are highly synergistic and at the L stage their functions most certainly co-operate, if not synergise. This suggests that the concerted action of these two immune evasion proteins contributes to protecting EBV infected cells from recognition during the IE and L stages of lytic cycle.

These findings raise some interesting points to consider. Firstly, these data pose the question as to why EBV would downregulate the expression of BNLF2a at the L stages of lytic cycle, when it is clearly such a potent immune evasion mechanism. This could perhaps be due to the action of BILF1 and other immune evasion mechanisms being sufficiently capable of protecting EBV infected cells from recognition at the L phase. Alternatively, the expression of too many immune evasion mechanisms contributing to the down regulation MHC class I levels could leave cells too vulnerable to NK cell destruction. The controlled expression of BNLF2a and BILF1 is perhaps an eloquent trait of EBV, in order to maximise protection from $CD8^+$ T cell recognition, while avoiding NK cell induced destruction. Moreover, perhaps the action of BNLF2a on TAP is not efficient at the L stage of lytic cycle. At this stage, which can last for up to 4 days, the level of viral transcripts are relatively low, due at least in part to the RNase activity of BGLF5. Consequently, one might anticipate less accumulation of viral

DRiPs and therefore, it may be that it is more efficient to target MHC class I molecules themselves, rather than the TAP transporter. In support of this scenario, the blocking of BGLF5 expression might be expected to boost antigen presentation. However, this was not supported by the experimental data presented in this thesis.

Another interesting point to consider is the fact that at the L stage of lytic cycle wild-type LCLs show less efficient antigen processing than at the IE stages, which results in decreased recognition of L-epitopes by $CD8^+$ T cells, compared to IE-epitope recognition, as shown by Pudney *et al* and further confirmed during the course of this Research project (Pudney *et al.*, 2005). Thus, one might assume that the knockdown of BILF1 expression would result in a much more exaggerated increase in recognition by L-epitope specific $CD8^+$ T cells than is seen for the increase in recognition of IE-epitopes presented by LCLs lacking in BNLF2a expression. However, this was not the case. In fact the increase in recognition of shBILF1-LCLs by L-epitope specific T cells was often comparable to the increase in recognition of shBNLF2a-LCLs by IE-epitope specific LCLs. This may suggest that there potentially could be other immune evasion mechanisms in action at the L stage of lytic cycle. A study carried out by other members of the lab identified at least two other E and L expressed viral genes which are capable of down-regulating MHC class I levels, when ectopically expressed (Dr Jianmin Zuo, personal communication). These new potential immune-evasion genes warrant further study to assess their mechanisms of action and whether either or both might preferentially modulate recognition by $CD8^+$ T cells specific for L-epitopes.

From these data a model is proposed whereby, upon EBV entry into lytic cycle, BNLF2a, the TAP inhibitor, is highly active at interfering with peptide transport into the ER and is able to protect these lytic cells from recognition by T cells, with the help of BILF1. Since BNLF2a

does not confer a complete block of TAP, perhaps BILF1, which is expressed slightly later than BNLF2a, plays the role of mopping up some of peptide loaded MHC class I molecules which are generated. During the E stage of lytic cycle the concerted action of BILF1 and BNLF2a are responsible for protecting cells from recognition and finally, at the L stages BILF1 is highly potent, acting in synergy with the less active BNLF2a immune evasion mechanism to confer blocking of peptide presentation, perhaps with the help of other, as of yet, unidentified immune evasion mechanisms. During this process, the diminished MHC class I synthesis imposed by BGLF5 appears to play little role in interfering with peptide presentation to T cells, despite its expression during the E and L phase. This supports the importance of investigating the effects of immune evasion mechanisms in the context of virus life cycle, rather than ectopic expression of these genes. The relative role of these immune evasion proteins during lytic cycle is shown in a schematic in Figure 7.1.

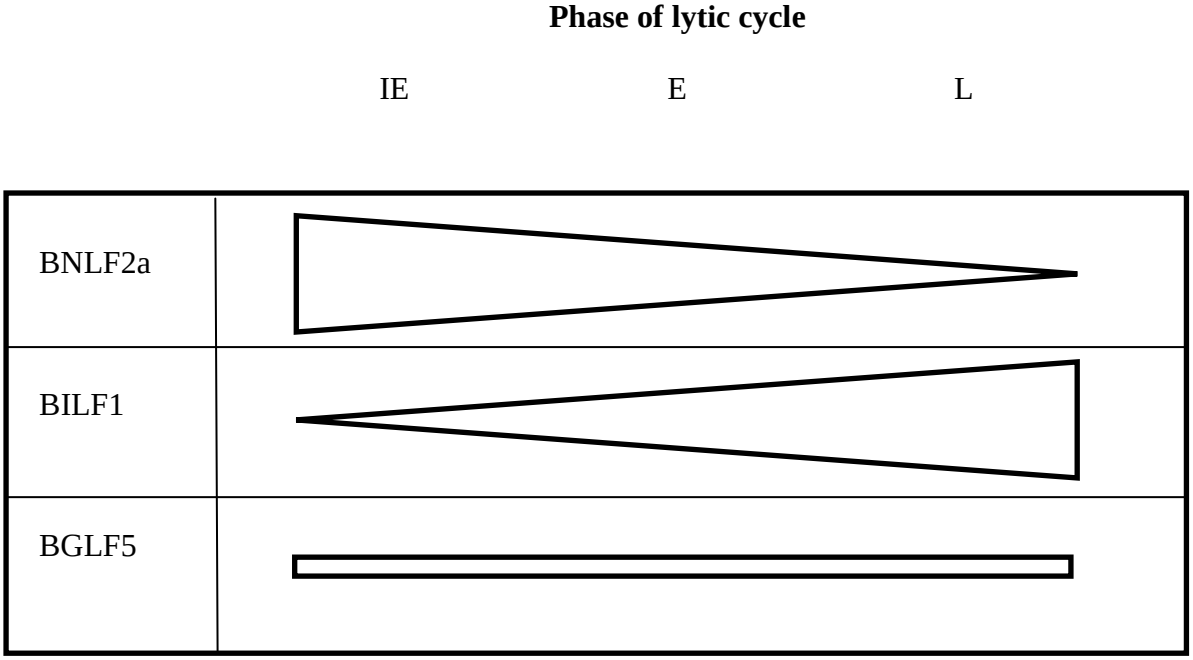


Figure 7.1. The relative roles of BNLF2a, BILF1 and BGLF5 in interfering with antigen presentation as lytic cycle progresses.

Diagram showing the strength of each immune evasion gene function at all stages of lytic cycle. BNLF2a is more potent at the IE time point and its effect diminishes as lytic cycle progresses. The potency of BILF1 increases as lytic cycle progresses. BGLF5 plays a minimal role throughout.

This thesis serves to highlight the importance of using functional T cell assays in order to investigate the effects of immune evasion mechanisms on the life cycle of EBV. If we consider only the level of MHC class I at the surface of infected cells, results may be misinterpreted. This is particularly evident in previously published experiments where the effects of ectopic expression of the viral genes was examined (Hislop *et al.,* 2007; Rowe *et al.,* 2007; Zuo *et al.,* 2009), in which relatively small changes in surface MHC class I expression are often associated with substantial inhibition of T cell recognition. Furthermore, LCLs derived by infection with recombinant EBV lacking BNLF2a expression show the same level of MHC class I surface expression during late phase lytic cycle, despite this protein still having a marginal effect on the presentation of epitopes at this stage. This is especially apparent when considering the data seen from knocking down BGLF5. Whereas ectopic expression of this gene clearly confers a reduction in MHC class I molecules, this does not translate in to diminished recognition by T cells. Thus measurement of total surface MHC class I expression does not necessarily reveal important qualitative differences in the repertoire of peptides and thus T cell recognition.

Another intriguing issue to consider is that BNLF2a, the TAP inhibitor, also appears to have dramatic effect on the presentation of epitopes defined as TAP-independent (Table 6.6). Thus, BNLF2a impairs presentation of the paradigmatic TAP-independent GLC epitope from the BMLF1 EBV protein as efficiently as it does presentation of the TAP-dependent TLD epitope from the BMRF1 protein. The critical experiment, which to our knowledge has not yet been reported, would be to determine whether BNLF2a is also active in the absence of TAP. In the first instance this could be done by ectopically expressing BNLF2a in the TAP negative cell line T2, alongside both TAP independent and TAP dependant antigen epitopes, to see if there is any difference in the effect of BNLF2a on the presentation of these peptides when TAP is

not expressed. Initial experiments performed suggest that the expression of BNLF2a from a vaccinia virus, in T2 cells, results in decreased recognition of both TAP independent (GLC) and TAP dependent (TLD) epitopes, despite the lack of TAP expression (personal communication, Dr V. Pudney). Thus it could be postulated that BNLF2a is able to target a yet unidentified TAP independent antigen processing pathway. However, this is complicated by the fact that T2-cells are latently infected with EBV (DeMars *et al.*, 1984), therefore if the results presented in chapter 5 hold true for T2-cells, BNLF2a may be expressed in these cells which would influence the above experimental design. It could be that any endogenous BNLF2a expression is insufficient to inhibit TAP however, if there is some function from this expression it could reduce the sensitivity of the assay described or mask any effect of ectopically expressed BNLF2a. It would therefore be necessary to knockdown the expression of BNLF2a in T2-cells, if it is seen to be expressed, in order to determine if it is functional. In addition, since T2-cells are tightly latent it would be necessary to establish new lines to examine the role of BNLF2a, in the absence of TAP, in lytic cycle. To do this, LCLs could be generated from individuals who suffer from a rare disease where they do not express TAP, known as bare lymphocyte syndrome ((Teisserenc *et al.*, 1997)), reviewed in (Gadola *et al.*, 2000)). If patients were obtained, LCLs would be generated using ΔBNLF2a and wild-type EBV. Or use shRNA to knockdown BNLF2a in wild-type LCLs from these patients or alternatively, knockdown the expression of TAP1/2 in wild-type LCLs. BNLF2a could also be ectopically expressed in wild-type LCLs derived from these patients, to determine if there is an effect of BNLF2a in the absence of TAP. Subsequently, BNLF2a pull down assays, followed by mass spectrometry identification of associated proteins could be carried out in order to identify exactly what BNLF2a is interacting with, in the absence of TAP.

During the course of this work it came to light that some genes that are currently considered lytic genes, namely BNLF2a and BNRF1, are actually expressed in lytic-incompetent cells (ΔBZLF1-LCLs). It is not entirely clear how BNLF2a is expressed during latency. One possibility is that BNLF2a transcripts are merely a read-through of the abundant LMP1 transcripts (Fig 7.2), in which case it does not necessarily follow that BNLF2a will be efficiently translated. This issue could be clarified by performing rapid amplification of the cDNA ends (RACE) to determine the full length transcript of BNLF2a expressed during latency, and thus determine the promoter usage for its expression and by more rigorous quantification of BNLF2a protein expression in latency and lytic cycle. The implications for BNLF2a in this respect are interesting from an immune evasion point of view. It could be that BNLF2a expressed in these latent cells, albeit to a much lesser extent, may well be protecting latent cells from recognition by $CD8^+$ T cells. It would be interesting to firstly, determine if the low level of BNLF2a which is expressed in these cells is conferring a level of antigen presentation inhibition. In addition, regardless of whether BNLF2a is expressed sufficiently to modulate antigen presentation in latency, it might be expressed sufficiently to provide targets of T cell recognition, which is shown in chapter 5 to be expressed to a high enough level to be recognised by BNLF2a specific T cells. This potential additional role of BNLF2a is even more impressive when considering the recent findings of Jochem *et al.*who published data showing that BNLF2a mRNA is contained within the EBV virion, and is subsequently translated immediately following infection and protects newly infected cells against recognition by T cells early in infection (Jochum *et al.*, 2012; Jochum *et al.*, 2012). These findings suggest that BNLF2a perhaps plays a more important role in the life cycle of EBV than originally thought.

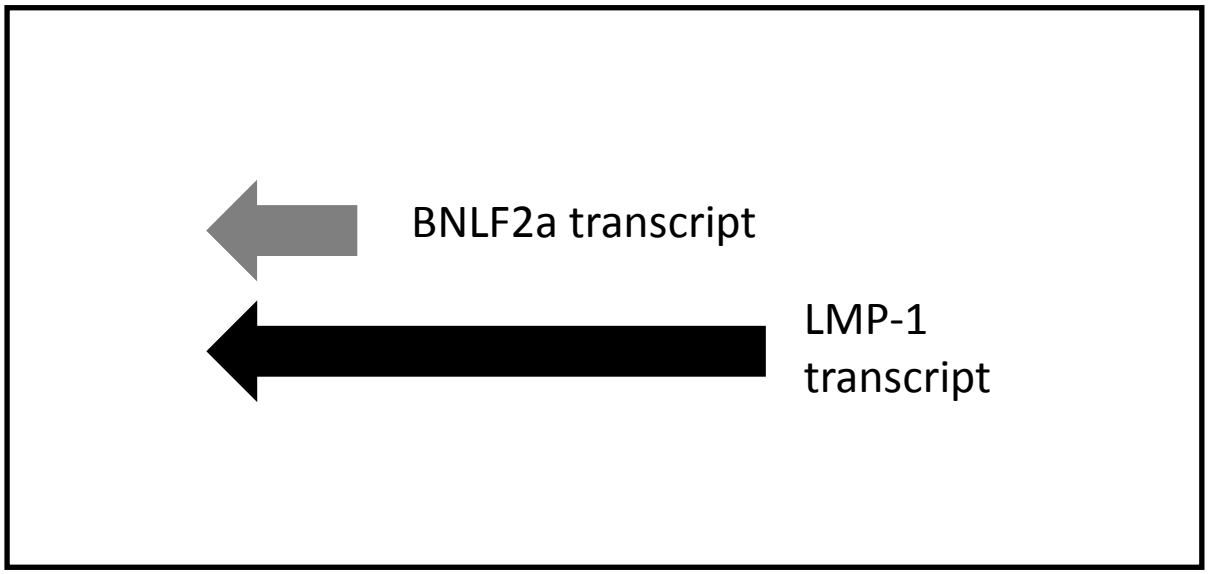


Figure 7.2 Transcripts of LMP1 and BNLF2a.

The expression of BNLF2a during lytic cycle utilises its own promoter. However, it is at located within the transcript of LMP1, thus its expression during latency could be due to read through of the LMP1 transcript.

The expression of BNRF1 in latency is interesting in the context of a recent study by Abbott *et al.* (unpublished), which included the work found in chapter 3 of this thesis. This study showed that BNRF1 is in fact a dominant target for $CD8^+$ T cells in healthy EBV carriers, thus BNRF1 expression in latency may be the reason it is such a frequent $CD8^+$ T cell target. If BNRF1 was also found to be expressed in other latent EBV infected cells, it could make for an attractive target for immunotherapy of EBV-associated disease. Moreover, these data bring to light the grey area which exists between latent and lytic cycle, suggesting that this may be more complicated than initially thought.

The work in this thesis has extended the earlier ectopic expression models of EBV immune evasion to a more physiologically relevant examination of evasion genes in the context of EBV lytic cycle; whilst these *in vitro* data help to develop an understanding of the role of immune-modulation in EBV persistence, *in vivo* confirmatory data is currently lacking. It would be most interesting to investigate the direct role of BNLF2a, BILF1 and BGLF5 *in vivo.* Our group are currently collaborating with Professor Christian Munz using the humanised mouse model developed by his group and infecting these mice with recombinant EBV, lacking in the expression of BNLF2a, BILF1 and BGLF5, this work is on-going. Taking into account the findings in this study it may be expected that ΔBNLF2a and ΔBILF1 virus infection would result in smaller tumour growth in these mice. It would be expected that there would be an increase in T cell responses, due to more efficient antigen processing and recognition by T cells and also a lower viral load, since presumably infected B-cells could be successfully recognised and eliminated by T cells. Alternatively, other animal models could be used such as rhesus macaque infection with the EBV-related lymphocryptovirus. To investigate the effects knocking down these genes has on disease progression and T cell responses.

REFERENCES

Abele, R. and R. Tampe (1999). "Function of the transport complex TAP in cellular immune recognition." Biochim Biophys Acta **6**(2): 405-419.

Ahn, K., A. Angulo, P. Ghazal, P. A. Peterson, Y. Yang and K. Fruh (1996). "Human cytomegalovirus inhibits antigen presentation by a sequential multistep process." Proc Natl Acad Sci U S A **93**(20): 10990-10995.

Ahn, K., A. Gruhler, B. Galocha, T. R. Jones, E. J. Wiertz, H. L. Ploegh, P. A. Peterson, Y. Yang and K. Fruh (1997). "The ER-luminal domain of the HCMV glycoprotein US6 inhibits peptide translocation by TAP." Immunity **6**(5): 613-621.

Ahn, K., T. H. Meyer, S. Uebel, P. Sempe, H. Djaballah, Y. Yang, P. A. Peterson, K. Fruh and R. Tampe (1996). "Molecular mechanism and species specificity of TAP inhibition by herpes simplex virus ICP47." EMBO J **15**(13): 3247-3255.

Akira, S., S. Uematsu and O. Takeuchi (2006). "Pathogen recognition and innate immunity." Cell **124**(4): 783-801.

Ameres, S., J. Mautner, F. Schlott, M. Neuenhahn, D. H. Busch, B. Plachter and A. Moosmann (2013). "Presentation of an immunodominant immediate-early CD8+ T cell epitope resists human cytomegalovirus immunoevasion." PLoS Pathog **9**(5): 23.

Andersson, M., S. Paabo, T. Nilsson and P. A. Peterson (1985). "Impaired intracellular transport of class I MHC antigens as a possible means for adenoviruses to evade immune surveillance." Cell **43**(1): 215-222.

Annels, N. E., M. F. Callan, L. Tan and A. B. Rickinson (2000). "Changing patterns of dominant TCR usage with maturation of an EBV-specific cytotoxic T cell response." J Immunol **165**(9): 4831-4841.

Apcher, S., C. Daskalogianni, F. Lejeune, B. Manoury, G. Imhoos, L. Heslop and R. Fahraeus (2011). "Major source of antigenic peptides for the MHC class I pathway is produced during the pioneer round of mRNA translation." Proc Natl Acad Sci U S A **108**(28): 11572-11577.

Apcher, S., C. Daskalogianni, B. Manoury and R. Fahraeus (2010). "Epstein Barr virus-encoded EBNA1 interference with MHC class I antigen presentation reveals a close correlation between mRNA translation initiation and antigen presentation." PLoS Pathog **6**(10): 1001151.

Apcher, S., A. Komarova, C. Daskalogianni, Y. Yin, L. Malbert-Colas and R. Fahraeus (2009). "mRNA translation regulation by the Gly-Ala repeat of Epstein-Barr virus nuclear antigen 1." J Virol **83**(3): 1289-1298.

Bell, M. J., R. J. Abbott, N. P. Croft, A. D. Hislop and S. R. Burrows (2009). "An HLA-A2-restricted T-cell epitope mapped to the BNLF2a immune evasion protein of Epstein-Barr virus that inhibits TAP." J Virol **83**(6): 2783-2788.

Beninga, J., K. L. Rock and A. L. Goldberg (1998). "Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase." J Biol Chem **273**(30): 18734-18742.

Bennett, E. M., J. R. Bennink, J. W. Yewdell and F. M. Brodsky (1999). "Cutting edge: adenovirus E19 has two mechanisms for affecting class I MHC expression." J Immunol **162**(9): 5049-5052.

Blake, N., S. Lee, I. Redchenko, W. Thomas, N. Steven, A. Leese, P. Steigerwald-Mullen, M. G. Kurilla, L. Frappier and A. Rickinson (1997). "Human CD8+ T cell responses to EBV EBNA1: HLA class I presentation of the (Gly-Ala)-containing protein requires exogenous processing." Immunity **7**(6): 791-802.

Bornkamm, G. W., C. Berens, C. Kuklik-Roos, J. M. Bechet, G. Laux, J. Bachl, M. Korndoerfer, M. Schlee, M. Holzel, A. Malamoussi, R. D. Chapman, F. Nimmerjahn, J. Mautner, W. Hillen, H. Bujard and J. Feuillard (2005). "Stringent doxycycline-dependent control of gene activities using an episomal one-vector system." Nucleic Acids Res **33**(16): e137.

Brown, J. C. and W. W. Newcomb (2011). "Herpesvirus capsid assembly: insights from structural analysis." Curr Opin Virol **1**(2): 142-149.

Buisson, M., T. Geoui, D. Flot, N. Tarbouriech, M. E. Ressing, E. J. Wiertz and W. P. Burmeister (2009). "A bridge crosses the active-site canyon of the Epstein-Barr virus nuclease with DNase and RNase activities." J Mol Biol **391**(4): 717-728.

Caldwell, R. G., J. B. Wilson, S. J. Anderson and R. Longnecker (1998). "Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals." Immunity **9**(3): 405-411.

Callan, M. F., L. Tan, N. Annels, G. S. Ogg, J. D. Wilson, C. A. O'Callaghan, N. Steven, A. J. McMichael and A. B. Rickinson (1998). "Direct visualization of antigen-specific

CD8+ T cells during the primary immune response to Epstein-Barr virus In vivo." J Exp Med **187**(9): 1395-1402.

Cancian, L., R. Bosshard, W. Lucchesi, C. E. Karstegl and P. J. Farrell (2011). "C-terminal region of EBNA-2 determines the superior transforming ability of type 1 Epstein-Barr virus by enhanced gene regulation of LMP-1 and CXCR7." PLoS Pathog **7**(7): 28.

Chang, Y. N., D. L. Dong, G. S. Hayward and S. D. Hayward (1990). "The Epstein-Barr virus Zta transactivator: a member of the bZIP family with unique DNA-binding specificity and a dimerization domain that lacks the characteristic heptad leucine zipper motif." J Virol **64**(7): 3358-3369.

Cohen, J. I. (2000). "Epstein-Barr virus infection." N Engl J Med **343**(7): 481-492.

Cohen, J. I., F. Wang, J. Mannick and E. Kieff (1989). "Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation." Proc Natl Acad Sci U S A **86**(23): 9558-9562.

Coscoy, L. and D. Ganem (2000). "Kaposi's sarcoma-associated herpesvirus encodes two proteins that block cell surface display of MHC class I chains by enhancing their endocytosis." Proc Natl Acad Sci U S A **97**(14): 8051-8056.

Croft, N. P., C. Shannon-Lowe, A. I. Bell, D. Horst, E. Kremmer, M. E. Ressing, E. J. Wiertz, J. M. Middeldorp, M. Rowe, A. B. Rickinson and A. D. Hislop (2009). "Stage-specific inhibition of MHC class I presentation by the Epstein-Barr virus BNLF2a protein during virus lytic cycle." PLoS Pathog **5**(6): 26.

Danchin, E., V. Vitiello, A. Vienne, O. Richard, P. Gouret, M. F. McDermott and P. Pontarotti (2004). "The major histocompatibility complex origin." Immunol Rev **198**: 216-232.

DeMars, R., C. C. Chang, S. Shaw, P. J. Reitnauer and P. M. Sondel (1984). "Homozygous deletions that simultaneously eliminate expressions of class I and class II antigens of EBV-transformed B-lymphoblastoid cells. I. Reduced proliferative responses of autologous and allogeneic T cells to mutant cells that have decreased expression of class II antigens." Hum Immunol **11**(2): 77-97.

El-Guindy, A., M. Ghiassi-Nejad, S. Golden, H. J. Delecluse and G. Miller (2013). "Essential role of Rta in lytic DNA replication of Epstein-Barr virus." J Virol **87**(1): 208-223.

Falk, K. and O. Rotzschke (1993). "Consensus motifs and peptide ligands of MHC class I molecules." Semin Immunol **5**(2): 81-94.

Farrell, P. J., D. T. Rowe, C. M. Rooney and T. Kouzarides (1989). "Epstein-Barr virus BZLF1 trans-activator specifically binds to a consensus AP-1 site and is related to c-fos." EMBO J **8**(1): 127-132.

Feederle, R., H. Bannert, H. Lips, N. Muller-Lantzsch and H. J. Delecluse (2009). "The Epstein-Barr virus alkaline exonuclease BGLF5 serves pleiotropic functions in virus replication." J Virol **83**(10): 4952-4962.

Feederle, R., M. Kost, M. Baumann, A. Janz, E. Drouet, W. Hammerschmidt and H. J. Delecluse (2000). "The Epstein-Barr virus lytic program is controlled by the co-operative functions of two transactivators." EMBO J **19**(12): 3080-3089.

Fingeroth, J. D., J. J. Weis, T. F. Tedder, J. L. Strominger, P. A. Biro and D. T. Fearon (1984). "Epstein-Barr virus receptor of human B lymphocytes is the C3d receptor CR2." Proc Natl Acad Sci U S A **81**(14): 4510-4514.

Fixman, E. D., G. S. Hayward and S. D. Hayward (1992). "trans-acting requirements for replication of Epstein-Barr virus ori-Lyt." J Virol **66**(8): 5030-5039.

Gabathuler, R. and S. Kvist (1990). "The endoplasmic reticulum retention signal of the E3/19K protein of adenovirus type 2 consists of three separate amino acid segments at the carboxy terminus." J Cell Biol **111**(5 Pt 1): 1803-1810.

Gadola, S. D., H. T. Moins-Teisserenc, J. Trowsdale, W. L. Gross and V. Cerundolo (2000). "TAP deficiency syndrome." Clin Exp Immunol **121**(2): 173-178.

Garstka, M., B. Borchert, M. Al-Balushi, P. V. Praveen, N. Kuhl, I. Majoul, R. Duden and S. Springer (2007). "Peptide-receptive major histocompatibility complex class I molecules cycle between endoplasmic reticulum and cis-Golgi in wild-type lymphocytes." J Biol Chem **282**(42): 30680-30690.

Gerlach, C., J. W. van Heijst, E. Swart, D. Sie, N. Armstrong, R. M. Kerkhoven, D. Zehn, M. J. Bevan, K. Schepers and T. N. Schumacher (2010). "One naive T cell, multiple fates in CD8+ T cell differentiation." J Exp Med **207**(6): 1235-1246.

Germain, R. N. and D. H. Margulies (1993). "The biochemistry and cell biology of antigen processing and presentation." Annu Rev Immunol **11**: 403-450.

Glosson, N. L., P. Gonyo, N. A. May, C. L. Schneider, L. C. Ristow, Q. Wang and A. W. Hudson (2010). "Insight into the mechanism of human herpesvirus 7 U21-mediated

diversion of class I MHC molecules to lysosomes." J Biol Chem **285**(47): 37016-37029.

Goldberg, A. L., P. Cascio, T. Saric and K. L. Rock (2002). "The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides." Mol Immunol **39**(3-4): 147-164.

Griffin, B. D., A. M. Gram, A. Mulder, D. Van Leeuwen, F. H. Claas, F. Wang, M. E. Ressing and E. Wiertz (2013). "EBV BILF1 evolved to downregulate cell surface display of a wide range of HLA class I molecules through their cytoplasmic tail." J Immunol **190**(4): 1672-1684.

Gruhler, A., P. A. Peterson and K. Fruh (2000). "Human cytomegalovirus immediate early glycoprotein US3 retains MHC class I molecules by transient association." Traffic **1**(4): 318-325.

Harty, J. T., A. R. Tvinnereim and D. W. White (2000). "CD8+ T cell effector mechanisms in resistance to infection." Annu Rev Immunol **18**: 275-308.

Henderson, R. A., H. Michel, K. Sakaguchi, J. Shabanowitz, E. Appella, D. F. Hunt and V. H. Engelhard (1992). "HLA-A2.1-associated peptides from a mutant cell line: a second pathway of antigen presentation." Science **255**(5049): 1264-1266.

Henderson, S., D. Huen, M. Rowe, C. Dawson, G. Johnson and A. Rickinson (1993). "Epstein-Barr virus-coded BHRF1 protein, a viral homologue of Bcl-2, protects human B cells from programmed cell death." Proc Natl Acad Sci U S A **90**(18): 8479-8483.

Hengel, H., J. O. Koopmann, T. Flohr, W. Muranyi, E. Goulmy, G. J. Hammerling, U. H. Koszinowski and F. Momburg (1997). "A viral ER-resident glycoprotein inactivates the MHC-encoded peptide transporter." Immunity **6**(5): 623-632.

Hewitt, E. W., L. Duncan, D. Mufti, J. Baker, P. G. Stevenson and P. J. Lehner (2002). "Ubiquitylation of MHC class I by the K3 viral protein signals internalization and TSG101-dependent degradation." EMBO J **21**(10): 2418-2429.

Hewitt, E. W., S. S. Gupta and P. J. Lehner (2001). "The human cytomegalovirus gene product US6 inhibits ATP binding by TAP." EMBO J **20**(3): 387-396.

Hickman, H. D., K. Takeda, C. N. Skon, F. R. Murray, S. E. Hensley, J. Loomis, G. N. Barber, J. R. Bennink and J. W. Yewdell (2008). "Direct priming of antiviral CD8+ T cells in the peripheral interfollicular region of lymph nodes." Nat Immunol **9**(2): 155-165.

Hislop, A. D., N. E. Annels, N. H. Gudgeon, A. M. Leese and A. B. Rickinson (2002). "Epitope-specific evolution of human CD8(+) T cell responses from primary to persistent phases of Epstein-Barr virus infection." J Exp Med **195**(7): 893-905.

Hislop, A. D., M. E. Ressing, D. van Leeuwen, V. A. Pudney, D. Horst, D. Koppers-Lalic, N. P. Croft, J. J. Neefjes, A. B. Rickinson and E. J. Wiertz (2007). "A CD8+ T cell immune evasion protein specific to Epstein-Barr virus and its close relatives in Old World primates." J Exp Med **204**(8): 1863-1873.

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

Hochberg, D., J. M. Middeldorp, M. Catalina, J. L. Sullivan, K. Luzuriaga and D. A. Thorley-Lawson (2004). "Demonstration of the Burkitt's lymphoma Epstein-Barr virus phenotype in dividing latently infected memory cells in vivo." Proc Natl Acad Sci U S A **101**(1): 239-244.

Horst, D., V. Favaloro, F. Vilardi, H. C. van Leeuwen, M. A. Garstka, A. D. Hislop, C. Rabu, E. Kremmer, A. B. Rickinson, S. High, B. Dobberstein, M. E. Ressing and E. J. Wiertz (2011). "EBV protein BNLF2a exploits host tail-anchored protein integration machinery to inhibit TAP." J Immunol **186**(6): 3594-3605.

Horst, D., D. van Leeuwen, N. P. Croft, M. A. Garstka, A. D. Hislop, E. Kremmer, A. B. Rickinson, E. J. Wiertz and M. E. Ressing (2009). "Specific targeting of the EBV lytic phase protein BNLF2a to the transporter associated with antigen processing results in impairment of HLA class I-restricted antigen presentation." J Immunol **182**(4): 2313-2324.

Hudson, A. W., P. M. Howley and H. L. Ploegh (2001). "A human herpesvirus 7 glycoprotein, U21, diverts major histocompatibility complex class I molecules to lysosomes." J Virol **75**(24): 12347-12358.

Hulpke, S., M. Tomioka, E. Kremmer, K. Ueda, R. Abele and R. Tampe (2012). "Direct evidence that the N-terminal extensions of the TAP complex act as autonomous interaction scaffolds for the assembly of the MHC I peptide-loading complex." Cell Mol Life Sci **69**(19): 3317-3327.

Ishido, S., C. Wang, B. S. Lee, G. B. Cohen and J. U. Jung (2000). "Downregulation of major histocompatibility complex class I molecules by Kaposi's sarcoma-associated herpesvirus K3 and K5 proteins." J Virol **74**(11): 5300-5309.

Jochum, S., A. Moosmann, S. Lang, W. Hammerschmidt and R. Zeidler (2012). "The EBV immunoevasins vIL-10 and BNLF2a protect newly infected B cells from immune recognition and elimination." PLoS Pathog **8**(5): 17.

Jochum, S., R. Ruiss, A. Moosmann, W. Hammerschmidt and R. Zeidler (2012). "RNAs in Epstein-Barr virions control early steps of infection." Proc Natl Acad Sci U S A **109**(21): 27.

Johannsen, E., E. Koh, G. Mosialos, X. Tong, E. Kieff and S. R. Grossman (1995). "Epstein-Barr virus nuclear protein 2 transactivation of the latent membrane protein 1 promoter is mediated by J kappa and PU.1." J Virol **69**(1): 253-262.

Karajannis, M. A., M. Hummel, I. Anagnostopoulos and H. Stein (1997). "Strict lymphotropism of Epstein-Barr virus during acute infectious mononucleosis in nonimmunocompromised individuals." Blood **89**(8): 2856-2862.

Karttunen, J. T., P. J. Lehner, S. S. Gupta, E. W. Hewitt and P. Cresswell (2001). "Distinct functions and cooperative interaction of the subunits of the transporter associated with antigen processing (TAP)." Proc Natl Acad Sci U S A **98**(13): 7431-7436.

Keating, S., S. Prince, M. Jones and M. Rowe (2002). "The lytic cycle of Epstein-Barr virus is associated with decreased expression of cell surface major histocompatibility complex class I and class II molecules." J Virol **76**(16): 8179-8188.

Kessler, J. H., S. Khan, U. Seifert, S. Le Gall, K. M. Chow, A. Paschen, S. A. Bres-Vloemans, A. de Ru, N. van Montfoort, K. L. Franken, W. E. Benckhuijsen, J. M. Brooks, T. van Hall, K. Ray, A. Mulder, Doxiadis, II, P. F. van Swieten, H. S. Overkleeft, A. Prat, B. Tomkinson, J. Neefjes, P. M. Kloetzel, D. W. Rodgers, L. B.

Hersh, J. W. Drijfhout, P. A. van Veelen, F. Ossendorp and C. J. Melief (2011). "Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes." Nat Immunol **12**(1): 45-53.

Khan, S., R. de Giuli, G. Schmidtke, M. Bruns, M. Buchmeier, M. van den Broek and M. Groettrup (2001). "Cutting edge: neosynthesis is required for the presentation of a T cell epitope from a long-lived viral protein." J Immunol **167**(9): 4801-4804.

Kloetzel, P. M. (2004). "Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII." Nat Immunol **5**(7): 661-669.

Koppers-Lalic, D., E. A. Reits, M. E. Ressing, A. D. Lipinska, R. Abele, J. Koch, M. Marcondes Rezende, P. Admiraal, D. van Leeuwen, K. Bienkowska-Szewczyk, T. C. Mettenleiter, F. A. Rijsewijk, R. Tampe, J. Neefjes and E. J. Wiertz (2005). "Varicelloviruses avoid T cell recognition by UL49.5-mediated inactivation of the transporter associated with antigen processing." Proc Natl Acad Sci U S A **102**(14): 5144-5149.

Koppers-Lalic, D., M. C. Verweij, A. D. Lipinska, Y. Wang, E. Quinten, E. A. Reits, J. Koch, S. Loch, M. Marcondes Rezende, F. Daus, K. Bienkowska-Szewczyk, N. Osterrieder, T. C. Mettenleiter, M. H. Heemskerk, R. Tampe, J. J. Neefjes, S. I. Chowdhury, M. E. Ressing, F. A. Rijsewijk and E. J. Wiertz (2008). "Varicellovirus UL 49.5 proteins differentially affect the function of the transporter associated with antigen processing, TAP." PLoS Pathog **4**(5): 1000080.

Kwun, H. J., S. R. da Silva, H. Qin, R. L. Ferris, R. Tan, Y. Chang and P. S. Moore (2011). "The central repeat domain 1 of Kaposi's sarcoma-associated herpesvirus (KSHV)

latency associated-nuclear antigen 1 (LANA1) prevents cis MHC class I peptide presentation." Virology **412**(2): 357-365.

Kwun, H. J., S. R. da Silva, I. M. Shah, N. Blake, P. S. Moore and Y. Chang (2007). "Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mimics Epstein-Barr virus EBNA1 immune evasion through central repeat domain effects on protein processing." J Virol **81**(15): 8225-8235.

Lacaille, V. G. and M. J. Androlewicz (1998). "Herpes simplex virus inhibitor ICP47 destabilizes the transporter associated with antigen processing (TAP) heterodimer." J Biol Chem **273**(28): 17386-17390.

Lampen, M. H., C. Hassan, M. Sluijter, A. Geluk, K. Dijkman, J. M. Tjon, A. H. de Ru, S. H. van der Burg, P. A. van Veelen and T. van Hall (2013). "Alternative peptide repertoire of HLA-E reveals a binding motif that is strikingly similar to HLA-A2." Mol Immunol **53**(1-2): 126-131.

Lautscham, G., T. Haigh, S. Mayrhofer, G. Taylor, D. Croom-Carter, A. Leese, S. Gadola, V. Cerundolo, A. Rickinson and N. Blake (2003). "Identification of a TAP-independent, immunoproteasome-dependent CD8+ T-cell epitope in Epstein-Barr virus latent membrane protein 2." J Virol **77**(4): 2757-2761.

Lautscham, G., S. Mayrhofer, G. Taylor, T. Haigh, A. Leese, A. Rickinson and N. Blake (2001). "Processing of a multiple membrane spanning Epstein-Barr virus protein for CD8(+) T cell recognition reveals a proteasome-dependent, transporter associated with antigen processing-independent pathway." J Exp Med **194**(8): 1053-1068.

Lautscham, G., A. Rickinson and N. Blake (2003). "TAP-independent antigen presentation on MHC class I molecules: lessons from Epstein-Barr virus." Microbes Infect **5**(4): 291-299.

Lee, S., J. Yoon, B. Park, Y. Jun, M. Jin, H. C. Sung, I. H. Kim, S. Kang, E. J. Choi, B. Y. Ahn and K. Ahn (2000). "Structural and functional dissection of human cytomegalovirus US3 in binding major histocompatibility complex class I molecules." J Virol **74**(23): 11262-11269.

Levitskaya, J., A. Sharipo, A. Leonchiks, A. Ciechanover and M. G. Masucci (1997). "Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigen 1." Proc Natl Acad Sci U S A **94**(23): 12616-12621.

Li, D., L. Qian, C. Chen, M. Shi, M. Yu, M. Hu, L. Song, B. Shen and N. Guo (2009). "Down-regulation of MHC class II expression through inhibition of CIITA transcription by lytic transactivator Zta during Epstein-Barr virus reactivation." J Immunol **182**(4): 1799-1809.

Long, H. M., T. A. Haigh, N. H. Gudgeon, A. M. Leen, C. W. Tsang, J. Brooks, E. Landais, E. Houssaint, S. P. Lee, A. B. Rickinson and G. S. Taylor (2005). "CD4+ T-cell responses to Epstein-Barr virus (EBV) latent-cycle antigens and the recognition of EBV-transformed lymphoblastoid cell lines." J Virol **79**(8): 4896-4907.

Long, H. M., G. S. Taylor and A. B. Rickinson (2011). "Immune defence against EBV and EBV-associated disease." Curr Opin Immunol **23**(2): 258-264.

Luka, J., B. Kallin and G. Klein (1979). "Induction of the Epstein-Barr virus (EBV) cycle in latently infected cells by n-butyrate." Virology **94**(1): 228-231.

Mackay, L. K., H. M. Long, J. M. Brooks, G. S. Taylor, C. S. Leung, A. Chen, F. Wang and A. B. Rickinson (2009). "T cell detection of a B-cell tropic virus infection: newly-synthesised versus mature viral proteins as antigen sources for CD4 and CD8 epitope display." PLoS Pathog **5**(12): 18.

Maini, M. K., N. Gudgeon, L. R. Wedderburn, A. B. Rickinson and P. C. Beverley (2000). "Clonal expansions in acute EBV infection are detectable in the CD8 and not the CD4 subset and persist with a variable CD45 phenotype." J Immunol **165**(10): 5729-5737.

Miller, C. L., A. L. Burkhardt, J. H. Lee, B. Stealey, R. Longnecker, J. B. Bolen and E. Kieff (1995). "Integral membrane protein 2 of Epstein-Barr virus regulates reactivation from latency through dominant negative effects on protein-tyrosine kinases." Immunity **2**(2): 155-166.

Miller, C. L., J. H. Lee, E. Kieff and R. Longnecker (1994). "An integral membrane protein (LMP2) blocks reactivation of Epstein-Barr virus from latency following surface immunoglobulin crosslinking." Proc Natl Acad Sci U S A **91**(2): 772-776.

Momburg, F., J. Roelse, G. J. Hammerling and J. J. Neefjes (1994). "Peptide size selection by the major histocompatibility complex-encoded peptide transporter." J Exp Med **179**(5): 1613-1623.

Nachmani, D., N. Stern-Ginossar, R. Sarid and O. Mandelboim (2009). "Diverse herpesvirus microRNAs target the stress-induced immune ligand MICB to escape recognition by natural killer cells." Cell Host Microbe **5**(4): 376-385.

Niedobitek, G., A. Agathanggelou, H. Herbst, L. Whitehead, D. H. Wright and L. S. Young (1997). "Epstein-Barr virus (EBV) infection in infectious mononucleosis: virus latency, replication and phenotype of EBV-infected cells." J Pathol **182**(2): 151-159.

Oliveira, C. C., P. A. van Veelen, B. Querido, A. de Ru, M. Sluijter, S. Laban, J. W. Drijfhout, S. H. van der Burg, R. Offringa and T. van Hall (2010). "The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects." J Exp Med **207**(1): 207-221.

Oresic, K., C. L. Ng and D. Tortorella (2009). "TRAM1 participates in human cytomegalovirus US2- and US11-mediated dislocation of an endoplasmic reticulum membrane glycoprotein." J Biol Chem **284**(9): 5905-5914.

Oresic, K. and D. Tortorella (2008). "Endoplasmic reticulum chaperones participate in human cytomegalovirus US2-mediated degradation of class I major histocompatibility complex molecules." J Gen Virol **89**(Pt 5): 1122-1130.

Pappworth, I. Y., E. C. Wang and M. Rowe (2007). "The switch from latent to productive infection in epstein-barr virus-infected B cells is associated with sensitization to NK cell killing." J Virol **81**(2): 474-482.

Paquet, M. E., M. Cohen-Doyle, G. C. Shore and D. B. Williams (2004). "Bap29/31 influences the intracellular traffic of MHC class I molecules." J Immunol **172**(12): 7548-7555.

Parish, I. A. and S. M. Kaech (2009). "Diversity in CD8(+) T cell differentiation." Curr Opin Immunol **21**(3): 291-297.

Pudney, V. A., A. M. Leese, A. B. Rickinson and A. D. Hislop (2005). "CD8+ immunodominance among Epstein-Barr virus lytic cycle antigens directly reflects the efficiency of antigen presentation in lytically infected cells." J Exp Med **201**(3): 349-360.

Qian, S. B., J. R. Bennink and J. W. Yewdell (2005). "Quantitating defective ribosome products." Methods Mol Biol **301**: 271-281.

Ragoczy, T., L. Heston and G. Miller (1998). "The Epstein-Barr virus Rta protein activates lytic cycle genes and can disrupt latency in B lymphocytes." J Virol **72**(10): 7978-7984.

Reits, E., J. Neijssen, C. Herberts, W. Benckhuijsen, L. Janssen, J. W. Drijfhout and J. Neefjes (2004). "A major role for TPPII in trimming proteasomal degradation products for MHC class I antigen presentation." Immunity **20**(4): 495-506.

Ressing, M. E., S. E. Keating, D. van Leeuwen, D. Koppers-Lalic, I. Y. Pappworth, E. J. Wiertz and M. Rowe (2005). "Impaired transporter associated with antigen processing-dependent peptide transport during productive EBV infection." J Immunol **174**(11): 6829-6838.

Ressing, M. E., D. van Leeuwen, F. A. Verreck, R. Gomez, B. Heemskerk, M. Toebes, M. M. Mullen, T. S. Jardetzky, R. Longnecker, M. W. Schilham, T. H. Ottenhoff, J. Neefjes, T. N. Schumacher, L. M. Hutt-Fletcher and E. J. Wiertz (2003). "Interference with T cell receptor-HLA-DR interactions by Epstein-Barr virus gp42 results in reduced T helper cell recognition." Proc Natl Acad Sci U S A **100**(20): 11583-11588.

Ressing, M. E., D. van Leeuwen, F. A. Verreck, S. Keating, R. Gomez, K. L. Franken, T. H. Ottenhoff, M. Spriggs, T. N. Schumacher, L. M. Hutt-Fletcher, M. Rowe and E. J. Wiertz (2005). "Epstein-Barr virus gp42 is posttranslationally modified to produce soluble gp42 that mediates HLA class II immune evasion." J Virol **79**(2): 841-852.

Reusch, U., W. Muranyi, P. Lucin, H. G. Burgert, H. Hengel and U. H. Koszinowski (1999). "A cytomegalovirus glycoprotein re-routes MHC class I complexes to lysosomes for degradation." EMBO J **18**(4): 1081-1091.

Robertson, E. S., J. Lin and E. Kieff (1996). "The amino-terminal domains of Epstein-Barr virus nuclear proteins 3A, 3B, and 3C interact with RBPJ(kappa)." J Virol **70**(5): 3068-3074.

Roeth, J. F., M. Williams, M. R. Kasper, T. M. Filzen and K. L. Collins (2004). "HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail." J Cell Biol **167**(5): 903-913.

Rowe, M., B. Glaunsinger, D. van Leeuwen, J. Zuo, D. Sweetman, D. Ganem, J. Middeldorp, E. J. Wiertz and M. E. Ressing (2007). "Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion." Proc Natl Acad Sci U S A **104**(9): 3366-3371.

Sadasivan, B., P. J. Lehner, B. Ortmann, T. Spies and P. Cresswell (1996). "Roles for calreticulin and a novel glycoprotein, tapasin, in the interaction of MHC class I molecules with TAP." Immunity **5**(2): 103-114.

Saric, T., C. I. Graef and A. L. Goldberg (2004). "Pathway for degradation of peptides generated by proteasomes: a key role for thimet oligopeptidase and other metallopeptidases." J Biol Chem **279**(45): 46723-46732.

Schubert, U., L. C. Anton, J. Gibbs, C. C. Norbury, J. W. Yewdell and J. R. Bennink (2000). "Rapid degradation of a large fraction of newly synthesized proteins by proteasomes." Nature **404**(6779): 770-774.

Semmling, V., V. Lukacs-Kornek, C. A. Thaiss, T. Quast, K. Hochheiser, U. Panzer, J. Rossjohn, P. Perlmutter, J. Cao, D. I. Godfrey, P. B. Savage, P. A. Knolle, W. Kolanus, I. Forster and C. Kurts (2010). "Alternative cross-priming through CCL17-CCR4-mediated attraction of CTLs toward NKT cell-licensed DCs." Nat Immunol **11**(4): 313-320.

Serwold, T., F. Gonzalez, J. Kim, R. Jacob and N. Shastri (2002). "ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum." Nature **419**(6906): 480-483.

Shresta, S., C. T. Pham, D. A. Thomas, T. A. Graubert and T. J. Ley (1998). "How do cytotoxic lymphocytes kill their targets?" Curr Opin Immunol **10**(5): 581-587.

Takada, K. and Y. Ono (1989). "Synchronous and sequential activation of latently infected Epstein-Barr virus genomes." J Virol **63**(1): 445-449.

Takeuchi, O. and S. Akira (2009). "Innate immunity to virus infection." Immunol Rev **227**(1): 75-86.

Teisserenc, H., W. Schmitt, N. Blake, R. Dunbar, S. Gadola, W. L. Gross, A. Exley and V. Cerundolo (1997). "A case of primary immunodeficiency due to a defect of the major

histocompatibility gene complex class I processing and presentation pathway." Immunol Lett **57**(1-3): 183-187.

Tellam, J., C. Smith, M. Rist, N. Webb, L. Cooper, T. Vuocolo, G. Connolly, D. C. Tscharke, M. P. Devoy and R. Khanna (2008). "Regulation of protein translation through mRNA structure influences MHC class I loading and T cell recognition." Proc Natl Acad Sci U S A **105**(27): 9319-9324.

Tey, S. K. and R. Khanna (2012). "Autophagy mediates transporter associated with antigen processing-independent presentation of viral epitopes through MHC class I pathway." Blood **120**(5): 994-1004.

Tomazin, R., A. B. Hill, P. Jugovic, I. York, P. van Endert, H. L. Ploegh, D. W. Andrews and D. C. Johnson (1996). "Stable binding of the herpes simplex virus ICP47 protein to the peptide binding site of TAP." EMBO J **15**(13): 3256-3266.

Tomkinson, B. and E. Kieff (1992). "Use of second-site homologous recombination to demonstrate that Epstein-Barr virus nuclear protein 3B is not important for lymphocyte infection or growth transformation in vitro." J Virol **66**(5): 2893-2903.

Tomkinson, B., E. Robertson and E. Kieff (1993). "Epstein-Barr virus nuclear proteins EBNA-3A and EBNA-3C are essential for B-lymphocyte growth transformation." J Virol **67**(4): 2014-2025.

Tovey, M. G., G. Lenoir and J. Begon-Lours (1978). "Activation of latent Epstein-Barr virus by antibody to human IgM." Nature **276**(5685): 270-272.

Trandem, K., J. Zhao, E. Fleming and S. Perlman (2011). "Highly activated cytotoxic CD8 T cells express protective IL-10 at the peak of coronavirus-induced encephalitis." J Immunol **186**(6): 3642-3652.

Uchida, J., T. Yasui, Y. Takaoka-Shichijo, M. Muraoka, W. Kulwichit, N. Raab-Traub and H. Kikutani (1999). "Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses." Science **286**(5438): 300-303.

Urier, G., M. Buisson, P. Chambard and A. Sergeant (1989). "The Epstein-Barr virus early protein EB1 activates transcription from different responsive elements including AP-1 binding sites." EMBO J **8**(5): 1447-1453.

van Endert, P. M., R. Tampe, T. H. Meyer, R. Tisch, J. F. Bach and H. O. McDevitt (1994). "A sequential model for peptide binding and transport by the transporters associated with antigen processing." Immunity **1**(6): 491-500.

van Gent, M., B. D. Griffin, E. G. Berkhoff, D. van Leeuwen, I. G. Boer, M. Buisson, F. C. Hartgers, W. P. Burmeister, E. J. Wiertz and M. E. Ressing (2011). "EBV lytic-phase protein BGLF5 contributes to TLR9 downregulation during productive infection." J Immunol **186**(3): 1694-1702.

Verweij, M. C., D. Koppers-Lalic, S. Loch, F. Klauschies, H. de la Salle, E. Quinten, P. J. Lehner, A. Mulder, M. R. Knittler, R. Tampe, J. Koch, M. E. Ressing and E. J. Wiertz (2008). "The varicellovirus UL49.5 protein blocks the transporter associated with antigen processing (TAP) by inhibiting essential conformational transitions in the 6+6 transmembrane TAP core complex." J Immunol **181**(7): 4894-4907.

Verweij, M. C., A. D. Lipinska, D. Koppers-Lalic, E. Quinten, J. Funke, H. C. van Leeuwen, K. Bienkowska-Szewczyk, J. Koch, M. E. Ressing and E. J. Wiertz (2011). "Structural and functional analysis of the TAP-inhibiting UL49.5 proteins of varicelloviruses." Mol Immunol **48**(15-16): 2038-2051.

Verweij, M. C., A. D. Lipinska, D. Koppers-Lalic, W. F. van Leeuwen, J. I. Cohen, P. R. Kinchington, I. Messaoudi, K. Bienkowska-Szewczyk, M. E. Ressing, F. A. Rijsewijk and E. J. Wiertz (2011). "The capacity of UL49.5 proteins to inhibit TAP is widely distributed among members of the genus Varicellovirus." J Virol **85**(5): 2351-2363.

Vrzalikova, K., M. Vockerodt, S. Leonard, A. Bell, W. Wei, A. Schrader, K. L. Wright, D. Kube, M. Rowe, C. B. Woodman and P. G. Murray (2011). "Down-regulation of BLIMP1alpha by the EBV oncogene, LMP-1, disrupts the plasma cell differentiation program and prevents viral replication in B cells: implications for the pathogenesis of EBV-associated B-cell lymphomas." Blood **117**(22): 5907-5917.

Wang, D., D. Liebowitz and E. Kieff (1985). "An EBV membrane protein expressed in immortalized lymphocytes transforms established rodent cells." Cell **43**(3 Pt 2): 831-840.

Wang, F., C. Gregory, C. Sample, M. Rowe, D. Liebowitz, R. Murray, A. Rickinson and E. Kieff (1990). "Epstein-Barr virus latent membrane protein (LMP1) and nuclear proteins 2 and 3C are effectors of phenotypic changes in B lymphocytes: EBNA-2 and LMP1 cooperatively induce CD23." J Virol **64**(5): 2309-2318.

Wei, M. L. and P. Cresswell (1992). "HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides." Nature **356**(6368): 443-446.

Wiertz, E. J., T. R. Jones, L. Sun, M. Bogyo, H. J. Geuze and H. L. Ploegh (1996). "The human cytomegalovirus US11 gene product dislocates MHC class I heavy chains from the endoplasmic reticulum to the cytosol." Cell **84**(5): 769-779.

Wiertz, E. J., D. Tortorella, M. Bogyo, J. Yu, W. Mothes, T. R. Jones, T. A. Rapoport and H. L. Ploegh (1996). "Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction." Nature **384**(6608): 432-438.

Wilkinson, G. W. and P. J. Lehner (2009). "Jenner's irony: cowpox taps into T cell evasion." Cell Host Microbe **6**(5): 395-397.

Williams, A. P., C. A. Peh, A. W. Purcell, J. McCluskey and T. Elliott (2002). "Optimization of the MHC class I peptide cargo is dependent on tapasin." Immunity **16**(4): 509-520.

Williams, M., J. F. Roeth, M. R. Kasper, R. I. Fleis, C. G. Przybycin and K. L. Collins (2002). "Direct binding of human immunodeficiency virus type 1 Nef to the major histocompatibility complex class I (MHC-I) cytoplasmic tail disrupts MHC-I trafficking." J Virol **76**(23): 12173-12184.

Woodberry, T., T. J. Suscovich, L. M. Henry, J. K. Davis, N. Frahm, B. D. Walker, D. T. Scadden, F. Wang and C. Brander (2005). "Differential targeting and shifts in the immunodominance of Epstein-Barr virus--specific CD8 and CD4 T cell responses during acute and persistent infection." J Infect Dis **192**(9): 1513-1524.

Yates, J., N. Warren, D. Reisman and B. Sugden (1984). "A cis-acting element from the Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells." Proc Natl Acad Sci U S A **81**(12): 3806-3810.

Yewdell, J. W. (2005). "Immunoproteasomes: regulating the regulator." Proc Natl Acad Sci U S A **102**(26): 9089-9090.

Yewdell, J. W., L. C. Anton and J. R. Bennink (1996). "Defective ribosomal products (DRiPs): a major source of antigenic peptides for MHC class I molecules?" J Immunol **157**(5): 1823-1826.

Yewdell, J. W. and J. R. Bennink (1999). "Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses." Annu Rev Immunol **17**: 51-88.

Yin, Y., B. Manoury and R. Fahraeus (2003). "Self-inhibition of synthesis and antigen presentation by Epstein-Barr virus-encoded EBNA1." Science **301**(5638): 1371-1374.

York, I. A., N. Bhutani, S. Zendzian, A. L. Goldberg and K. L. Rock (2006). "Tripeptidyl peptidase II is the major peptidase needed to trim long antigenic precursors, but is not required for most MHC class I antigen presentation." J Immunol **177**(3): 1434-1443.

York, I. A., A. X. Mo, K. Lemerise, W. Zeng, Y. Shen, C. R. Abraham, T. Saric, A. L. Goldberg and K. L. Rock (2003). "The cytosolic endopeptidase, thimet oligopeptidase, destroys antigenic peptides and limits the extent of MHC class I antigen presentation." Immunity **18**(3): 429-440.

York, I. A., C. Roop, D. W. Andrews, S. R. Riddell, F. L. Graham and D. C. Johnson (1994). "A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes." Cell **77**(4): 525-535.

Zeidler, R., G. Eissner, P. Meissner, S. Uebel, R. Tampe, S. Lazis and W. Hammerschmidt (1997). "Downregulation of TAP1 in B lymphocytes by cellular and Epstein-Barr virus-encoded interleukin-10." Blood **90**(6): 2390-2397.

Zuo, J., A. Currin, B. D. Griffin, C. Shannon-Lowe, W. A. Thomas, M. E. Ressing, E. J. Wiertz and M. Rowe (2009). "The Epstein-Barr virus G-protein-coupled receptor contributes to immune evasion by targeting MHC class I molecules for degradation." PLoS Pathog **5**(1): 2.

Zuo, J., L. L. Quinn, J. Tamblyn, W. A. Thomas, R. Feederle, H. J. Delecluse, A. D. Hislop and M. Rowe (2011). "The Epstein-Barr virus-encoded BILF1 protein modulates immune recognition of endogenously processed antigen by targeting major histocompatibility complex class I molecules trafficking on both the exocytic and endocytic pathways." J Virol **85**(4): 1604-1614.

Zuo, J., W. Thomas, D. van Leeuwen, J. M. Middeldorp, E. J. Wiertz, M. E. Ressing and M. Rowe (2008). "The DNase of gammaherpesviruses impairs recognition by virus-specific CD8+ T cells through an additional host shutoff function." J Virol **82**(5): 2385-2393.

Zuo, J., W. A. Thomas, T. A. Haigh, L. Fitzsimmons, H. M. Long, A. D. Hislop, G. S. Taylor and M. Rowe (2011). "Epstein-Barr virus evades CD4+ T cell responses in lytic cycle through BZLF1-mediated downregulation of CD74 and the cooperation of vBcl-2." PLoS Pathog **7**(12): 22.

zur Hausen, H., F. J. O'Neill, U. K. Freese and E. Hecker (1978). "Persisting oncogenic herpesvirus induced by the tumour promotor TPA." Nature **272**(5651): 373-375.