T CELL IMMUNITY TO KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS LATENT PROTEINS

by

SHEREEN SABBAH

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Abstract

T cell immunity is important for the control of Kaposi's sarcoma-associated herpesvirus (KSHV) disease, yet little is known about KSHV-specific immunity in healthy donors. Screening PBMCs from such donors by ELISpot analysis identified weak responses to the KSHV latent antigens; antigens expressed in the virus associated pathologies. We generated T cell clones to the latent proteins LANA and vFLIP and determined whether they recognised target cells. CD8+ clones poorly recognised targets expressing vFLIP or LANA, through mechanisms which reduce target protein synthesis: vFLIP used rare codons in the mRNA encoding this protein, while deleting the acidic repeat of LANA increased its recognition. We then examined whether LANA-specific CD4+ T cells recognised B cells expressing or fed LANA protein. These were recognised, however most KSHV-infected cell lines, in the form of primary effusion lymphoma (PEL) lines, were not. PELs express vIRF3 which inhibits promoter function of the HLA class II transactivator CIITA. Expressing CIITA from a different promoter restored CD4+ T cell recognition of PELs. This study suggests CD8 recognition of the latent antigens tested is inefficient due to the innate properties of the targets but that CD4 T cells can effectively recognise targets if the immune evasion mechanisms are bypassed.

DEDICATION

This thesis is dedicated to my sister Deleen,

I am blessed to have you in my life, thank you for everything.

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Abbreviations

3-MA	3-Methyladenine
AEP	Asparingyl endopeptidase
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
APS	Ammonium persulphate
β2m	β2 microglobulin
BFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
C	Constant
CD	Cluster of differentiation
CCR7	Chemokine receptor 7
CIITA	Class II transactivator
CLIP	Class II-associated invariant chain peptide
CMA	Chaperone-mediated autophagy
CMV	Cytomegalovirus
CPM	Counts per minute
CTLs	Cytotoxic T lymphocytes
D	Diversity
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
Dox	Doxycycline
DRiPs	Defective ribosomal products

ds	double-stranded
E	Early
EBV	Epstein barr virus
ECL	Enhanced Chemiluminescence
ER	Endoplasmic reticulum
ERAAP	Endoplasmic reticulum associated with antigen processing
FasL	Fas Ligand
FCS	Foetal calf serum
FPLC	Fast protein liquid chromatography
GAr	Glycine-alanine repeats
GILT	γ-IFN-inducible lysosomal thiol reductase
GM-CSF	Granulocyte macrophage-colony stimulating factor
HA	Haemagglutinin antigen
HAART	Highly active antiretroviral therapy
HEK 293	Human embryonic kidney epithelial cells
HFF	Human foreskin fibroblasts
HHV-8	Human herpes virus-8
HINGS	Heat-inactivated goat serum
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigens
HMEC-1	Human microvascular endothelial cells
HMVEC-d	Human dermal microvascular endothelial cells
HPV	Human papilloma virus
HRP	Horse radish peroxidase
HSC70	Heat shock cognate protein 70

Hu	Humanised
HUVEC	Human umbilicial vein endothelial cells
HVS	Herpes virus saimiri
IKKγ	Inhibitor κB kinase-γ
IL	Interleukin
IE	Immediate early
IF	Immunofluorescence
IgE	Immunoglobulin E
IRF	Interferon regulatory factor
J	Joining
L	Ligand
LC3	Light chain 3
LCLs	EBV transformed B cells or lymphoblastoid cell lines
LNGFR	Low-affinity nerve growth factor receptor
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Latent
LAMP-2a	Lysosome-associated membrane protein-2a
LANA	Latency-associated nuclear antigen
li	Invariant chain
LL	Late lytic
LMP	Latent membrane protein
IFN	Interferon
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting

MCD	Multicentric castleman’s disease
MHC	Major histocompatibility complex
MIIC	MHC class II compartment
MIR	Modulator of immune recognition
MOI	Multiplicity of infection
mRNA	Messenger RNA
MVA	Modified virus Ankara
NeoR	Neomycin phosphotransferase II
NGFR	Nerve Growth Factor Receptor
NK	Natural killer
NO	Nitric oxide
ORF	Open reading frame
OSM	Oncostatin-M
OVA	Ovalbmin
PAMPS	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBS/T	PBS/Tween
PCR	Polymerase chain reaction
PEL	Primary effusion lymphoma
PHA	Phytohaemaglutanin
PLC	Peptide loading complex
PRRs	Pattern-recognition receptors
RAP	Replication associated protein
Rb	Retinoblastoma

RT	Room temperature
RTA	Lytic switch protein
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
shRNA	Small hairpin ribonucleic acid
siRNA	Small interfering ribonucleic acid
SFC	Spot forming cells
SOX	KSHV shutoff and exonuclease protein
TAP	Transporter associated protein
Tc's	Cytotoxic T cells
TCRs	T cell receptors
Tet	Tetracycline
TGF-β	Transforming growth factor-β
T_H1	T Helper 1 cells
T_H2	T Helper 2 cells
T_H3	T Helper 3 cells
TLRs	Toll-like receptors
TNF	Tumour necrosis factor
TNFR-1	Tumour necrosis factor receptor-1
T_R1	T regulatory cells 1
Tregs	Regulatory T cells
Ub	Ubiquitin
UV	Ultraviolet
V	Variable
vCCL	Viral CC chemokines

vCyclin	Viral Cyclin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vFLIP	Viral FLICE-like inhibitory protein
vIL-6	Viral interleukin-6
vIRF	Viral interferon regulatory factor
vIRF3	Viral interferon regulatory factor 3
Wt	Wildtype

Chapter 1

Introduction

1.1 Viral immunity

1.1.1 Viral infection

Viruses are small infectious agents that require the host cellular machinery for replication. Virus infection of a host cell is usually comprised of two stages: attachment and penetration. The virus will bind specific receptors on the surface of a host cell and enter by receptor-mediated endocytosis or membrane fusion, uncoating and replicating itself by the synthesis of viral nucleic acid and proteins. Viruses use different strategies to maintain themselves and infect new hosts, for example the flu virus will replicate rapidly to invade a new host before it can be cleared by the original host's immune system. Other viruses have adapted to maintain long term colonisation of their hosts. Herpesviruses, for example, upon infection of a host cell can restrict their viral gene expression and enter a latent state, thereby evading recognition by the immune system, however occasionally they will reactivate into the virus productive cycle and lytic replication will ensue to produce new virions with the aim of infecting other cells and new hosts. By contrast, the human papilloma virus (HPV) constantly produces infectious virions to sustain itself in the host. However, HIV, a virus relatively recently introduced into the human population and yet to reach a stable virus-host relationship, contradicts these theories as it gradually destroys each of its hosts cell, producing more infectious virions to invade new hosts. The immune system has developed a number of defence strategies and mechanisms to combat and control these events, involving both the innate and adaptive immune response.

1.1.2 Innate immunity to viruses

The innate immune system is the front line of defence once the physical barriers of the body have been breached. It is made up of both cellular and soluble components. These cellular components have evolved to recognise pathogen-associated molecular patterns (PAMPs) through the expression of pattern-recognition receptors (PRRs) on host cells, the major group of which are the toll-like receptors (TLRs). Activation of PRRs induces the release of cytokines inducing an inflammatory response. A viral infection can induce the production of type-I interferons such as interferon-α (IFN-α) or interferon-β (IFN-β) by any cell in the body. One of the ways this has been shown to occur is through the detection of viral RNA as it enters the cytosol by a cytosolic receptor known as retinoic acid inducible gene I (RIG-I) trigerring a type-I interferon (IFN)-mediated response (Hirata et al., 2007). While type-II interferons such as interferon-γ (IFN-γ) are only produced by another component of the innate immune response, natural killer cells (NK), as well as cells of the adaptive immune response such as T cells. Interferons limit viral replication within infected cells by inducing an antiviral state in cells and induce increased MHC Class I expression on the surface of non-infected neighbouring cells protecting them from attack by NK cells. The main function of NK cells is to kill virus-infected cells, these are recognised through altered levels of ligands expressed on the surface of the infected cell which stimulate activating and inhibitory receptors on the NK cell.

1.1.3 Adaptive immunity to viruses

The innate immune system provides an immediate response to invading pathogens, allowing time for the activation of the effectors of the adaptive immune system, namely T and B lymphocytes. Dendritic cells (DC's) are potent activators of the T

cell response and similar to phagocytic cells, they take up antigens or pathogens via their PRRs such as the C-type lectin DC-SIGN or other TLRs (reviewed in (Geijtenbeek et al., 2002; Pulendran et al., 2001)). However unlike other phagocytes such as macrophages, DC's migrate to the local lymph node, in which they express fragments of the pathogen on their surface MHC molecules, presenting them to T cells initiating the generation of a specific adaptive immune response. There are two types of adaptive immune response: humoral and cell-mediated immune response.

1.1.3.1 Humoral immune response

Humoral immunity is mediated by antibodies produced from plasma cells which are derived from B lymphocytes. Antibodies are Y shaped molecules which, in early B cell subsets, are present on the surface of B cells as surface bound receptors. The arms of these molecules have two identical antigen-binding sites, which are highly diverse from one B cell to another. This is the result of somatic gene segment recombination of the immunoglobulin genes, a process that occurs within the germinal centre of lymph nodes, selecting B cells with the highest affinity receptor to access opsonised antigen presented by follicular dendritic cells. To produce high affinity isotype switched antibodies usually requires the aid of the CD4+ helper T cells, which recognise specific peptide fragments presented by B cells (Parker, 1993). The T cells provide cytokines and costimulation through molecules such as CD40-CD40 ligand (CD40L) to drive B cell proliferation and differentiation, mounting a clonal response into either plasma or memory cells.

Antibodies secreted in response to invading pathogens such as viruses bind to viral antigens acting as opsonins and also can activate the complement system for direct

lysis of the pathogen as well as opsonise the antigen for recognition by phagocytes (Schifferli et al., 1989). Antibodies also bind to toxins or viral antigens, blocking them from entering and infecting host cells, in a process known as neutralisation (Roost et al., 1995). Immunological memory for a particular antigen can be carried for many years as a result of long-lived memory B cells clones, which can reactivate upon encounter of the specific antigen. While producing antibodies is the sole effector function of B cells, T cells however have a number of effector functions.

1.1.3.2 Cell-mediated immune responses

Viruses replicate inside cells and although antibodies have recently been found to be capable of inhibiting virus replication within the cell, their contribution to intracellular control of virus is currently unappreciated (Mallery et al., 2010). However T cells have been found to have a well established role in the control of virally-infected cells. This takes place through a direct interaction between the cells bearing the specific antigen, which is presented in the form of a peptide fragment by major histocompatibility complex (MHC) proteins on the cell surface to the T cells. The T cells scan these complexes using their T cell receptor (TCR) present on their cell surface. This interaction and recognition by the respective T cells induces their effector function, helping to prevent viral replication and subsequent spread of the virus in the infected host.

T cell development

The earliest progenitors of T cells are produced in the bone marrow, they do not develop and undergo selection until they are in the thymus. Two lineages of T cells develop in the thymus, these can be discriminated on the basis of their TCRs; the γδ T

cells and the αβ T cells, which make up the majority of T cell repertoire. Similar to B cells, T cells also develop TCRs specific for antigen, this occurs as the T cell precursors (thymocytes) pass through the thymus. The TCR genes consist of a variable (V) and constant (C) domains, which are encoded by separate exons. The sequence of the V domain exon is determined through a process of gene rearrangement, which mediates the process of variable, diversity (D) and joining (J) gene sequences or VDJ. The random union of VDJ sequences determines the sequence of the functional V domain for the TCR β and δ genes, while the rearrangement of the V and J genes determines the sequence for the TCR α genes. Following successful gene rearrangement, generating thymocytes with a broad diversity of specificities, the specificity of the TCR expressed on its surface will determine its fate.

The thymocytes are subjected to the processes of positive and negative selection to generate a repertoire of peripheral T cells that are functional and not autoreactive. During positive selection, it is important that thymocytes can recognise peptides bound by self MHC on the cortical epithelial cells present within the thymus, in order to be selected for further differentiation (Sha et al., 1988). At the time of positive selection, thymocytes express both CD4 and CD8 co-receptor molecules. All mature thymocytes that recognise peptides bound to MHC class II molecules will ultimately express CD4 receptors on their surface, while those that express CD8 receptors on their surface will ultimately recognise peptide presented by MHC Class I molecules. Therefore, at the end of positive selection mature thymocytes will express only one of these co-receptors.

During negative selection, thymocytes with TCRs that strongly recognise self antigens presented by MHC Class I or MHC Class II on the surface of DCs or macrophages, will be eliminated by apoptosis to prevent the likelihood of encountering the same peptides once they are mature T cells (Kappler et al., 1987). The paradox that recognition of self MHC:self peptide by the TCRs on the thymocytes can lead to two different outcomes still remains to be resolved but has been proposed to be related to the affinity of TCR-peptide-MHC interactions delivering qualitatively different signals (Hogquist et al., 1994; Thien et al., 2005). However, once these selection processes have taken place, the thymocyte can leave the central lymphoid tissues and enter the periphery as naïve T cells until they encounter their cognate antigen.

T cell activation and memory

Once the naïve T cells have undergone thymic selection, they recirculate between the blood and peripheral lymphoid tissues, such as the lymph nodes and Peyer's patches in search of their cognate antigen. This preferential homing of naïve T cells to the lymphoid tissues requires the expression of chemokines and their cognate receptors on the T cells, as well as adhesion molecules, including selectins, integrins and corresponding vascular ligands (Ebert et al., 2005). Naïve T cells can recognise antigen in the T cell areas of the secondary lymphoid organs presented on the surface of an antigen presenting cell (APC) bound to a self MHC molecule in a MHC:peptide complex.

The most potent APCs are dendritic cells (DCs). Immature DCs in the tissues take up antigen at sites of infection, travel to local lymphoid tissues, under the guidance of

chemokine expression and mature to present epitopes from their captured antigens to T cells. For T cell activation by DCs, two signals are required; the first from the interaction between the TCR and the MHC:peptide complex and the second through signals derived from co-stimulatory molecule stimulation (Reis e Sousa, 2004). This helps to regulate appropriate stimulation of epitope-specific T cells. The second signals typically involve co-stimulation through the CD28 molecule, that is present on most human T cells especially naive T cells, which binds to the B7 glycoprotein family members B7.1 (CD80) or B7.2 (CD86) on the surface of the APC (Bour-Jordan et al., 2002). Once the naïve T cell is activated it expresses a number of proteins that contribute towards its proliferation and differentiation, such as CD40 and CD40L, which belong to the tumour necrosis factor (TNF) family of receptors and ligands. These proteins also have an important role in its effector function once fully differentiated.

T cells can be divided into three main classes, specialised to deal with the recognition of different targets. Cytotoxic T cells, which express the CD8 co-receptor, are capable of directly killing virally infected or foreign cells which display epitope-peptides by MHC class I on their surface. The T helper cells, T_H1 and T_H2, both express the CD4 co-receptor and recognise peptides presented by surface MHC Class II molecules. T_H1 cells have been proposed to activate macrophages, enabling them to remove microorganisms such as bacteria more efficiently, as well as activating B cells to produce strongly opsonising antibodies and also have cytotoxic potential. Through the cytokine profile that T_H2 cells show, these have been proposed to provide help to B cells, driving differentiation, antibody production and isotype switching.

Following the resolution of an infection, the majority of armed effector T cells die from apoptosis or become anergic, as the antigens that elicited the response are no longer present at the level needed to sustain the T cells (Callan et al., 2000). However, a minority of the effector T cells as well as activated naïve T cells may become memory T cells, these remain and confer protection upon rechallenge with the antigen or recrudescence of a latent infection (Hislop et al., 2002; Amyes et al., 2003). Memory CD8+ T cells exhibit a different phenotype to that of naïve T cells, they can be distinguished based on their expression of homing lymphoid markers such as CD62 ligand (CD62L) and chemokine receptor 7 (CCR7), as well as the integrin LFA-1, with the memory CD8+ T cells expressing an LFA-1(high)CCR7(low) phenotype (Faint et al., 2001).

These memory T cells can be divided into two functionally distinct populations based on their CCR7 expression. The first of which are the effector memory T cells, these are CCR7-, express receptors for migration to peripheral tissues and show immediate effector function upon encounter with the same pathogen, rapidly producing cytokines such as IFN-γ, IL-4 and IL-5. While the second population are the central memory cells, these are CCR7+ and typically express CD62L, these cells can therefore recirculate more easily to the lymph nodes but lack immediate effector function (Sallusto et al., 1999). More recent studies have shown that these two populations are not from independent lineages but rather differentiate in a linear fashion. Following the clearance of antigen from an acute infection of vaccination, effector CD8 T cells will initially differentiate into CCR7- effector memory cells and, with the continued absence of antigen, the cells will undergo further differentiation into CCR7+ central memory cells (Wherry et al., 2003). These memory cells will persist at constant

numbers for many years, as a homeostatic balance exists between the slow proliferation, survival and death of these cells (Gourley et al., 2004).

The CD8+ T cell response

CD8+ T cells recognise peptides presented by MHC I molecules present on the surface of all nucleated cells in the body. CD8+ T cells are known as cytotoxic T cells (Tc's) for their ability to kill mainly virally infected cells or cells that are otherwise damaged or dysfunctional. The primary mechanism through which CD8+ T cells act is through the release of lytic granules upon the recognition of their target peptide. The release of the proteins from these granules is calcium-dependent, these include perforin, granzymes and granulysin (reviewed in (Blott et al., 2002; Barry et al., 2002)). Perforin, forms a structure on target cell-membranes creating transmembrane pores. The second component of the lytic granules are the granzymes, these consist of at least three proteases, behaving as destructive enzymes. Finally, granulysin induces apoptosis and has anti-microbrial action (Lieberman, 2003).

However, Tc's can continue killing their target cells following calcium depletion, suggesting a different mechanism of action. One such mechanism involves an interaction between the Fas ligand (FasL) protein expressed on the surface of the Tc's and Fas expressed on the target cells, leading to the activation of caspases and subsequent apoptosis of the target cells. Tc's also release the cytokines IFN-γ, TNF-α and TNF-β, all of which contribute to host defence. IFN-γ directly inhibits viral replication, increases expression of MHC Class I in infected cells and activates macrophages recruiting them to the site of infection as effector cells or APCs. TNF-α and TNF-β, synergise with IFN-γ in recruiting macrophages, they are also directly

involved in target cell killing through interaction with TNFR-1.

The CD4+ T cell response

CD4+ T cells recognise peptides presented by cells expressing surface MHC Class II, these include B cells, DC's and macrophages. CD4+ T cells are divided into various subtypes, based on their cytokine expression and functions in vivo. The T_H1 cells also known as the inflammatory T cells, help activate macrophages to kill intracellular microorganisms such as mycobacteria. Macrophages require two signals for activation, the first of which is IFN-γ produced by the T_H1 cells upon the recognition of the peptide:MHC II complex on the surface of macrophages. The second signal sensitises the macrophages to respond to IFN-γ, this is provided by the interaction between the CD40 ligand (CD40L) expressed on the surface of the T_H1 cells and CD40 on the macrophage. Moreover, IFN-γ produced by the T_H1 cells can synergise with TNF-α produced by the activated macrophages, aiding the production of reactive nitrogen metabolite nitric oxide (NO), which has broad antimicrobial activity (Munoz-Fernandez et al., 1992). In addition to these functions, activated T_H1 cells produce pro-inflammatory cytokines, such as interleukin-2 (IL-2) inducing T cell proliferation and interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF), inducing macrophage differentiation in the bone marrow. More recent studies have focussed on the cytotoxic potential of these cells with effectors found that can kill in a perforin dependent manner (Haigh et al., 2008). As well as exerting effector functions through Fas-dependent mechanisms as FasL is expressed on the surfaces of T_H1 and some T_H2 cells (Green et al., 2003).

The T_H2 cells, also known as the helper T cells are essential for B cell activation and

proliferation, they also facilitate the activation of mast cells. Upon the recognition of their specific ligand on the surface of a B-cell, T_H2 cells produce interleukin-4 (IL-4). The interaction between IL-4 and CD40L synergistically acts to drive the clonal expansion of B-cells (Valle et al., 1989), IL-4 is also a regulator of mast cell and eosinophil function. Following several rounds of proliferation of the clonal B cells, T_H2 cells secrete interleukin-5 (IL-5) and interleukin-6 (IL-6), which contribute to the subsequent B-cell differentiation into antibody-secreting plasma cells, particularly immunoglobulin E (IgE) antibody expressing plasma cells (Del Prete, 1992). An additional cytokine released by T_H2 cells is interleukin-10 (IL-10), which upregulates surface MHC Class II on macrophages and inhibits the T_H1 response (Mosmann et al., 1991).

The fate of a CD4+ T-cell is not predetermined: it is unknown whether it will differentiate into a T_H1 or T_H2 lineage. The decision of the progeny that the naïve CD4+ T cell will take, is made during the clonal expansion following the initial encounter with antigen. However, the factors that determine this differentiation remain unclear. It is generally thought to be a combination of the co-stimulators used to drive the response, the nature of peptide:MHC ligand and the cytokine milieu elicited by the response, primarily IFN-γ, interlukin-12 (IL-12) and IL-4. These cytokines can have contrasting effects, the development of T_H1 cells is enhanced by IL-12 but inhibited by IL-4, while the differentiation into T_H2 cells is stimulated by IL-4 and inhibited by IL-12 and their proliferation inhibited by IFN-γ (reviewed in (Mosmann et al., 1996)).

In contrast to the helper CD4+ T cells an additional subset of CD4+ T cells, the

regulatory CD4+ T cells (Tregs), are distinguished from T helper cells based on their expression of CD4, CD25 (the α chain of the IL-2 receptor) and the forkhead/winged helix transcription factor gene FoxP3 (Fontenot et al., 2003). Tregs play an important role in controlling the magnitude and duration of T cell responses through the secretion of cytokines such as IL-10 and TGF-β, both of which can inhibit T cell proliferation. Tregs also have a role in maintaining tolerance as during positive selection in the thymus, some autoreactive T cells may not be deleted as the autoantigen is not available. Tregs on encounter of an autoantigen will release inhibitory cytokines IL-10 and transforming growth factor (TGF-β), inhibiting all surrounding autoreactive T cells, irrespective of their precise autoantigen specificity. This process is known as regulatory tolerance (reviewed in (Sakaguchi, 2000)).

Other regulatory CD4+ T cell subsets have also been found, however unlike the CD4+ CD25+ Tregs, these are CD4+ CD25- cells and include the T_H3 and T_R1 cells found in the mucosal immune system. The T_H3 cells secrete IL-4, IL-10 and can be distinguished from the T_H2 cells by their production of TGF-β, while the T_R1 cells share a similar cytokine profile but can be distinguished from the T_H3 cells by their inability to secrete IL-4. The exact origin and role of these regulatory cells is unclear, however a lack of these cells has been linked to autoimmune disease in the gut, as IL-10 has been shown to have an important role in maintaining intestinal tolerance to normal enteric antigens and in the systemic tolerance to self antigens (reviewed in (Roncarolo et al., 2000)).

More recently an additional subset of helper T cells known as the T_H17 cells have been identified, which challenges the well-established T_H1/T_H2/Treg paradigm

(reviewed in (Peck et al., 2010)). The T_H17 cells are an IL-17 producing subset of CD4+ effector T cells and in contrast to the CD4+ CD25+ Treg cells, the T_H17 have been shown to have a crucial role in the production of autoimmune tissue injury (Park et al., 2005; Bettelli et al., 2006). There is evidence to suggest that the proinflammatory cytokine milieu that contributes to T_H17 cell differentiation and maintenance is characterisitic to that of the autoimmune process, such as the cytokines: TGF-β, IL-6, IL-1β, IL-21 and IL-23 (Veldhoen et al., 2006; Serada et al., 2008; Sutton et al., 2009; Korn et al., 2007). It was initially thought that the cytokine IL-23 was responsible for the differentiation of T_H17 cells, however it was later established that their differentiation is induced by IL-6 and TGF-β (Bettelli et al., 2006) and in the absence of IL-6, by IL-21 and TGF-β (Korn et al., 2007). The cytokine TGF-β has been shown to be essential in driving the differentiation of both the Treg and T_H17 cell lineages. More recently TGF-β combined with IL-4 has been shown to induce the differentiation of an additional T helper cell subset which predominantly produce the cytokine IL-9 and have subsequently been named the T_H9 cells. An exact role for these T_H9 cells is unclear, however they have been proposed to have a role in regulating chronic allergic inflammation (reviewed in (Jager et al., 2010)).

1.2 Antigen processing and presentation

Antigens can be derived from many sources including pathogens but also self proteins. However, as T cells can only recognise antigens presented on the surface of APCs in the context of MHC molecules, these peptides need to be processed and subsequently presented in these molecules. Processing of the peptides occurs using one of two pathways, the MHC class I processing pathway or the MHC class II

processing pathway. The MHC molecules are host glycoproteins encoded by a large cluster of genes, in humans these are referred to as human leucocyte antigens (HLA), which are very polymorphic in the human population. These are located on the loci of chromosome six, the molecules encoded on these loci are respectively termed MHC class I molecules HLA-A, -B, -C, -D, -E and –G alleles and the MHC class II molecules HLA-DR, -DQ, and –DP. Each of the HLA molecules can bind to a range of peptides derived from antigens and present them for T cell recognition.

1.2.1 MHC class I processing and presentation

MHC class I is expressed on the surface of all nucleated cells and conventionally presents peptides derived from endogenously expressed antigens. These are degraded in the cytosol by a multi-subunit protease known as the proteasome into small peptides. These peptides are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), where they are loaded onto MHC class I molecules with the help of chaperone proteins. Upon the formation of the peptide;MHC class I complex, they are presented on the surface of the cell for recognition by CD8+ T cells (Bouvier, 2003). The processes involved in MHC class I processing and presentation are illustrated in figure 1.1 and discussed further in this chapter.

Structure of MHC class I molecules

The distinct structure of the MHC class I molecule allows it to serve its function in antigen presentation, binding peptides and activating different subsets of T cells. The MHC class I molecule is a heterodimer of two polypeptide chains, the α chain bound non-covalently to the smaller highly conserved β2 miroglobulin (β2m). The α chain

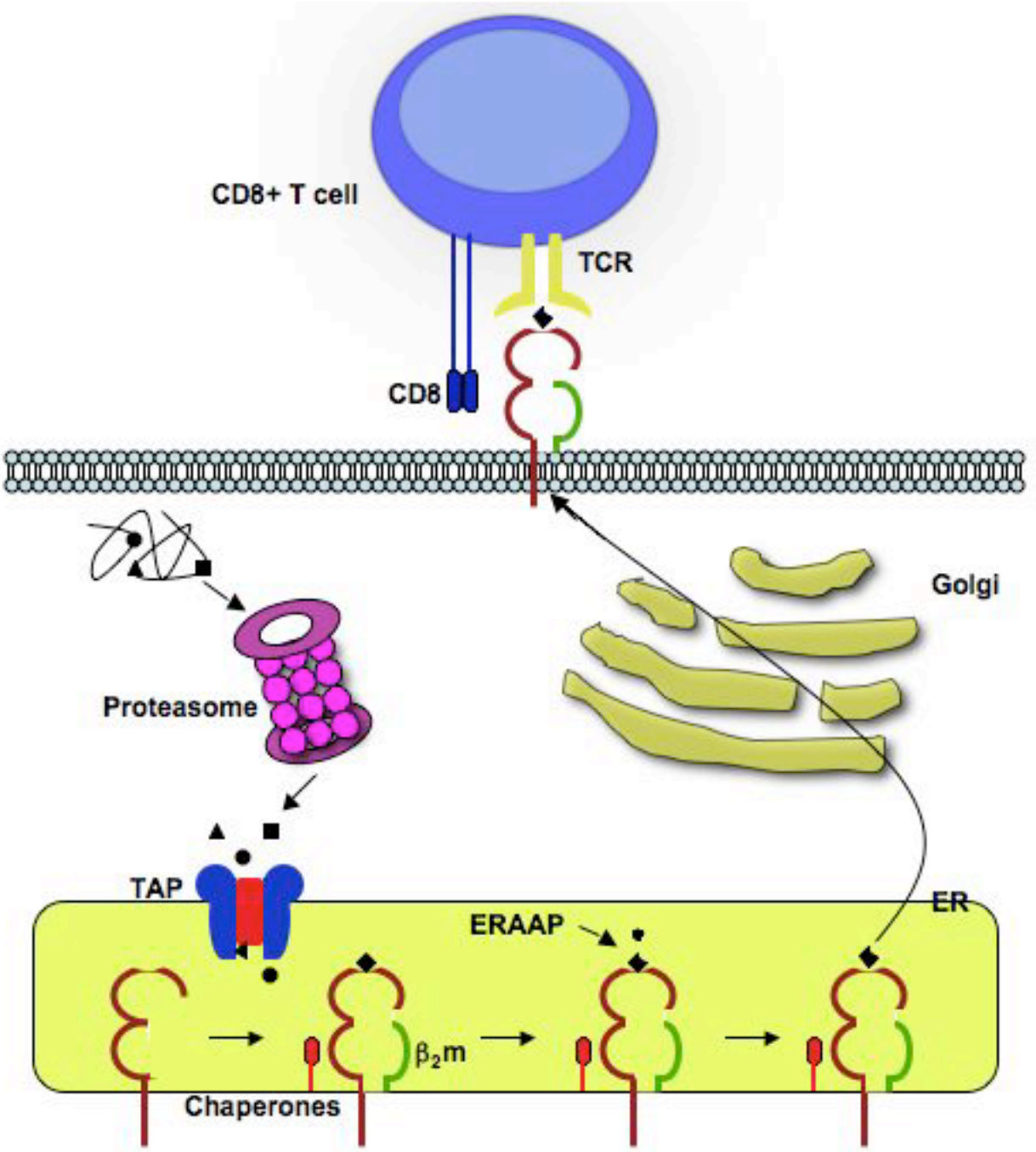


Figure 1.1 MHC class I antigen presentation: the classical pathway. Endogenous proteins are degraded into peptide fragments within the cytosol by the proteasome. Peptides are translocated into the ER by TAP and loaded onto nascent MHC class I molecules with the assistance of the chaperone molecules of the PLC. The aminopeptidase ERAAP trims the N-terminus of the peptide before or after initial MHC class I loading. The peptide:MHC class I complexes are transported from the ER through the Golgi to the cell surface for CD8+ T cell recognition.

spans the membrane and folds into three domains: α_1, α_2 and α_3. The α_1 and α_2 domains fold together to form the peptide-binding groove of the molecule. While the α_3 domain and β2m have a folded structure similar to that of an immunoglobulin domain. The most polymorphic part of the MHC class I molecule is the peptide-binding groove, which confers the unique peptide and TCR binding properties of each MHC molecule (reviewed in (Madden, 1995)).

There are six pockets in the peptide-binding groove, of variable shape and composition, which have preferences for certain amino acids, binding the specific amino acid side chains of antigenic peptides. The specific amino acids from each peptide responsible for the stable binding are referred to as the anchor residues. As a result of these unique binding pockets, MHC class I molecules show preferences for peptides with certain amino acids in anchor positions and different MHC class I molecules will then bind and present different peptides. As expression of the HLA genes are codominant, each allele is expressed binding and presenting different arrays of peptides (Falk et al., 1991). As the amino and carboxy termini of the peptides are enclosed within the peptide-binding groove, this restricts the length of the peptide that can bind, therefore peptides that bind to the MHC class I molecule are usually 8-10 amino acids long (Bouvier et al., 1994).

Peptide generation

The peptides that bind to MHC class I for CD8+ T cell recognition are derived from endogenous proteins, most commonly from intracellular sources including viruses that have overtaken the cellular machinery. Proteins in the cells are continuously being degraded and replaced with newly synthesised proteins, as part of the normal protein

turnover within the cell. The majority of endogenous proteins obtain or acquire signals for degradation through the attachment of poly-ubiquitin chains (Ub) to free amino groups on the target proteins. This signals that the proteins are to be degraded using the multi catalytic protease complex, the proteasome. All eukaryotes possess 20S proteasomes, these are complex barrel-like structures that contain a catalytic core and can digest proteins into oligopeptides. Poly-ubiquitinated proteins are recognised by the 19S regulatory subunit of the proteasome, which deubiquitylates, unfolds and threads substrates into the 20S proteasome, producing peptides ranging from 4-30 amino acids in length (reviewed in (Yewdell, 2007)).

The proteasome can exist in two forms: the constitutively active proteasome found in all cells and the immunoproteasome found in cells stimulated with interferons. In the immunoproteasome three of the constitutively expressed 20S proteasome subunits are replaced with the interferon-inducible counterparts bli (LMP2), b5i (LMP7) and b2i (MECL-1). This results in changes to the specificity of the proteasome, altering the cleavage of polypeptides resulting in the production of peptides with carboxy-terminal residues which are preferred for binding to MHC Class I molecules and transport by TAP. An additional change elicited by the release of IFN-γ is the expression of the PA28 proteasome-activator molecule, this binds to either or both ends of the proteasomal cylinder, opening up the cylinder and subsequently increasing the rate of efflux of peptides (Toes et al., 2001). Therefore, degradation of proteins by the immunoproteasome results in an increased generation of peptides with different peptide sequences.

The half-life of endogenous proteins can vary from minutes to up two weeks, with the

average half-life being approximately 1-2 days. However, virally infected cells can be recognised by T cells within an hour of virus infection (Yewdell et al., 2007). As such it has been suggested that viral epitope-peptides are preferentially derived from a distinct subset of translated products that are short-lived proteins which are rapidly degraded after synthesis rather than from long-term stable proteins (Qian et al., 2006). These short lived polypeptides have been termed defective ribosomal products (DRiPs). These are mainly derived from viral (or cellular) proteins that have been unable to achieve a stable conformation, as a result of errors during their synthesis or folding (Yewdell et al., 1996).

Formation of the MHC class I-peptide complex

Following proteasomal degradation, the peptides generated are translocated by the TAP proteins from the cytosol and into the endoplasmic reticulum (ER) for loading onto the nascent MHC class I molecules. TAP mediated transport into the ER is a multistep process, requiring both MHC-encoded TAP proteins TAP1 and TAP2. These form a heterodimeric complex that spans the ER membrane and hydrolysis of ATP by TAP leads to the opening and closing of the pore, allowing peptides to be translocated from the cytosol and into the ER lumen (Neefjes et al., 1993). It has also been shown that the TAP complex shows some specificity for the peptide it will transport, preferring peptides between 8 and 16 amino acids in length, with 9-12 amino acid residues suited best, possessing hydrophobic or basic residues at the carboxy-terminus (reviewed in (Uebel et al., 1999)). The majority of MHC class I peptides are supplied by TAP translocation, however in some circumstances peptides have been successfully presented by MHC class I molecules in the absence of the TAP complex (Wei et al., 1992). This has been highlighted in a number of studies

using TAP-deficient cell lines leading to the identification of TAP-independent epitopes within the γ1 human herpesvirus, Epstein Barr virus (EBV) (Khanna et al., 1996; Lautscham et al., 2001; Bell et al., 2009).

Once in the ER, although the appropriate C-termini residues on the peptides were generated in the cytosol, the peptides that are too long to bind to MHC class I molecules are trimmed by an IFN-γ inducible ER aminopeptidase associated with antigen processing (ERAAP) at the N-termini (reviewed in (Wearsch et al., 2008)). The importance of ERAAP in antigen processing is highlighted by a study carried out on ERAAP-knockout mice, in which many unstable and 'unedited' peptides were bound to MHC class I molecules that were capable of eliciting CD8+ T cell responses (Hammer et al., 2007). The mechanism of action of ERAAP is still a matter of debate, as it is unclear whether it acts pre- or post- binding to the MHC class I molecule (reviewed in (Wearsch et al., 2008)).

Peptides in the ER bind to nascent β_2m-associated MHC class I molecules, which have been assembled with the assistance of the ER-resident accessory proteins calnexin, calreticulin, tapasin and Erp57. Newly synthesised MHC class I α chains that enter the ER membranes bind to the chaperone protein calnexin, which has a central role in the folding of many immunological proteins. Upon the binding of the α chain to β_2m, the heterodimer dissociates from calnexin and binds to calreticulin, a component of the peptide loading complex (PLC), with a similar chaperone function (Radcliffe et al., 2002). The remaining components of this complex, the TAP-associated protein tapasin and Erp57, a thiol oxidoreductase, form a bridge between MHC class I molecule and TAP, enhancing the loading of antigenic peptides onto the

α:β_2m heterodimer (reviewed in (Williams et al., 2002)). The binding of peptides increases the stability of the MHC class-I complex, with the affinity between the heavy chain and β_2m being increased ten-fold, as well as the affinity of heavy chains for peptides increasing dramatically in the presence of β_2m (Elliott, 1991). Following peptide-binding, the peptide:MHC complex is released from the PLC and leaves the ER through the golgi apparatus and to the plasma membrane, for surveillance by their specific CD8+ T cells.

Alternative routes of MHC class I processing: the exogenous pathway

It is generally accepted that antigens presented on the surface of MHC class I molecules are derived from endogenous antigens, usually synthesised within the cell itself that is presenting the antigen. However, a study conducted by Bevan dating back to 1976 identified an alternative route for deriving MHC class I antigens from an exogenous source, this has been referred to as 'cross-presentation' and the subsequent activation of CD8+ T cells is referred to as 'cross-priming' (Bevan, 1976). This phenomenon provides an explanation for the ability of uninfected APCs to initiate a CD8+ T cell response against the infectious agent.

Since the first evidence presented by Bevan's study, Albert et al. have provided further evidence that the APCs that are mainly responsible for cross-presentation of antigen from the virus-infected apoptotic cells are the DCs (Albert et al., 1998). Subsequent studies have been carried out demonstrating this phenomenon, viral proteins were expressed by transformed, transfected cells or within transgenic mice and their cross-presentation was shown to rely on DCs for the presentation of several viral antigens derived from a number of viruses such as influenza virus (Sigal et al.,

2000), vaccinia virus and poliovirus (Sigal et al., 1999).

Cross-presentation is thought to occur by three potential mechanisms. The first of which is that antigens could be degraded by endosomal proteases and loaded onto MHC Class I molecules in the endosomes, this has been referred as proteasome- and TAP-independent crosspresentation (Gromme et al., 1999; Reid et al., 1990). The second mechanism involves the transport of endocytosed antigen from the endosome and into the cytosol, where it is degraded by the cytoplasmic proteases and subsequently transported by TAP into the ER (Ackerman et al., 2004). In the third proposed mechanism, antigens are degraded in the cytosol and reimported into the endosome by TAP molecules present in the endosomal membrane, where MHC I loading will take place (Albert et al., 1998).

Furthermore, autophagy has been implicated in the sequestration and delivery of antigens for cross presentation. Autophagy is a cellular process that degrades long-lived, misfolded or damaged proteins and larger cytoplasmic components, it is induced under conditions of stress in the cell, such as starvation. A study by Li et al. has shown the importance of autophagy in the presentation of tumour antigens or the model antigen OVA, inhibition of autophagy by 3-methyladenine (3-MA) almost completely abolished cross-presentation. While autophagy induction by cell starvation or treatment with rapamycin greatly enhanced cross-presentation (Li et al., 2008), an additional study by Uhl et al. highlighted the importance of autophagy in the cross-priming of viral and tumour-specific CD8+ T cells; the inhibition of autophagy-related gene Atg5 expression in virus-infected fibroblasts resulted in the inhibition of cross-presentation of the antigen-specific CD8+ T cells (Uhl et al., 2009).

1.2.2 MHC class II processing and presentation

MHC class II is only expressed on the surface of particular cells, mainly the APCs, these include B cells, macrophages and DCs. However, class II expression can be induced on different cell types such as fibroblasts and endothelial cells by IFN-γ treatment. Peptides that are presented on the surface of cells expressing MHC class II molecules are usually derived from exogenously acquired antigens. These antigens are degraded in the endolysosomal compartment of the cell by proteases under acidic conditions and loaded onto nascent MHC class II molecules; thereafter the peptide:MHC class II complex are transported onto the surface of the cell for recognition by CD4+ T cells. The processes involved in MHC class II processing and presentation are illustrated in figure 1.3 and discussed further in this chapter.

Structure of MHC class II molecules

The MHC class II molecule is composed of two transmembrane glycoprotein chains, α and β, both of which are MHC encoded. Each chain is composed of two extracellular domains (α1 and β1) and two membrane proximal domains (α2 and β2). Figure 1.4 illustrates the structure of the MHC class II molecule. The extracellular domains α1 and β1 fold to form the peptide-binding groove in a very similar structure to the MHC class I molecule (Brown et al., 1988). In contrast to MHC class I molecules, the ends of the peptide-binding grooves in MHC class II molecules are more open and peptides that bind can protrude from the groove. This characteristic allows MHC class II molecules to bind peptides of variable length and sequence; MHC class II molecules predominantly bind peptides between 13-17 residues (Rudensky et al., 1991), with peptides as long as 30 residues being identified (Engelhard, 1994).

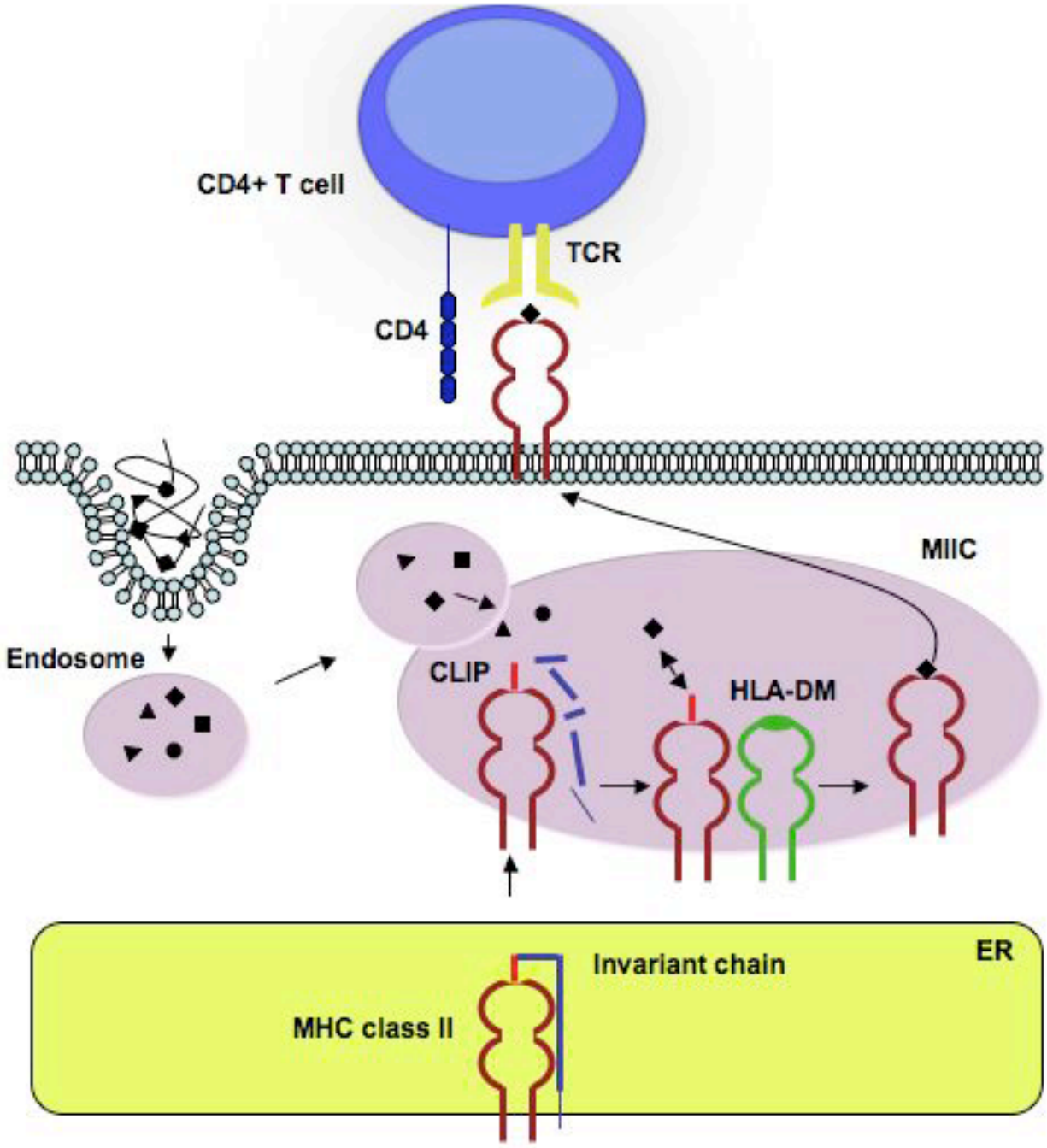


Figure 1.3 MHC class II antigens presentation: the classical pathway. Exogenous proteins are endocytosed by the plasma membrane and degraded in the endocytic pathway into peptides. MHC class II molecules are assembled in the ER with the chaperone invariant chain and transported into the MIIC. In the MIIC, the invariant chain is degraded leaving CLIP fragment in the peptide-binding groove, with the aid of the chaperone molecule HLA-DM, CLIP is exchanged for the antigenic peptide. The assembled peptide:MHC class II complexes are transported to the cell surface for CD4+ T cell recognition.

Peptide generation

Peptides presented on the surface of MHC class II molecules are usually derived from exogenous proteins. Soluble antigens are internalised by APCs through either clathrin-dependent receptor-mediated endocytosis or clathrin-independent macropinocytosis and perhaps alternatively from particulate antigens that have been engulfed through receptor-mediated phagocytosis (Robinson et al., 2002). Following uptake into the cell, proteins become enclosed in endocytic vesicles and enter the endocytic pathway, which is composed of three main components: the early endosomes, the late endosomes and the lysosomes. The endosomes become increasingly more acidic as they progress into the interior of the cell, eventually fusing with lysosomes to form the endolysosomal compartment. This acidic environment is essential for the unfolding and degradation of the proteins; GILT (γ-IFN-inducible lysosomal thiol reductase) is the enzyme responsible for the reduction of the intra- and inter-molecular disulphide bonds, a process which is necessary for the complete denaturation and unfolding of the protein (Lennon-Dumenil et al., 2002). Following this denaturation, the cysteine, serine and aspartic proteases cathepsins B, D, S and L, as well as enzymes related to the cysteine protease papain, such as cathepsins H, L, S, F, Z and AEP (asparingyl endopeptidase) are expressed in APCs for the digestion of proteins. The majority of these proteases are optimal at an acidic pH and have broad substrate specifity, with the exception of AEP, which cleaves only after asparagines residues (Watts, 2001). Cathepsins S and L have been implicated as the predominant proteases involved in the processing of vesicular antigens (Pluger et al., 2002; Hsieh et al., 2002).

Formation of the MHC class II-peptide complex

The biosynthetic pathway of MHC class II molecules begins with translocation into the ER whereby it associates with a chaperone protein known as the MHC class II-associated invariant chain (Ii), to form the nanomeric $(\alpha\beta I)_3$ complexes (Marks et al., 1990). The Ii serves a number of distinct and crucial roles in the transport and assembly of the MHC class II:peptide complex. Its initial role is to assist in the correct folding of the nascent MHC class II molecules, preventing the aggregation of unfolded MHC class II molecules in the ER (Bonnerot et al., 1994). Its second role is to stabilise the MHC class II molecule, and prevent it from prematurely binding any peptides in the ER. This function is primarily achieved through the blocking of the peptide-binding groove with a short fragment of the Ii referred to as the class II-associated invariant chain peptide (CLIP) (Hiltbold et al., 2002). Thirdly, a signal motif in the cytoplasmic tail of the Ii targets the delivery of the MHC class II molecules to the endosomal compartment (Anderson et al., 1992). Once the MHC class II-Ii complex enters the endocytic pathway, it is retained for 2-4hrs, during which the Ii is cleaved by proteases such as cathepsin S leaving only the CLIP fragment bound to the MHC class II molecule.

The loading of MHC class II molecules with antigenic peptides occurs in an endosomal compartment late in the endosomal pathway, referred to as the MHC class II compartment (MIIC). Within the MIIC, a specialised MHC class II-like chaperone molecule HLA-DM, catalyses the removal of CLIP and the subsequent loading of the MHC class II molecules with antigenic peptides (Sherman et al., 1995). HLA-DM also catalyses the release of unstably bound or low-affinity peptides, a process often referred to as 'peptide editing', increasing the likelihood that the MHC class

II:peptide complexes formed are maintained long enough on the surface of the APC for recognition by CD4+ T cells (Pu et al., 2004). The importance of HLA-DM in MHC class II presentation, has been highlighted in a number of studies that have shown that mutant B-cell lines which have a defect in HLA-DM, often fail to bind peptides derived from the internalised proteins and arrive at the cell surface with CLIP peptide still bound to the surface of the MHC class II molecule (Weenink et al., 1997; Riberdy et al., 1992).

HLA-DM activity can be regulated by a second atypical MHC class II molecule HLA-DO, which is predominantly expressed in the intracellular vesicles of B cells and thymic epithelial cells. HLA-DO is a negative regulator of HLA-DM, it elicits its effects by binding to and inhibiting HLA-DM from catalysing the removal of CLIP from MHC class II molecules, preventing the binding of antigenic peptides (Jensen, 1998). HLA-DO also acts by restricting the pH range at which HLA-DM is usually functional (Liljedahl et al., 1998). However, during an inflammatory response the production of IFN-γ increases the expression of HLA-DM, subsequently overcoming the inhibitory effects of HLA-DO (Albanesi et al., 1998). Upon the exchange of CLIP-peptide for a target peptide-epitope on the surface of the MHC class II molecules, the complex is transported from the MIIC to the cell surface by mechanisms that are still undefined, for recognition by CD4+ T cells.

Alternative routes of MHC class II processing: the endogenous pathway

Antigens presented by the MHC class II pathway are usually derived from exogenous sources of protein acquired by APCs, however it is now well established that endogenous cytosolic or nuclear derived antigens can be presented by MHC class II

molecules. The analysis of natural ligands eluted from MHC class II molecules has revealed that a large number of MHC Class II peptides are derived from endogenous proteins that intersect the class II pathway (Chicz et al., 1993; Rammensee et al., 1999). A number of theories have been proposed for why endogenous proteins are able to access the class II pathway. Firstly, the MHC class II molecules may directly form complexes with unfolded proteins in the ER and this complex is subsequently transported to the endosome, where it is cleaved by proteolysis and presented on the cell surface for CD4+ T cell recognition. In this theory the binding of premature protein may precede the association of the MHC class II molecule with the li chain. This pathway has been shown to favour transmembrane or secreted proteins, such as the influenza-derived haemagglutinin (HA) antigen (Aichinger et al., 1997). The second theory proposes a TAP-dependent mechanism for the import of cytosolic proteins into the ER and onto MHC class II molecules, this has been referred to as MHC class II molecules 'hijacking' the MHC class I pathway (Lechler et al., 1996). A study by Malnati et al. has shown that influenza-HA derived peptides are processed in a TAP-dependent manner for CD4+ T cell recognition, the peptides that are presented by MHC class II molecules using this pathway are usually short cytosolic peptides. However in the same study, a TAP-independent mechanism for the transport of the HA-derived antigens was also identified (Malnati et al., 1992). This finding has been supported by a number of other studies suggesting that TAP plays no role in the presentation of endogenous antigens by the MHC class II pathway, this can occur in a proteasome-dependent (Lich et al., 2000) or proteasome-independent manner (Dissanayake et al., 2005).

Autophagy has also been reported to play an important role in the presentation of

endogenous antigens using the MHC class II pathway. APCs are constitutively undergoing autophagy, of which chaperone-mediated autophagy (CMA) (Zhou et al., 2005) and macroautophagy (Paludan et al., 2005) have been implicated in the presentation of peptides for CD4+ T cell recognition. In CMA, proteins carrying signal peptides for sorting into lysosomes, are specifically transported into lysosomes by the concerted action of the lysosome-associated membrane protein (LAMP-2a) and lysosomal heat shock cognate protein 70 (HSC70). In contrast to other autophagic pathways, CMA does not require vesicle formation or major changes in the lysosomal membrane. CMA has been implicated in the enhanced presentation of the autoantigens GAD and SMA, with over-expression of LAMP2a increasing the presentation of these peptides (Zhou et al., 2005). CMA has found to be upregulated during prolonged metabolic stress, while macroautophagy is the early stress response pathway (Kaushik et al., 2008).

Macroautophagy is the major route of degradation of cytoplasmic constituents. During macroautophagy a double membrane vacoule forms around a region of cytoplasm termed the autophagosome, which goes on to fuse with lysosomes and late endosomes for degradation of the autophagosomal contents (reviewed in (Lunemann et al., 2009)). The first study that implicated macroautophagy in antigen presentation was conducted by Brazil et al. on the presentation of a model endogenously expressed protein, the C5 complement protein, by the class II processing pathway. The use of the autophagy inhibitor 3-methyladenine (3-MA), abrogated the presentation of endogenous C5, indicating the importance of lysosomal degradation in the generation of peptides for MHC class II presentation (Brazil et al., 1997). More recent studies have emphasised a role for macroautophagy in the presentation of endogenous

peptides, demonstrated by their sensitivity to autophagy inhibitors such as 3-MA and another PI3-kinase inhibitor wortmannin in the processing of the cytosolic protein neomycin phosphotransferase II (NeoR) (Nimmerjahn et al., 2003). In addition to these inhibitors, downregulation of genes expressed in the autophagy process, such as Atg12 by siRNA mediated silencing, reduced the MHC class II presentation of epitopes from EBV's genome maintenance protein EBNA1 (Paludan et al., 2005). Furthermore, it has been shown that MHC class II presentation to CD4+ T cells can be enhanced, by the direct targeting of antigens into the autophagy pathway. Schmid et al. demonstrated that the fusion of the influenza MP1 to the autophagosome marker Atg8/LC3, strongly increased MHC class II presentation to MP1-specific CD4+ T cells (Schmid et al., 2007).

1.3 Kaposi's sarcoma-associated herpesvirus (KSHV)

The herpesvirus family is a group of large and complex viruses with double-stranded (ds) linear DNA genomes. Historically these were divided into three main subfamilies α, β and γ grouped according to their biological properties; predominantly their cellular tropism. Kaposi's sarcoma-associated herpes virus (KSHV) is a lymphotrophic γ-herpes virus, also known as human herpes virus 8 (HHV8), belonging to the γ2 or rhadinovirus genus. KSHV was first discovered in 1994 by Chang et al. using representational difference analysis (a PCR-based technique) on restricted cellular DNA from a Kaposi's sarcoma tissue biopsy which had herpesvirus sequence homology (Chang et al., 1994). Soon after, the entire double stranded linear DNA viral genome was cloned apart from a 3-kb sequence near the right end of the genome, revealing sequence homology with EBV and herpes virus saimiri (HVS) (Russo et al., 1996). KSHV is the second human herpes virus identified, following

EBV, which shows oncogenic potential. It is linked to the establishment of the malignancies Kaposi's sarcoma (KS) and the B cell malignancies, primary effusion lymphoma (PEL) and Multicentric castleman's disease (MCD) (reviewed in (Bouvard et al., 2009)).

1.3.1 KSHV infection

Unlike other human herpes viruses, KSHV infection is not ubiquitous amongst the general healthy population. Seroprevalence is highest in sub-Saharan Africa at more than 50%, while in the Mediterranean region has intermediate seroprevalence at 10-30%, mostly among elderly men. While the seroprevalence in northern Europe, Asia and the United States is lowest at less than 10% (reviewed in (Uldrick et. al, 2011)). Routes of transmission for KSHV are unclear, however there is evidence for oral and sexual route of transmission as KSHV incidence is very high among homosexual men with HIV. However, among heterosexuals a main mode of transmission is yet to be identified, there is evidence for both sexual and non-sexual routes of transmission. In western countries, the incidence of KSHV infection is higher in women co-infected with HIV and commercial sex work suggesting a sexual route of transmission (Bestetti et al., 1998) (reviewed in (Schulz, 2000)). While in African countries with endemic KS such as the Gambia, Uganda and Cameroon, KSHV incidence is found in a large number of pre-pubescent children, suggesting a nonsexual horizontal mode of transmission (reviewed in (Geraminejad et al., 2002)). Furthermore, over the past three decades there has been an increased incidence of KSHV infection in organ transplant recipients receiving immunosuppressive therapy; in countries where classic KS can occur such as Saudi Arabia, KS is the most common post transplant tumour in renal transplant recipients (Qunibi et al., 1988).

KSHV mediated pathogenesis is enhanced in the presence of a number of growth factors and inflammatory cytokines. These are responsible for endothelial cell recruitment, activation of lymphocytes, and the reactivation of KSHV. Some of the growth factors that have an essential role in promoting KSHV infection include: IFN-γ, Tissue necrosis factor (TNF), IL-1, IL-6, basic fibroblast growth factor (BFGF), and oncostatin-M (OSM) (reviewed in (Bryan et al., 2005)). The growth factor that plays a significant role in KSHV pathogenesis is vascular endothelial growth factor (VEGF); it has been implicated in a number of processes such as differentiation, migration, cell survival and activation of oncogenesis. VEGF has also been reported to be upregulated along with its receptor (VEGFR) in tumour angiogenesis and proliferation (Ferrara, 2004; Masood et al., 2002) (reviewed in (Uldrick et al., 2011)).

In vivo, KSHV DNA and transcripts have been detected in B cells, endothelial cells, monocytes, epithelial cells and keratinocytes, suggesting KSHV has a wide cellular tropism. KSHV undergoes both lytic and latent replication but primarily establishes a latent state of expression in two cell types, endothelial cells and B cells. To date, it has been difficult to establish a KSHV infected endothelial cell line from KS lesions as KS cells grow poorly in culture, or the cells rapidly lose the viral genome (Lebbe et al., 1997; Ganem, 2007). However, human B cells and monocytes appear to be reservoirs of latent infection (Ambroziak et al., 1995). Furthermore, cells with B-cell characteristics have been established from the PEL biopsies, such as the tumour line BC-1 (Cesarman et al., 1995). The majority of these lines are co-infected with EBV and similar to EBV transformed B cells (LCLs), only 1-3% of the PELs will spontaneously reactivate and enter lytic cycle (reviewed in (Chandran, 2010)).

In vitro KSHV infects endothelial cells (Blackbourn et al., 2000), epithelial cells, fibroblasts (Matthews et al., 2011), and more recently primary B cells (Rappocciolo et al., 2008), tonsillar B cells (Myoung et al., 2011) and EBV-negative Burkitt lymphoma cell lines (Myoung et al., 2011) resulting in a mainly latent infection. However, primary non-stimulated B cells show a low percentage of infection with KSHV and unlike EBV infection of B cells, KSHV infection does not result in the immortalisation or transformation of B cells into LCLs (Myoung et al., 2011). However, lytic replication has been reported in activated and tonsillar B cells (Rappocciolo et al., 2008; Myoung et al., 2011). In other cases however, *in vitro* KSHV infection of adherent target cells such as human dermal microvascular endothelial cells (HMVEC-d), human umbilicial vein endothelial cells (HUVEC), human foreskin fibroblasts (HFF) and human embryonic kidney epithelial cells (HEK 293 cells) results mainly in the expression of the latent genes with very little lytic replication occurring within the cells. Therefore studying KSHV lytic replication in these cells requires induction of the virus into lytic cycle using chemicals such as phorbol ester or by expression of KSHV lytic switch gene, the lytic cycle open reading frame 50 (RTA) (Krishnan et al., 2004; Lagunoff et al., 2002).

The KSHV genome encodes 87 ORFs and, similar to EBV during lytic replication, viral genes are expressed in a temporally regulated cascade: the immediate early (IE), early (E) and late lytic (LL), which encompass lytic cycle gene expression and latent (L) gene expression. The most important immediate early gene is ORF50 which encodes the lytic switch protein RTA and is the central event in the induction of lytic cycle gene expression through its action as a transcriptional activator (Lukac et al., 1999). Deletion of RTA from the viral genome has been shown to inactivate

spontaneous or chemically triggered induction of lytic replication (Xu et al., 2005). The early lytic genes encode for a large class of viral proteins that prepare the cell for DNA replication or viral gene expression. The regulators of viral gene expression in this class of proteins include the post transcriptional regulator MTA, encoded by ORF57 (mRNA transcript accumulation) (Sandri-Goldin, 2001) and replication associated protein (RAP) or K-bZIP encoded by ORF-K8 (Polson et al., 2001). Whereas, the late lytic genes encode predominantly for viral structural proteins, these include envelope and capsid proteins such as ORF65, which has shown to be essential for capsid assembly (Perkins et al., 2008).

1.3.2 KSHV latency

In contrast to gene expression profiles seen during lytic cycle replication, KSHV which has entered a latent form of replication shows a highly restricted pattern of gene expression. In these forms of infection, the viral genome is maintained in a circularised episomal state and viral gene expression does not result in virion production. However in these forms of infection, the viral genome and cell viability is maintained, preserving the potential for virion production upon reactivation (reviewed in (Dourmishev et al., 2003)). The major latency viral transcripts expressed in all KSHV-infected cells and malignancies encode the genome maintenance protein latency-associated nuclear antigen (LANA), the viral cyclin (vCyclin), the anti-apoptototic multifunctional protein viral FLICE-like inhibitory protein (vFLIP) and Kaposin (reviewed in (Tempera et al., 2010)). In the case of the PELs and infected cells in MCD a different form of latency is seen with at least two additional proteins, the viral IL-6 and the immunomodulatory and anti-apoptotic protein vIRF3 being

expressed. More recently ORF-K1 has also been described as a latency protein which mimics signaling through the B-cell receptor (Chandriani et al., 2010).

LANA, encoded by ORF73, is a large multifunctional protein that is localised to the nucleus of latently infected cells where it is responsible for maintaining the KSHV viral genome. It has three major domains: An N-terminal region that has been associated in chromatin attachment and co-repressor recruitment, a central region that is highly acidic, and finally a C-terminal region that is involved in DNA binding and oligomerisation (Renne et al., 2001). LANA has also been associated with the dysregulation of many pathways that play a role in cell growth and survival promoting tumourigenesis. Friborg et. al. showed that LANA interacts with tumour suppressor p53, repressing its transcription and ability to induce apoptosis, implying a contribution of LANA to KSHV induced oncogenesis (Friborg et al., 1999). LANA also binds to the tumour suppressor Retinoblastoma (Rb) protein resulting in its inactivation, and the consequent upregulation of E2F dependent genes in LANA transfected cells (Radkov et al., 2000). Furthermore, LANA has been shown to modulate Wnt signalling. Wnt-1 stimulates proliferation of primary endothelial cells *in vitro* and LANA can interfere with this, thereby promoting KSHV induced growth deregulation (Osborne et al., 1999; Fujimuro et al., 2003).

A second KSHV latency protein that interferes with cell growth regulatory pathways is vCyclin, encoded by ORF72, the viral homolog of cellular cyclin D, which binds and activates cdk6. The vCyclin-cdk6 complex phosphorylates Rb overcoming Rb-mediated growth arrest induced by cdk-inhibitors, which in turn activates S phase genes allowing cell cycle progression and cell transformation that may contribute to

KS formation (Swanton et al., 1997). It has proven difficult to culture cell lines expressing vCyclin as they regularly undergo apoptosis, especially if the cdk6 levels are high, as the vCyclin-cdk6 complex phosphorylates the antiapoptotic factor bcl2 resulting in its inactivation (Ojala et al., 1999; Ojala et al., 2000). Furthermore, vCyclin expression in primary cells leads to cytokinesis defects and polyploidy, activating p53, however the loss of functional p53 allowed the cells to survive in the presence of high levels of vCyclin, highlighting the oncogenic potential of vCyclin (Verschuren et al., 2002).

The third latency protein transcribed from the major latency locus is vFLIP, encoded by ORF71/K13, the viral homologue to cellular FADD-like interleukin-1β converting enzyme inhibitor protein (FLIP). It has been consistently shown that KSHV vFLIP expression can upregulate the anti-apoptotic transcription factor NF-κB by binding to inhibitor κB kinase-γ (IKKγ) or NEMO, this complex activates IKK leading to the phosphorylation of IKB and release of active NF-κB (Liu et al., 2002). This activation of NF-κB also induces the release of various cytokines such as IL-8 in endothelial cells that have been implicated in KS pathogenesis (Sun et al., 2006). vFLIP is also responsible for the spindle cell morphology of endothelial cells elicited by KSHV infection (Grossmann et al., 2006). More recently vFLIP has been reported to have a role in the suppression of autophagy by preventing Atg3 the E2-like enzyme from binding and processing LC3 (Lee et al., 2009).

Kaposins are encoded from the alternatively spliced ORF K12, the most abundant transcript expressed in KSHV latently infected cells. The Kaposin proteins are expressed at low levels in uninduced PEL cells and in KS spindle cells, but are highly

upregulated during lytic replication (Sadler et al., 1999). Kaposin mRNA codes for at least 3 proteins: kaposin A, B, and C. Kaposin A is present on both the cell surface and intracellular membranes and has transforming ability in rodent fibroblasts (Tomkowicz et al., 2002; Muralidhar et al., 1998). Kaposin B increases the expression of cytokines by blocking the degradation of messenger RNAs (mRNAs), by binding to and activating the kinase MK2 which is a major target of the p38 pathway that is involved in regulating the stability of a specific class of cytoplasmic mRNA, preventing their turnover and enhancing their accumulation (McCormick et al., 2005). Kaposins A and B, as well as latent gene vFLIP, may contribute to the inflammatory microenvironment of KS.

Unlike the majority of KSHV-infected spindle cells seen in KS lesions, infected PEL and MCD cells additionally express vIL-6. This viral protein shows sequence and functional homology to human IL-6, an important B cell growth and differentiation factor (Parravicini et al., 1997). However, vIL-6 may have a broader tropism and is not subjected to the same regulation as human IL-6. vIL-6 is able to bind the gp130 co-receptor independently of the human IL-6 gp80 receptor, allowing signal transduction upon vIL-6 binding, in the absence of the specific IL-6 receptor. vIL-6 protects PELs from the antiviral effects of IFN-α, which down-regulates the surface expression of gp80 but not gp130 (Chatterjee et al., 2002; Osborne et al., 1999).

An additional latent protein that is expressed in PELs and MCD is vIRF3 or LANA2 encoded by ORF K10.5. vIRF3 is a multifunctional protein that has been shown to inhibit type I interferon signaling, is required for the survival of PELs (Wies et al., 2008), inhibits p53 function (Rivas et al., 2001) and more recently in PELs has been

shown to inhibit promoter function of the HLA class II transactivator CIITA, decreasing expression of surface MHC class II and IFN-γ production (Schmidt et al., 2011).

1.3.3 KSHV pathogenesis

KSHV has been identified as the causative agent of the endothelial pathology KS and B cell lymphoproliferative disorders PEL and MCD (Bouvard et al., 2009). KS has been identified as the most common KSHV-associated malignancy; it is the most frequently reported cancer in HIV infected individuals and one of the commonest cancers affecting men and children in sub-Saharan Africa (Masood et al., 2002). KS is a highly vascular tumour that originates from endothelial cells resulting in inappropriate endothelial cell proliferation and inflammatory lesions that are localised in the skin and visceral organs. There are 4 main types of KS: Classic KS, AIDS-related KS, African-endemic KS, and iatrogenic KS (reviewed in (Geraminejad et al., 2002)). Classic KS mainly affects elderly men of Mediterranean, eastern-European or Jewish descent. Lesions are mainly localised to the lower extremities, generally does not disseminate and is mostly an indolent disease. AIDS-related KS is however extremely aggressive with lesions spreading rapidly and viscerally, affecting major organs such as the lungs. The African-endemic form of KS most often affects young men with disseminated lymphadenopathy. Finally, iatrogenic KS develops in immunosuppressed patients following organ transplantation, the lesions are reduced upon the relaxation of immunosuppression (reviewed in (Bryan et al., 2005; Mesri et al., 2010)).

The second malignancy linked to KSHV infection is primary effusion lymphoma (PEL), a rare form of monoclonal B cell lymphoma thought to be of post germinal centre origin, that most often occurs in HIV-AIDS patients and has a highly aggressive course (Schulz, 2001). PEL mainly manifests in body cavities; it is very rare to see lymphodenopathy and solid tumours, however soft tissue masses and infiltration into body cavity tissues has been reported (Rivas et al., 2001). In vitro, PEL cell lines have been established and have been an essential tool to studying KSHV latency in a B cell background (Brander et al., 2000; Cesarman et al., 1995; Renne et al., 1996; Cannon et al., 2000).

A second B cell proliferative disorder associated with KSHV infection is Multicentric Castleman's disease (MCD), characterised by proliferation of B cells in a microenvironment of excess interleukin 6 (IL-6) (Parravicini et al., 1997). KSHV infection is found in almost 100% of cases of reported HIV positive related MCD, and 40-50% of cases that are HIV negative (Soulier et al., 1995). Clinical exacerbations of MCD correlate with KSHV replication and high viral loads in the blood (Parravicini et al., 1997). MCD when seen in individuals such as AIDS patients follows a relapsing remitting course, the risk of relapse appearing to correlate with virus load (Stebbing et al., 2011), sometimes requiring chemotherapy or B cell depleting treatments such as administration of Rituximab (Guihot et al., 2005; Bower, 2010).

High viral loads during MCD disease flares suggest that immune control of the virus is lost (Lallemand et al., 2000). This has also been suggested for individuals with iatrogenic KS and AIDS-related KS, as restoration of the immune system in these immunocompromised individuals with treatment of HAART or relaxation of

immunosuppression has shown regression of these malignancies (Bihl et al., 2007; Duman et al., 2002). These observations suggest an important role for the immune response in the control of the pathogenesis of this virus.

1.3.4 Immunity to KSHV

Similar to all other herpesviruses, KSHV infection persists in the host for life where it can enter either of two transcriptional programs: lytic and latent cycles. During latency, a passive strategy for immune evasion, a minimal number of gene products are expressed, decreasing the number of antigens available for immune surveillance. However, during lytic replication, the majority of viral proteins are expressed and these require more active immune evasion strategies to modulate both the innate and adaptive immune response to the virus. Indeed, KSHV has amassed a formidable repertoire of genes capable of moderating immune recognition and function, with a recent annotation of the viral genome ascribing potential immunomodulatory function to 22 genes of a total of 87 (Rezaee et al., 2006).

1.3.4.1 The innate immune response

The first line of defense against virus infection following the entry of the virus into the host is the production of interferons, IFN-α and IFN-β. These induce a number of autocrine and paracrine effects which limit viral replication, increase the expression of surface MHC class I, inhibit cell proliferation and increase apoptosis (Randall et al., 2008).

The interferon regulatory factor (IRF) family are transcription mediators of viral, bacterial and IFN-induced signaling pathways in humans which regulate the IFN-

responsive genes: IRF-1, 3 and 7 have been implicated in transactivation of the IFN-α and -β genes. KSHV encodes four genes which have limited homology to the cellular IRFs, called the vIRF genes. These genes are mainly expressed during the lytic cycle, however vIRF-1 and vIRF-3 have also been detected during latency (Rivas et al., 2001). It has been hypothesised that these genes have evolved to subvert the cellular IRF signaling pathways. vIRF-1, encoded by ORF K9, negatively regulates IFN signaling in the cell by suppressing the transcriptional activity of cellular IRF-1 and IRF-3 (reviewed in (Offermann, 2007)). Overexpression of vIRF1 in lymphatic endothelial cells has also been shown to downregulate surface MHC class I expression (Lagos et al., 2007). vIRF-2, translated from K11 and K11.5, inhibits both IRF-3 and transactivation of the IFN-α and IFN-λ driven signaling pathways (Fuld et al., 2006). vIRF-3, in addition to its other functions mentioned previously, interacts with IRF-7 inhibiting its DNA binding activity and subsequent IFN-α production (Joo et al., 2007). Finally, a role for vIRF-4 in regulating the IFN antiviral response is yet to be determined. A number of other proteins have been shown to have a role in regulating the IFN-mediated antiviral response: the virion-associated protein ORF45 which blocks the transactivation of IRF-7 and replication (Zhu, 2002; Honda et al., 2005), RTA negatively regulates IRF-7 by targeting it for proteasomal-mediated degradation, inhibiting the activation of the type I IFNs (Yu et al., 2005), the K8-encoded K-bZIP protein interacts with IRF-3 and prevents its binding to IFN-β promoter, preventing IFN-β transcription (Lefort et al., 2007).

An essential component of antiviral immunity is the TLR recognition of KSHV. A recent study has shown that KSHV infection of monocytes can upregulate the TLR3 pathway inducing the expression of TLR3-specific cytokines such as IFN-β1, CXCL-

10 and CCL2 (West et al., 2008). The infection of endothelial cells with KSHV results in the immediate suppression of TLR4 expression, it has been shown that levels of TLR4 expressed on cells *in vitro* inversely affects the susceptibility of cells to KSHV infection (Lagos et al., 2008). Furthermore, the role of TLR9 in gammaherpes immunity was highlighted in a mouse model of MHV-68, DCs derived from TLR9 -/- mice secreted lowers level of the cytokines IL-12, IL-6 and IFN-α compared to DCs from wildtype mice (Guggemoos, et al. 2008). KSHV also encodes three chemokine homologue proteins viral CCchemokines - vCCL-1, vCCL-2 and vCCL-3 during lytic replication, they have various immune regulatory functions, however collectively these cytokines play a role in polarizing the adaptive immune response towards a T_H2-like or humoral response, most likely reducing the efficiency of the antiviral response (reviewed in (Rezaee et al., 2006)).

1.3.4.2 The adaptive immune response

The B cell immune response

The adaptive immune response to KSHV infection involves both the humoral and cell-mediated immune response. Currently systematic surveys of antibody responses to all KSHV ORF products are being conducted, to definitively identify the targets of the antibody response in healthy donors (Whitby, personal communication). However, the main mapped ORF targets of the antibody response have been identified as LANA (Kellam et al., 1997), K8.1 (Raab et al., 1998) and ORF65 (Simpson et al., 1996).

A role for neutralising antibodies in the control of KSHV infection has been

suggested in a study which compared neutralising antibody levels between healthy KSHV infected donors and donors with KS, showing significantly lower levels of neutralising antibodies in the cohort with KS (Kimball et al., 2004). An additional study carried out by Sullivan et al. has shown that in HIV infected homosexual men HAART increases the KSHV-specific humoral immune response, coincident with clearance of viremia and regression of KS (Sullivan et al., 2010).

The T cell immune response

Cytotoxic T Lymphocytes (CTLs) play a key role in the control of viral infections, especially as effectors of long-term immune surveillance against viruses in the infected host. Unlike some primary infections with EBV that result in infectious mononucleosis with a rapid expansion of activated CD8+ T cells specific for a number of lytic proteins (Maini et al., 2000; Pudney et al., 2005), little is known about primary KSHV infection. Primary infection in children may be associated with an acute febrile illness and a maculopapular rash (Andreoni et al., 2002), while lymphadenopathy has been reported in men who have sex with men, at the time of seroconversion (Casper et al., 2002). Goudsmit et al. investigated the events that occur surrounding the time of seroconversion and provided evidence to support the assumption that KSHV seroconverters have primary infections, as the majority of seroconverters showed detectable levels of virus close to the point of conversion (Goudsmit et al., 2000).

Where T cell responses have been studied in primary KSHV infection has been in carefully monitored HIV infected patients. Here these patients showed non-specific symptoms at the time of seroconversion with no gross alterations in circulating levels

of CD4 and CD8 T cells. Analysis of the specificity of the T cell response elicited suggested a broad repertoire of T cell responses was made, targeting epitopes derived from early and late expressed proteins (Wang et al., 2001). The frequency of responses reached peak levels at one to two years following seroconversion before decreasing again.

To date, a few minimal epitopes have been identified to which CD8+ and CD4+ T cell responses in KSHV infected individuals are directed. A detailed analysis of KSHV specific T cell responses has proven to be very difficult as a result of its very large genome encoding 87 ORFs. Furthermore, the responses that have been detected are generally much weaker to KSHV epitopes in comparison to EBV epitopes. The *ex vivo* detection of these requires several stimulations with the target antigens and the application of professional antigen presenting cells to extract any noticeable response (Fabiola Micheletti et al., 2002; Robey et al., 2009; Lepone et al., 2010).

Despite the difficulty in detecting the epitopes responsible for KSHV responses, a number of epitopes have been identified, the majority of which are HLA-A*0201 restricted and derived from lytic proteins (Wang et al., 2001; Wang et al., 2002; Ribechini et al., 2006; Fabiola Micheletti et al., 2002) and more recently to the latent proteins (Lepone et al., 2010; Guihot et al., 2006). The first MHC Class I specific epitope found to induce a CD8+ T cell response in HLA-A2 individuals was specific to the Kaposin A protein encoded by ORF K12, the optimal CTL epitope LLNGWRWRL (Brander et al., 2001). Soon after a second HLA-A2 restricted epitope was identified in the lytic antigen glycoprotein H (gH), encoded by ORF22, the CTL optimal epitope FLNWQNLLNV. CTL cultures raised against both peptides

could efficiently target HLA-A*0201 matched PEL cells following a series of peptide stimulations, indicating that these two epitopes are targets of CTL-mediated immunity (Fabiola Micheletti et al., 2002).

It is very difficult to be confident that these epitopes are immunogenic in vivo, as the CTL responses *ex vivo* were very weak in comparison to the closely related γ-herpesvirus EBV. This difference was highlighted in a study that directly compared CTL responses between various latent and lytic EBV and KSHV proteins using an overlapping peptide library spanning their sequences in an IFN-γ ELISpot. The average EBV response was almost ten-fold higher, emphasising the weakness of KSHV responses compared to EBV (Bihl et al., 2007).

The size of the CTL response has been shown to vary between different KSHV infected cohorts, depending on: presence of KS, immunosuppression in post-transplant recipients, HIV co-infection and if the HIV patients are receiving HAART treatment. These are all factors that have been shown to influence the frequency of T cell responses to KSHV proteins. A study carried out by Woodberry et al. showed T cell responses to LANA and ORF65 were more frequently seen in HIV co-infected individuals. Furthermore, a higher detectable T cell response has been reported in subjects with active KS compared to those without KS, the highest cellular immune responses were found at time points of highest KSHV DNA loads, suggesting viral loads are driving the T cell response (Woodberry et al., 2005). However, these findings are in contrast to a different study that compared KSHV specific CTL responses between transplant recipients, AIDS-related KS patients on HAART and patients with classic KS and found that KSHV CTL responses were least frequent in

AIDS-related KS (Lambert et al., 2006). Furthermore, a number of studies have also found that CTL responses detected in patients without KS or in control of their KS had more frequent and diverse CTL responses than those with progressive disease (Lambert et al., 2006; Bihl et al., 2009; Guihot et al., 2006).

The CD8+ T cell immune response in MCD patients has also been investigated in a comparison between MCD patients co-infected with HIV and asymptomatic HIV co-infected individuals, using IFN-γ ELISpot with 56 peptides representing a number of KSHV latent and lytic proteins. The screen revealed a similar magnitude of responses between MCD patients and asymptomatic carriers and also identified a new 10 amino acid long HLA-B*7 restricted CD8+ epitope in the K15 protein. These results imply that unlike in KS, MCD does not appear to lack a functional KSHV CD8+ T cell immune response (Guihot et al., 2008).

A number of molecular mechanisms have been reported that might account for the weak T cell responses. The down-regulation of MHC class I molecules on the surface of B cells infected with KSHV through the DC-SIGN receptor has been reported (Rappocciolo et al., 2008). Proteins encoded by KSHV have been shown to have the capacity to downregulate surface MHC class I: these are the K3 and K5 proteins, also known as modulators of immune recognition (MIR1) and MIR2, respectively. These function through the ubiquitination of the MHC class I molecules cytoplasmic tail, subsequently targeting it for proteasomal degradation (Coscoy et al., 2001). The proximity of the cellular proteins varies depending on their proximity to the transmembrance domain, with MIR2 preferentially ubiquitinating residues closer to the membrane than MIR1 (Cadwell et al., 2008). MIR2 also downregulates other

components of the immune synapse such as ICAM-1 and PECAM *in vitro* and this effect is directly correlated to the levels of MIR2 expressed during KSHV infection (Adang et al., 2007). The K3 and K5 proteins are mainly expressed during the lytic cycle, however recent studies have found that K5 can be expressed in endothelial cells up to 5 days later following KSHV infection (Krishnan et al., 2004). An additional target of MIR1 and MIR2 is the IFN-γ receptor which is also ubiquituinated and targeted for degradation, preventing IFN-γ from exerting its anti-viral effects which include the upregulation the antigen processing and presentation machinery for both the MHC class I and class II pathways (Li et al., 2007).

An additional lytic protein which also inhibits CD8+ T cell recognition of targets, is the KSHV shutoff and exonuclease (SOX) protein, encoded by ORF37. Its EBV homologue is BGLF5 and both function using a similar mechanism, which involves the acceleration of degradation of host mRNA, inhibiting all host-cell gene expression within 12hr of lytic reactivation and consequently inhibiting antigen presentation (Zuo et al., 2008).

The latency protein LANA is functionally analogous to the EBV genome maintenance protein EBNA1, both of which possess a central repeat domain. In EBNA1 this repeat domain has been shown to retard protein translation and degradation, limiting the antigen supply for presentation for T cell recognition (Levitskaya et al., 1995; Yin et al., 2003). The acidic repeat domain within LANA has also been shown to reduce the rate of translation and increase protein stability, consequently limiting the source of viral antigens for presentation in a similar fashion to EBNA1 (Kwun et al., 2007;Zaldumbide et al., 2007).

1.4 Aims of this project: To examine the T cell response to the KSHV latent proteins

As described above, a number of studies have examined the T cell response to KSHV within a clinical setting. The majority of work to date has been focused on T cell responses in HIV co-infected individuals, post transplant recipients or patients with KS. More importantly, when immune control is restored in these patient groups, either through the administration of HAART or relaxation of immunosuppression, these malignancies can regress, implying a significant role for T cell immunity in the control of KSHV pathogenesis (Bihl et al., 2007; Duman et al., 2002). Few therapeutic strategies exist to control KSHV disease and progression, this is partly due to the lack of a well-characterised T cell repertoire and being able to determine whether these will be effective *in vivo*. As mentioned above, the virus has developed numerous immune evasion strategies to hinder its recognition by the host immune system. The aim of this project was to examine the T cell response to latent antigens in healthy KSHV-infected donors, which are controlling their virus infection, to establish a repertoire of latent T cell target epitopes from these donors and develop well-characterised T cell clones to these newly identified target epitopes. These will then be used to ask whether T cells specific to these epitopes can effectively respond to *in vitro* antigen expressing targets or infected cells including the malignant PELs. An understanding of which viral proteins can be efficiently targeted by the immune response or what immune evasions may be employed to avoid or inhibit this recognition may help further the understanding of the pathogenesis of this oncogenic virus and the development of therapeutic interventions.

Chapter 2

Material and Methods

2.1 Tissue culture media and reagents

RPMI-1640 supplemented with 2mM L-glutamine (Sigma), stored at 4°C and used for washing cells and the culture of B cells.

Foetal calf serum (FCS) (PAA Laboratories) stored at -20°C in 100ml aliquots until use.

Penicillin-streptomycin solution (Invitrogen) containing 5000IU/ml penicillin and 5000μg/ml streptomycin used at a final dilution of 1 in 100 in culture medium.

Standard cell culture medium was used for the culturing of Epstein-Barr virus (EBV) transformed lymphoblastoid cell lines (LCLs), primary effusion lymphoma lines (PELs) and other B cell lines. This media contained RPMI-1640 supplemented with 10% FCS and penicillin-streptomycin solution.

HMEC-1 cell culture medium was used for the culture of the cell line human microvascular endothelial cell-1 (HMEC-1). Culture medium contained M199 (Gibco) supplemented with 20% FCS, 2.5μg/ml amphotericin B, 1μg/ml hydrocortisone, 10ng/ml epidermal growth factor (all from Sigma-Aldrich).

EBM-2 (Lonza Clonetics) Endothelial cell basal medium-2 which contains no growth factors cytokines, or supplements was used for KSHV infection of HMEC-1 cells.

DMEM supplemented with 2mM L-glutamine (Sigma), stored at 4°C and used for the culture of epithelial cell lines.

Opti-MEM1 (Invitrogen) was stored at 4°C and used on cell lines which were used in transfection experiments.

Human serum (TCS Biologicals) was stored in 50ml aliquots at -20°C.

Phosphate buffered saline (PBS) was made by dissolving 1 Dulbecco A tablet (Oxoid) per 100ml of distilled water; the solution was autoclaved for sterility if necessary.

Recombinant interleukin-2 (IL-2) (Peprotech) stored at -20°C and used at 50 IU per ml.

Cyclosporin A (Sandimmun, Sandoz) was reconstituted in RPMI 1640 to give a final concentration of 10μg/ml and stored at 4°C.

Doxycycline (dox) was prepared from doxycycline hyclate powder (Sigma) by dissolving in distilled water to give a final concentration of 100μg/ml. The solution was sterilised through a 0.22μM filter and stored at 4°C in the dark.

MLA-144 supernatant (MLA) was obtained from the Gibbon-derived MLA-144 cell line, which spontaneously releases IL-2. The cells were grown in standard cell medium for 2 weeks without further feeding. The supernatant was then harvested by centrifugation at 300xg for 10mins, followed by filtration through Millipore steritop 0.22μM filter membrane by vacuum suction. The filtered MLA supernatant was stored at -20°C in 60ml aliquots.

T cell cloning and culture medium contained RPMI 1640 supplemented with L-glutamine, 30% MLA, 10% FCS, 1% human serum, 1% penicillin and streptomycin and 50IU per ml of recombinant human IL-2, stored at 4°C until use.

Synthetic peptides synthetic 15-mer peptides overlapping by 10 amino acids with the neighbouring peptides, generated by Mimotopes, were dissolved in dimethyl sulfoxide (DMSO) (Sigma) for 30mins at RT then stored at -20°C. These peptides spanned the KSHV proteins Kaposin, vFLIP, vCyclin and LANA, excluding the acidic repeat region. Sequences of the peptides were based on the BC-1 strain of virus.

Biuret assay was used to determine the peptide concentration (Doumas, 1975). Twenty μl peptide solution was added to 100μl biuret reagent in a 96-well V-bottom plate (Nunc). The plate was incubated for 30mins at RT to allow colour development, and then centrifuged at 200xg for 5mins. One hundred μl of the supernatant from each well was transferred into 96-well flat bottom plate, and the absorbance was measured at 540nm using an automated microplate reader (Bio-Rad). Peptide concentration was

calculated against a standard curve of bovine serum albumin (0-40mg/ml) incubated with the biuret reagent, measured in the same assay.

Trypan blue staining was carried out to determine the number of viable cells within a cell culture. The cell culture was mixed 1:1 with trypan blue (Sigma) and the number of unstained cells/ml was determined using a haemocytometer.

Mycoplasma testing was routinely carried out on cultured cells using MycoAlert® Mycoplasma detection kit (Cambrex). The testing was carried out according to the manufacturer's instructions.

Cryopreservation and recovery of cryopreserved cells

Cells to be cryopreserved were pelleted by centrifugation at 300xg for 6 mins, the supernatant was removed and the cells were resuspended in 1ml of freezing medium: RPMI 1640 supplemented with 20% FCS and 10% DMSO. The cells were transferred into sterile 1.5ml plastic cryovials (Nunc) and placed in a Mr Frosty (Nalgene), containing isopropanol and placed in a -80°C freezer. For long term storage, the frozen cells were transferred into a freezer containing liquid nitrogen. Cells were recovered from such storage by thawing in a 37°C waterbath and then transferring the cells into a 15ml tube (Starstedt) followed by the dropwise addition of 10mls of the appropriate growth medium. The cells were pelleted by centrifugation at 300xg for 6mins and resuspended in the appropriate growth medium for culture.

2.2 Generation and culture of cell lines

Blood donors

Blood was obtained from 100 donors recruited at the Medical Research Council in Fajara, the Gambia. The donors were tested for HIV1 and 2, Hepatitis B, and Hepatitis C and confirmed to be negative before taking 20mls of blood for the study. Blood was also obtained from healthy laboratory personnel. Buffy coats were obtained from the Birmingham National Blood Service. All experiments were conducted with ethical approval from the South Birmingham Local Research Ethics Committee (REC reference 06/Q2707/300) and The Gambia Government/MRC Laboratories Joint Ethics Committee.

HLA typing

The HLA status of the donors was confirmed through PCR-based DNA typing by the Anthony Nolan trust, Histocompatibility laboratories in Hampstead, London.

Isolation of peripheral blood mononuclear cells (PBMCs)

PBMCs were obtained from peripheral blood collected either by venous puncture into heparinised syringes, or from buffy coat preparations. Blood donations were diluted 1:1 in RPMI 1640 medium (Sigma) and layered onto lymphoprep (Axis-shield) before centrifugation at 300xg for 30 minutes, with no braking applied. The PBMCs were harvested from the lymphoprep interface and washed three times with RPMI 1640. The PBMCs were counted and if being stored were separated into 5 x 10^6 cell aliquots, resuspended in freezing medium and frozen, or used immediately.

Generation of LCLs

LCLs were generated in vitro by EBV-infection of B cells within PBMC preparations using the B95.8 strain of the virus. One million PBMCs were pelleted by centrifugation, 500μl of 100x concentrated virus supernatant from the virus producer cell line B95.8 was added to the cells and incubated overnight at 37°C and 5% CO_2. The following day, the cells were washed with RPMI 1640 and resuspended in standard medium with the addition of 0.25μg/ml of Cyclosporin A and cultured in a 48-well plate (Iwaki). The B cell's transformation into LCLs was monitored and proliferating cells expanded accordingly until they could be maintained in a 25cm^2 flask (Iwaki), after which they were split and fed twice a week with standard medium.

2.3 Generation of T cell clones

Establishment of peptide-specific polyclonal T cell cultures by peptide stimulation

KSHV-specific T cells were initially expanded from PBMC preparations using a protocol based on Lalvani et al (Lalvani et al., 1997). Here 1-10x10^6 PBMCs were pelleted by centrifugation and pulsed with overlapping 15-mer peptides grouped into pools for the relevant KSHV proteins at a concentration of 5 μM for 1hr 30mins at 37°C and 5% CO_2, resuspending the mixture every 20 mins. The cells were subsequently washed and cultured in 1ml of RPMI 1640 medium supplemented with 10% FCS, and 25ng/ml of IL-7 (Peprotech) in a 24-well plate (Iwaki). IL-2 (Peprotech) was added at a final concentration of 10 IU/ml to the medium every 3 days up until day 7, when the cells were harvested for IFN-γ capture magnetic sort cloning.

IFN-γ capture magnetic sort cloning

Epitope-specific T cells were isolated by IFN-γ capture and magnetically sorted from the peptide stimulated polyclonal T cell cultures. PBMCs were restimulated for 1 hr with 5μM of the relevant peptide pools, washed and incubated at 37^{o}C for 3 hours in RPMI 1640 supplemented with 5% human serum. The cells were harvested and the IFN-γ producing cells isolated using an IFN-γ secretion assay-cell enrichment and detection kit (Miltenyi-Biotec) according to the manufacturer's instructions. Following the incubation, the cells were washed in MACS buffer (PBS, 1% BSA and 2mM EDTA), resuspended in 80μl of RPMI/10% FCS with 20μl of IFN-γ catch reagent added. The cells were then incubated on ice for 5mins, after which 10mls of pre-warmed RPMI/10% FCS was added and incubated at 37°C for 45mins on a cell roller. These were then washed in MACS buffer, resuspended in 80μl of MACS buffer and 20μl of IFN-γ detection antibody added. After a 10 minute incubation on ice, the cells were washed, resuspended in 80μl of MACS buffer and 20μl of magnetic microbeads added. The cells were then placed at 4°C for 15mins (shaking every 5mins). The cells were washed one final time and resuspended in 500μl of MACS buffer before being applied to a MACS MS column within a magnetic field. After extensive washing, retained labeled cells were collected by removing the column from the magnetic stand and flushing it with 1ml of MACS buffer. The isolated IFN-γ producing cells were then cloned by limiting dilution cloning.

Limiting dilution cloning

T cell clones were generated by limiting dilution cloning using the magnetically sorted IFN-γ producing cells isolated as described above. These cells were seeded at 0.3 and 3 cells per well in 96-well round-bottom tissue culture plates (Iwaki), with γ-

irradiated mixed allogeneic buffy coat feeder cells which had been activated with 10μg/ml phytohaemaglutanin (PHA) overnight, at 10^5 cells per well. Cultures were seeded in T cell medium supplemented with the anti-CD3 monoclonal antibody OKT3 (Insight Biotechnology LTD) at 30 ng/mL, in a total volume of 100μl per well. After 1 week a further 100μl of T cell medium was added to each well.

Identification of epitope-specific clones

After 2-3 weeks depending on the growth of the microcultures, proliferating cells were selected and expanded using T cell medium and γ-irradiated buffy coat feeder cells into 24 well plates (Nunc). The wells were subsequently screened for peptide specificity by IFN-γ ELISA. In this assay, 50μl of cells from each clone were removed into duplicate wells in a 96-well V-bottom plate and washed 3 times in RPMI 1640. The cell pellets were then resuspended in 100μl of culture medium containing 1μM of the relevant peptide pool mixture, or an equivalent concentration of DMSO solvent. These cultures were then incubated overnight at 37°C and 5% CO_2 and supernatants tested for the specific release of IFN-γ by ELISA the following day. Peptide-specific clones were identified as those giving increased IFN-γ release to the peptide pool mixture compared to DMSO alone. Peptide-specific clones were then assayed using this procedure against the individual peptides within reactive pools to identify the stimulating peptide. These T cell clones were maintained in culture by feeding twice a week with T cell medium.

2.4 Immunological assays

ELISpot assays for detection of IFN-γ release

Ninety six well Millipore plates (Bedford, MA) with Immobilon-P polyvinylidene difluoride membranes were coated with 15μg/ml of an anti-IFN-γ monoclonal antibody D1K (Mabtech, Stockholm) diluted in PBS for 3hr at RT, after which the plate was washed 6 times with RPMI 1640 and blocked for 30mins with standard medium. Four hundred thousand PBMCs were added to duplicate wells in 100μl of standard medium, to which pooled peptides were added at a final concentration of 5μM peptide. The plate was incubated overnight at 37°C and 5% CO_2. The following day the cells were removed and the plates were washed 6 times in PBS containing 0.05% Tween 20 (PBS/T). Fifty μl of 1μg/ml biotinylated anti-IFN-γ mAb was added to the wells for 2-4hrs and washed again 6 times with PBS/T. Fifty μl of streptavidin-conjugated alkaline phosphatase was added to the wells for 1-2hrs and the plates were washed again 6 times with PBS/T. The chromogenic substrate 5-bromo-4chloro-3-indolyl phosphate and nitro blue tetrazolium was added to the wells to reveal IFN-γ produced by individual cells as dark spots, where each spot is representative of one reactive T cell. The reaction was stopped 15mins later by washing the plate with tap water and allowing it to dry. The number of spots were read using an AID automated ELISpot reader and data transformed to give the number of spot forming cells 10^6 PBMC.

Chromium release assays

Cytotoxicity of the T cell clones was assessed in standard 5hr chromium release assays at known effector/target ratios. Target LCLs were placed in 15ml tubes (Starstedt) and pelleted by centrifugation at 300xg for 6 mins, the cells were then

pulsed with 10-fold dilutions of target peptide ranging from 10^{-7}M to 10^{-12}M or the peptide solvent DMSO as a negative control. The cells were labeled with 50μCi of ^{51}Cr and incubated for 90mins at 37°C and 5% CO_2, shaking every 30mins. During this time, the effector T cells were counted, pelleted and resuspended in RPMI and 10% FCS. The T cells were plated out in triplicate at two dilutions of 2500 and 5000 T cells in 100 μl per well in 96 well V-bottom plates and incubated at 37°C and 5% CO_2. The ^{51}Cr labelled LCLs were then washed twice in RPMI 1640 at 300xg for 6mins and resuspended in 1ml of LCL medium. These were counted and 2500 target LCLs in 100μl of RPM1 and 10% FCS added to the 96 well V-bottom plates containing the two dilutions of effector T cells. This gave effector:target ratios of 1:1 and 2:1. For each ^{51}Cr labelled target LCL, separate aliquots of cells were lysed with 1% sodium-dodecyl sulphate (SDS) to represent 100% lysis, while ^{51}Cr labelled target LCLs incubated in media alone were included to measure values for spontaneous lysis. The plates were centrifuged at 30xg for 4mins and incubated for 5 hours at 37°C and 5% CO_2. Following the incubation, the plates were centrifuged again and 100μl of supernatant per well was harvested into LP2 tubes and the counts per minute (CPM) radiation emitted counted in a γ-radiation counter. The percentage specific lysis was calculated using the CPM values as follows: (lysis by CTL - spontaneous lysis)/(total lysis in 1% SDS - spontaneous lysis) x 100.

IFN-γ enzyme-linked immunosorbent assay (ELISA)

IFN-γ ELISA was used to measure IFN-γ release from effector T cells upon recognition of their epitopes presented by target cells. As described below, effector-target combinations were cultured in triplicate for 18 hours after which the supernatants were assayed for IFN-γ by ELISA. Here 96-well Maxisorp plates (Nunc)

were coated with 50µl of mouse anti-human IFN-γ diluted to 0.75µg/ml in coating buffer and incubated overnight at 4°C. The following day the antibody was removed from the plates and excess protein binding sites blocked for 1 hr at RT in 200µl of blocking buffer (PBS/T containing 1% bovine serum albumin (BSA)). The plates were then washed 6 times in PBS/T and 50µl of culture supernatant from the T cell assay either neat or diluted (1 in 10) was added to the wells. To quantify the IFN-γ released, recombinant IFN-γ (R&D Systems) was added to triplicate wells at a range of doubling dilutions from 2000pg/ml to 31.25pg/ml. The plates were incubated for 2-4hrs at RT, after which the supernatants were discarded and the plates washed 6 times with PBS/T. The wells then had added to them 50µl of biotinylated mouse anti-human IFN-γ mAb at 0.75µg/ml diluted in blocking buffer and these incubated for 1-2hrs at RT. The plates were then washed again 6 times in PBS/T followed by a 30min incubation at RT with 50µl per well of ExtrAvidin peroxidase conjugate (Sigma) diluted 1/1000 in blocking buffer. Finally, the plates were washed 8 times in PBS/T followed by the addition of 100µl of 3,3',5,5'-tetramethylbenzidine solution containing peroxide (TebuBiotech) and left at RT to allow the reaction to develop. To stop this reaction, 100µl of 1M hydrochloric acid was added and the absorbance of each well measured at 450nm with wavelength correction of 655nm, using an automated plate reader (Bio-Rad). IFN-γ release from each T cell was quantified from a standard curve generated from the dilutions of recombinant IFN-γ added to each assay.

Standard T cell assay

In a standard T cell assay, $5x10^4$ HLA-matched or HLA-mismatched target cells were incubated with $5x10^3$ T cells in a 96 well V-bottom plate. To serve as a positive

control in T cell assays, aliquots of target cells were pulsed with either 5μM of epitope peptide or an equivalent dilution of peptide solvent DMSO as a negative control for 1 hr at 37°C. These were then extensively washed before use in assays. T cells were co-cultured with target cells in triplicate and after overnight incubation at 37°C and 5% CO_2, 50μl of supernatant from each well was tested by ELISA for IFN-γ.

Modified vaccinia Ankara (MVA) virus infection assays

Target LCLs and PELs were infected with recombinant MVA viruses expressing invariant chain tagged EBV or control antigens at a multiplicity of infection (moi) of 10 for 2.5 hours. These were then washed several times prior to incubation with EBV antigen-specific T cells in standard T cell assays. Recognition was assessed by IFN-γ ELISA the following day.

Flow cytometry staining

Between 2 $x10^5$ and 3 $x10^5$ cells were placed in 5ml polystyrene round-bottom tubes (BD Falcon), pelleted by centrifugation and washed once in PBS with 2% FCS at 250xg for 4mins. Cells were resuspended and incubated for 30mins on ice in the dark with a pre-titrated concentration of primary antibody (see Table 2.1) or IgG isotype matched control diluted in PBS and 2% FCS. If an unlabelled primary antibody was used, cells were washed again with PBS and 2% FCS and incubated for 30mins on ice with the appropriate fluorochrome conjugated secondary antibody (see Table 2.1). The cells were then washed in PBS and 2% FCS and resuspended in 2% PFA diluted in PBS and 2% FCS. The samples were kept at 4°C in the dark until analysis was performed using an XL-MCL flow cytometer (Beckman Coulter) or an LSR-II

Table 2.1 Antibodies

	Target	Clone Name	Origin	Applications	Dilutions	Company/ Reference
Primary Antibodies	LANA1 (Repeat)	N/A	Rat	WB IF	1/1000 1/100	Autogen Bioclear
	LANA1 (N'-terminus)	AT4C11	Mouse	WB	1/1000	Abnova
	LANA2 (vIRF-3)	CM-A807	Mouse	WB	1/1000	Abcam
	HA (Hemagglutinin peptide)	3F10	Rat	WB	1/1000	Roche
	FLAG	M2	Mouse	WB	1/1000	Sigma
	Actin	AC-74	Mouse	WB	1/10000	Sigma
	Rat CD2	OX34	Mouse	FC	1/100	ATCC
	CLIP	CerCLIP	Mouse	FC	1/50	BD Pharmingen
	Mouse anti-human HLA-DR, DP, DQ	Tu39	Mouse	FC	1/50	BD Pharmingen
	Mouse IgG1 Isotype Control	MOPC-21	Mouse	FC	1/50	BD Pharmingen
	Mouse IgG2a Isotype Control	G155-178	Mouse	FC	1/50	BD Pharmingen
	Anti-human HLA-DR L243	HB-55	Mouse	FC	1/50	ATCC
	Anti-human HLA-DQ	SPV-L3	Mouse	FC	1/50	Serotech
	Anti-human HLA-A,B,C	W6/32	Mouse	FC	1/50	Abova
Secondary Antibodies	Goat anti-Mouse IgG:HRP	N/A	Goat	WB	1/1000	Sigma
	Goat anti-mouse IgM+IgG+IgA(H+L) FITC	N/A	Goat	FC	1/50	Southern Biotech
	Goat anti-rat Alexa Fluor 594	N/A	Goat	IF	1/100	Invitrogen
	Goat anti-human IgM FITC	N/A	Goat	IF	1/50	Sigma
Conjugated Antibodies	Mouse anti-human CD8 FITC	RPA-T8	Mouse	FC	1/50	BD Pharmingen
	Mouse anti-human CD4 FITC	RPA-T4	Mouse	FC	1/50	BD Pharmingen
	Mouse anti-human HLA-A,B,C APC	W6/32	Mouse	FC	1/20	BioLegend

Application Abbreviations
WB = Western blot
IF = Immunofluorescence
FC = Flow cytometry

cytometer (Becton Dickinson). Flow cytometry data was analysed using Flowjo software (Treestar).

LANA protein

LANA protein for T cell recognition experiments was derived from BCBL-1 cells and provided by Dr Andrew Hislop. In brief, nuclear proteins were extracted from $500x10^6$ BCBL-1 cells using a procedure as described (Shore et al., 2006). LANA proteins were enriched from these eluates by FPLC. Here the nuclear proteins were transferred into a low salt buffer of 0.2M NaCl, 0.05M Tris pH8.0 using PD-10 columns (GE Healthcare) before being loaded onto a Mono Q anion exchange column (GE Healthcare). Protein fractions were eluted from the column using a NaCl gradient from 0.2M to 1M. Eluted fractions were tested for the presence of LANA by western blot analysis. Nuclear extracts from the KSHV- and EBV-negative Burkitts lymphoma line DG-75 were similarly processed to provide a control antigen.

Protein feeding assay

LCLs and PELs were pelleted by centrifugation, washed and resuspended in 500 μl of serum free AIM-V media (Gibco) in a 48 well plate (Iwaki). Purified protein was added to the cells to final concentrations of 80μg/ml and 40μg/ml and incubated overnight at 37°C and 5% CO_2. The following day the cells were washed in RPMI 1640 and incubated with CD4+ T cells in a standard T cell assay, followed by IFN-γ ELISA.

2.5 Clone characterisation

CD4/CD8 staining

Expanded T cell clones were analysed for CD4/CD8 status by flow cytometry staining. Approximately, $3x10^5$ T cells were placed in a 5ml FACS tube (Falcon) and washed with 2.5ml of PBS/2%FCS. Fifty µl of diluted PE-conjugated anti-CD4, PE-conjugated anti-CD8 (Serotec) mAbs and PE-conjugated IgG1 as an isotype control was added to different tubes of cells and incubated on ice for 30mins in the dark. The cells were then washed again in 2.5ml of PBS and 2%FCS and resuspended in 200µl of PBS and 2%FCS and 200µl of 4% paraformaldehyde (Sigma) in PBS and analysed using the XL2 flow cyotmeter (Beckman Coulter).

Testing functional avidity

Each T cells clone's autologous lymphoblastoid cell line (LCL) was sensitised with cognate peptide at 10-fold dilutions ranging from 10^{-5}M to 10^{-13}M for 1hr at 37^{o}C, washed and incubated with the T cell clone overnight. IFN-γ secreted by the T cells was quantified by ELISA and the functional avidity determined as the dose of peptide eliciting 50% maximal IFN-γ secretion.

HLA restriction of T cell clones

HLA restriction of T cell clones was performed by assaying these against a panel of peptide sensitised LCLs which shared one or two HLA-alleles with the T cell in question. The LCLs were pulsed with the clone's specific peptide at 5µM for 1 hour, washed 4 times with RPMI 1640, resuspended in RPMI 1640 medium supplemented with 10% FCS and incubated overnight with the T cell clone in a standard assay. IFN-γ release from the T cells was measured by IFN-γ ELISA to identify LCLs capable of

stimulating the T cells and thus identify which HLA molecule was responsible for presentation of the peptide.

2.6 Establishment of stably transfected FL-LANA, LANAΔAcid & LANAΔAcid li-tet target lines

LANA expression plasmids

LANA constructs were ectopically expressed using derivatives of the pRTS-CD2 plasmid (Kelly et al., 2009) which had been transfected into target cell lines. This construct contains the EBV origin of replication and constitutively expresses the rat CD2 molecule allowing maintenance and selection respectively in EBV negative cell lines. This vector also contains a bi-directional tetracycline responsive promoter, which allows inducible expression of inserted genes as well as the reporter molecules GFP and the truncated nerve growth factor receptor (NGFR). LANA sequences from the BCBL-1 strain of virus were inserted into this plasmid to generate a construct expressing full length LANA (FL-LANA), using standard techniques. LANA constructs were also created in which the acidic repeat sequence was removed (LANAΔacid) and a derivative of this construct was further engineered to incorporate the first 80 amino acids of the MHC class II invariant chain sequence at the amino terminus of this protein to direct it into the lysosomal compartment (LANAΔacid li) (Sanderson et al., 1995). All three LANA constructs were tagged at the C-terminus with the commonly used epitope tag HA from the influenza virus hemagglutinin protein for protein visualisation.

Transfection of LCLs and PELs with DNA plasmids

DNA plasmids were introduced into the LCLs and PELs by electroporation. To achieve maximum transfection efficiency, cells were passaged 24hrs prior to electroporation to ensure they were in the optimal growth phase. Ten million LCLs or PELs were washed once in PBS and once in Optimem by centrifugation at 300xg for 6 mins and then resuspended in 300µl Optimem containing 10µg of plasmid DNA. The cells were transferred into a 4mm sterile electroporation cuvette (Geneflow), electroporation was perfomed using a Bio-Rad electroporation apparatus at 230V and at a capacitance of 975µF. The cells were immediately transferred into a 6 well plate (Iwaki) containing 8ml of pre-warmed RPMI and 20%FCS and were incubated at 37°C and 5% CO_2.

Selection of transfected cells expressing rat-CD2 (rCD2)

Twenty four hours post-transfection, viable cells were isolated by layering onto lymphoprep and centrifuging at 300xg for 30mins with no brake. The viable cells were isolated from the lymphoprep layer and washed twice in MACS buffer. The vector positive cells were then enriched by magnetic activated cell sorting (MACS), selecting on the constitutively expressed rat CD2 marker. Transfected cells were resuspended in 10µg/ml of anti-rat CD2 mAb OX34 (ATCC) and incubated on ice for 30mins. Following the incubation, the cells were washed twice in MACS buffer and resuspended in 80µl MACS buffer with 20µl of rat anti-mouse IgG2a+b microbeads added and incubated for 15mins at 4°C. The cells were then washed once in MACS buffer and positively selected by magnetically sorting with MACS LS columns (Miltenyi Biotech) according to the manufacturer's instructions. The cells were placed

in a 96 well round bottom plate (Iwaki) in 200μl of RPMI and 20% FCS and expanded as necessary.

Induction of protein expression from doxycycline (dox) tet-regulated plasmids

Protein expression from tet-regulated plasmids was induced by the addition of the tetracycline analogue dox to the cell culture medium. Dox was added at dilutions ranging from 2μg/ml (maximum induction) down to 0.75ng/ml (minimum induction) in order to titrate and control protein expression levels.

KSHV FLIP (vFLIP) DNA expression plasmids

KSHV FLIP (vFLIP) constructs were kindly provided by Dr Priya Bellare, The University of California San Francisco (UCSF), USA. The following constructs included two vFLIP sequences: the wildtype vFLIP sequence referred to as Wt vFlip and the vFLIP with codon usage optimised using the human codon usage referred to as Hu vFLIP**.** Both sequences contained at the amino terminus FLAG tag and were expressed in pcDNA3.1 vectors, with expression driven by the CMV promoter in the vector and an internal ribosome entry site (IRES) GFP for measuring transfection efficiency through GFP fluorescence. The well-known EBV HLA-B*08011 restricted epitope FLRGRAYGL (FLR) sequence derived from the EBNA3A latent protein, was inserted at the carboxy terminus of the vFLIP constructs to act as a model antigen in T cell recognition studies.

Transfection of attached cell lines with DNA plasmids

The epithelial cell line HEK-293 and melanoma cell line MJS were transfected with KSHV expressing DNA plasmids prior to use in T cell recognition assays. The cells

were split into a 24-well plate (Iwaki) at a density of $2x10^5$ cells per well and incubated overnight in 500µl of standard medium with no antibiotics at 37°C and 5% CO_2. The following day, 1.2µg of plasmid DNA was added to 50µl of Opti-MEM1 (Invitrogen) while 2µl of transfection reagent Lipofectamine 2000 (Invitrogen) was mixed with 50µl of Opti-MEM1 and both incubated for 5mins at RT. The DNA and Lipofectamine were then mixed together and incubated for a further 20mins at RT, after which a further 50µl of Opti-MEM1 was added to the mixture prior to addition to the cells. The medium was then removed from the cells and replaced with 150µl of the DNA and lipofectamine mixture and incubated for 5-6 hours at 37°C and 5% CO_2. Following on from the incubation, 500µl of standard medium without antibiotics was added to the cells and these incubated for 24-48 hours prior to being used as targets in subsequent T cell assays.

Cytometric analysis of transfection efficiency

One to two hundred thousand of the pRTS-CD2 transfected cells were analysed by flow cytometry for expression of GFP after dox induction. The cells were washed twice with PBS and 2% FCS at 250xg for 4mins and resuspended in 2% paraformaldeyhde in PBS and 2% FCS. The GFP expression was determined using the XL-MCL flow cytometer (Beckman Coulter).

Enrichment of LNGFR expressing transfected cells

Transfected LCLs or PELs were induced with dox for at least 24hrs prior to selection for low-affinity nerve growth factor receptor (MACSelect™ LNGFR System), expressed by the pRTS-CD2 vectors upon induction. The cells were pelleted by centrifugation at 160xg for 10mins and resuspended in 320µl of MACS buffer and

80µl of LNGFR beads (Miltenyi Biotec) to every 10^7 cells and incubated for 15mins on ice. Following the incubation, the cells were topped up to 2ml with MACS buffer and magnetically sorted using MACS LS columns (Miltenyi Biotec) according to the manufacturer's instructions. The LS column was removed from the magnet and the positively selected population was flushed through with 5ml of MACS buffer. The cells were pelleted, washed once in RPMI 1640 and resuspended in standard medium, after which the cells are either used directly in a standard T cell assay or maintained in culture for later use.

KSHV infection of HMEC-1

Human microvascular endothelial cells (HMEC-1) which have successfully been immortalised with a plasmid expressing simian virus 40 A gene product, large T antigen (Ades et al., 1992). These cells were generously donated from Professor Gerard Nash, The University of Birmingham. HMEC-1 cells were split one day prior to infection and placed in a 24-well plate (Nunc) at a density of $2x10^5$ cells per well in 1ml of HMEC-1 medium. Titrated recombinant KSHV virus (rKSHV.219) derived from the engineered JSC-1 strain (Vieira et al., 2004), kindly provided by Professor David Blackbourn The University of Birmingham, was used to infect the cells. The virus was used at an MOI of 10. The virus was prepared by diluting the virus particles in 320µl of EBM-2 medium (Lonza Clonetics) for each well of cells. The HMEC-1 medium was removed from the wells and the diluted virus EBM-2 mixture was added gently to the cells. The cells were then infected by spinoculation at 160xg at 32°C for 30mins followed by 90min incubation at 37°C and 5% CO_2. Following the incubation, the virus was gently removed from the wells, the cells washed with 400µl of EBM-2 medium and cultured in 400µl of HMEC-1 medium for 72hrs at 37°C and

5% CO_2. The efficiency of KSHV infection of the cells was determined by the levels of GFP fluorescence by flow cytometry as this indicates successful latent KSHV infection. The infected cells were subsequently used in standard T cell recognition assays with LANA clones and recognition measured the following day by IFN-γ ELISA.

2.7 Antigen detection

Protein sample preparation for polyacrylamide gel electrophoresis (PAGE) and western blot analysis

Cells were lysed in 9M urea buffer (9M urea, 0.075M Tris/HCl pH 7.5) and sonicated for 15seconds. Protein concentration was determined using the Bradford determination reagent (BioRad) according to manufacturer's instructions. Twenty μg of each protein sample was prepared by adding a quarter of the volume of 4x SDS gel sample buffer (0.0625M Tris-HCl pH 6.8, 2%SDS, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue) (Laemmli et al., 1970) and 1M DTT. Protein samples were then denatured at 100°C for 5mins and solubilised proteins were separated by SDS-PAGE. Eight and twelve % acrylamide gels were used containing 100mM Tris-HCl, 0.1% SDS, and 0.01% TEMED, diluted with water to give the appropriate percentage gel. Acrylamide polymerisation was initiated through the addition of ammonium persulphate (APS) to a final concentration of 0.02%. Gels were assembled using PROTEAN II Biorad apparatus according to manufacturer's instructions in 1x Electrophoresis buffer. The denatured samples were loaded onto the gel and into one well the 6μl of the See Blue pre-stained standard (Invitrogen) as a protein molecular weight marker, electrophoresis was performed using standard protocols (Laemmli, 1970).

Western blot and antigen detection

Following electrophoresis, separated proteins were transferred to nitrocellulose membranes. In brief, individual gels were laid onto a nitrocellulose membrane (Bio Trace NT, Gelman Sciences), sandwiched between three pieces of 3MM blotting filter paper (Whatmann) and two blotting sponge pads and placed in plastic cassettes, all of which had been pre-soaked in transfer buffer (0.01M 3-(Cyclohexylamino)-1-propanesulfonic acid (CAPS, Sigma) pH1, 10% V/V methanol in deionised water). The blotting cassette (Bio-Rad) was assembled according to the manufacturer's instructions in the order of a sponge, three pieces of filter paper, gel, nitrocellulose membrane, another three pieces of filter paper and a sponge, from black (cathode) to the white (anode). The assembled blotting cassettes and an ice block for cooling were inserted into the transfer tank filled with transfer buffer. Proteins were transferred to the membranes at 100V for 1hr, or 20V overnight. Following transfer, the blots were placed in a blocking agent (5% skimmed milk powder in PBS/T) overnight at 4°C or for 1hr at RT. The membrane was then incubated in the appropriate primary antibody (see Table 2.1) diluted in PBS/T containing 5% milk for 1 hr at RT with agitation. The membrane was washed 4 times with PBS/T and incubated with an appropriate diluted secondary horseradish peroxidase (HRP) conjugated antibody (see Table 2.1). The membrane was then washed as above. An enhanced Chemiluminescence (ECL) detection kit (Amersham Biosciences) and Amersham Hyperfilm (GE Healthcare) were used to visualise the HRP bound proteins.

Immunofluorescence staining

To check the KSHV status of the study participants, plasma was isolated from the blood samples of each of the donors and heat inactivated at 56°C for 30mins.

Multispot microscope slides (Hendley-Essex) had added to them either 100 000 KSHV-infected BCBL-1 PEL cells or 100 000 KSHV negative BJAB cells. Slides were dried and fixed for 30mins in at -20°C acetone:methanol (1:1). Slides were blocked with 30µl of 10% heat-inactivated goat serum (HINGS) for 20mins at RT. This was then rinsed and replaced with 30µl of either donor plasma tested at 3 dilutions of 1/20, 1/40 and 1/100 or monoclonal LANA antibody as a positive control or monoclonal HA antibody as a negative control (see table 2.1), all diluted in 10% HINGS in PBS. The slides were then incubated at 37°C for 1hr in a humidified chamber. Following the incubation, slides were washed twice in PBS for 10mins with gentle stirring to remove any unbound antibody. Excess PBS was removed from the slides around the multispot wells with a cotton bud and 30µl of the appropriate fluorochrome conjugated secondary antibody diluted in 10% HINGS and PBS (see Table 2.1) added. Slides were incubated again at 37°C for 1hr in a humidified chamber. Following two 10mins washes in PBS, slides were dried between the multispot well, and a drop of 10% glycerol was added to each well and immediately mounted with a coverslip. Slides were examined on a Nikon E600 UV microscope.

Chapter 3

Identification and characterisation of T cell responses to Kaposi's sarcoma-associated herpesvirus latent proteins in healthy donors

Kaposi's sarcoma-associated herpesvirus (KSHV) is a γ-herpesvirus that has come to prominence through its association with the vascular tumour Kaposi's sarcoma (KS) and the B cell lymphoproliferative disorders primary effusion lymphoma (PEL) and the plasmablastic form of Multicentric Castleman's disease (MCD). Evidence from immunosuppressed patients suggests that cellular immunity is crucial to the prevention and control of KSHV-associated malignancies (Osmond et al., 2002; Boshoff et al., 2002). Thus, KS can be seen in transplant recipients receiving immunosuppressive therapy or HIV patients who have progressed to AIDS (Frances et al., 2000). Importantly, control of KSHV malignancies can occur upon restoration of T cell immune function by relaxation of immunosuppression or HAART therapy, respectively (Wilkinson et al., 2002). Although immunity appears important in the control of KSHV-associated disease, little is known about the T cell targets of the immune response, the size of such responses and their biological effectiveness in controlling disease in healthy KSHV-infected donors.

A subset of viral genes are consistently expressed in the KSHV-associated malignancies; mostly the latent genes (reviewed in (Geraminejad et al., 2002)). These genes include the genome maintenance protein LANA, the viral FLICE like inhibitory protein vFLIP, that has an anti-apoptotic function, the viral cyclin which can disrupt

the cell cycle, as well as Kaposin (Dittmer et al., 1998; Li et al., Guasparri et al., 2004; Sarid et al., 1999). The products of these genes are potential targets of the immune response and thus may be important in immune mediated control of KSHV infection and disease. However, few T cell targets have been identified in these proteins (Bryan et al., 2005). Furthermore, where these have been measured, mostly in immunocompromised donors, weak T cell responses have been detected ex vivo compared to responses made to the closely related γ-herpesvirus Epstein-Barr virus (Fabiola Micheletti et al., 2002; Bihl, et al. 2007).

The lack of known KSHV-specific T cell targets makes studying the immune response in KSHV-infected donors, healthy or otherwise, much more challenging. A number of studies have attempted to fill this void by investigating the size of the T cell response to KSHV-antigens in immunocompromised donors such as HIV-infected donors or patients with KSHV disease. HIV-infected patients with KS disease have very weak or no detectable responses to LANA and a number of lytic proteins, while patients receiving HAART who are controlling their disease did generally elicit responses towards the KSHV proteins tested (Lambert et al., 2006; Guihot et al., 2006). Furthermore, an additional study has shown that the administration of HAART to these immunocompromised patients over a period of time, decreased KSHV viral loads and KSHV-specific responses increased (Bourboulia et al., 2004).

Several other studies have focused on identifying HLA-A*0201 restricted responses, since this HLA molecule possesses a well-defined binding motif, allowing the use of peptide prediction algorithms, and is the most common HLA type in the Caucasian

population (Wang et al., 2001; Wang et al., 2002; Ribechini et al., 2006; Fabiola Micheletti et al., 2002). However, this method of defining targets immediately excludes the many donors' who are not HLA-A*0201 positive and prevents identification of epitopes presented by other HLA types. A more favourable approach of measuring the size of responses to proteins without identifying epitopes involves screening KSHV-infected donors PBMCs with all possible overlapping synthetic peptides spanning one protein, assayed in one pool. This method of screening gives a measure of the global response to those proteins, but makes it difficult to track individual epitope specific responses within donors (Woodberry et al., 2005; Bihl et al., 2007).

The following study was designed to identify KSHV-specific latent responses taking these variables into account, thereby increasing the likelihood of identifying a wider range of T cell responses. Firstly, the study focused on identifying T cell responses within KSHV-infected healthy donors who showed no obvious KSHV disease and were likely effectively controlling their infection. Secondly, all the donors were recruited regardless of their HLA type, increasing the likelihood of identifying the maximal number of epitopes. Finally, the approach used to screen the donors and identify responses increased the chances of identifying both CD8 and CD4 T cell responses.

3.1 ELISpot screening for KSHV latent protein T cell responses

To characterise the T cell response to KSHV latent antigens in healthy donors and identify T cell epitopes, 30 healthy Gambian donors were recruited to this study who were HIV, Hepatitis B and Hepatitis C negative with no obvious KSHV disease.

Blood specimens were collected from each donor and plasma isolated and tested for evidence of KSHV-specific antibodies. Immunofluorescence assays were performed using the plasma on the KSHV infected PEL line BCBL-1, to detect LANA-specific responses as described in the materials and methods (Gao et al., 1996). Fourteen donors were identified as being KSHV positive and 16 donors as KSHV negative by this assay (data not shown).

PBMCs from the KSHV seropositive and KSHV seronegative donors, as controls, were then used in IFN-γ ELISpot assays to identify KSHV-specific T cell responses. Here PBMCs from the donors were stimulated with a series of 237 15mer peptides (overlapping by 10 amino acids) spanning the sequences of the four well-defined latency proteins namely LANA (excluding the acidic repeat sequence), vFLIP, vCyclin and Kaposin, to screen for responses. The ELISpot assays were carried out as described in Chapter 2 with the peptides combined into 18 pools in sequential order, each containing 12-13 peptides, to minimise the number of PBMCs required due to the limited number of cells. The peptide pools were added to 4 x 10^5 PBMCs per well in duplicate in the ELISpot assays. The magnitude of the responses were calculated from the mean of the duplicate wells with the result adjusted to no. of spots/10^6 cells. The PBMCs were incubated with phytohaemagglutinin (PHA) as a positive control and with the peptide solvent DMSO as a negative control.

Figure 3.1A shows examples of the results of these ELISpot assays from two seronegative donors KS08 and KS23, with the results presented as graphs of number spots per 10^6 cells versus peptide pools. PBMCs from these donors induced few or no

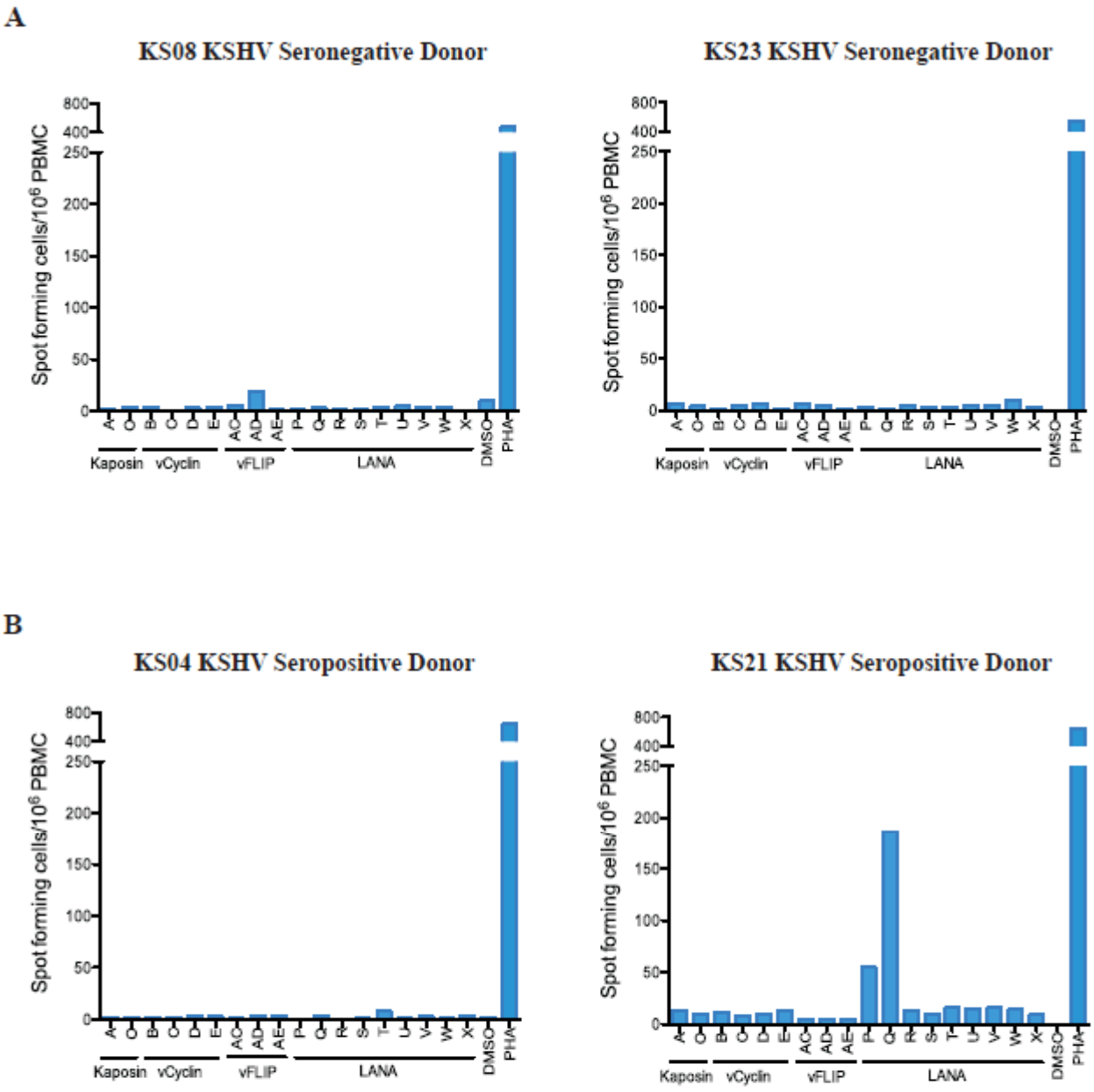


Figure 3.1 Examples of ELISpot responses to KSHV latent proteins in KSHV positive and negative donors. The KSHV latent peptide pools spanning the amino acid sequences of Kaposin, vCyclin, vFLIP and LANA were added to the PBMCs in the ELISpot assay that had been plated at 4 x 10^5 cells per well, the magnitude of the response calculated from the mean of duplicate wells and the result adjusted to no. of spots/10^6 cells. The PBMCs were incubated with phytohaemagglutinin (PHA) as a positive control and with the peptide solvent DMSO as a negative control. (A) ELISpot responses from two KSHV negative donors, KS08 and KS23. (B) ELISpot responses from two KSHV positive donors, KS04 and KS21.

spots in response to stimulation with all of the peptide pools. Similarly, figure 3.1B shows results from assays conducted on PBMCs of two KSHV seropositive donors, donor KS21 and KS04. PBMCs from KS21 show a clear response to the LANA peptide pools, P and Q. However, PBMCs for donor KS04 showed no obvious response to any of the latent antigen peptide pools. This latter pattern of result was representative of the majority of KSHV seropositive donors responses in the ELISpot screens. Figures 3.2A and B show the ELISpot responses to the peptide pools for each of the different antigens for the seronegative and seropositive donors respectively. Overall, PBMCs from seronegative donors generally showed no or weak responses to stimulation with the KSHV latent peptide pools. PBMCs from the majority of the seropositive donors also showed no or weak responses to these peptide pools, despite all of the donors PBMCs being capable of producing IFN-γ in response to the T cell mitogen PHA. Statistical analysis was conducted using the Wilcoxon two-sample test using SAS software (SAS) and statistical differences were seen when comparing responses made by seropositive versus seronegative donors to pools Q, T and W ($p \leq 0.03$), however the weakness of these responses questions the biological significance of these statistics.

Figures 3.3A and B shows the breakdown of the responses that were made by the donors, with each of the different coloured boxes representing a range of the number of spot forming cells (SFC) produced per 10^6 PBMCs in response to each of the peptide pools. The white boxes represent 0-20, the yellow boxes represent 21-40, the blue boxes 41-60 and finally the red boxes represent over 60 SFC/10^6 in response to an individual peptide pool. Of the KSHV seropositive donors, six made responses which were mostly weak but noticeably higher than the responses made by KSHV

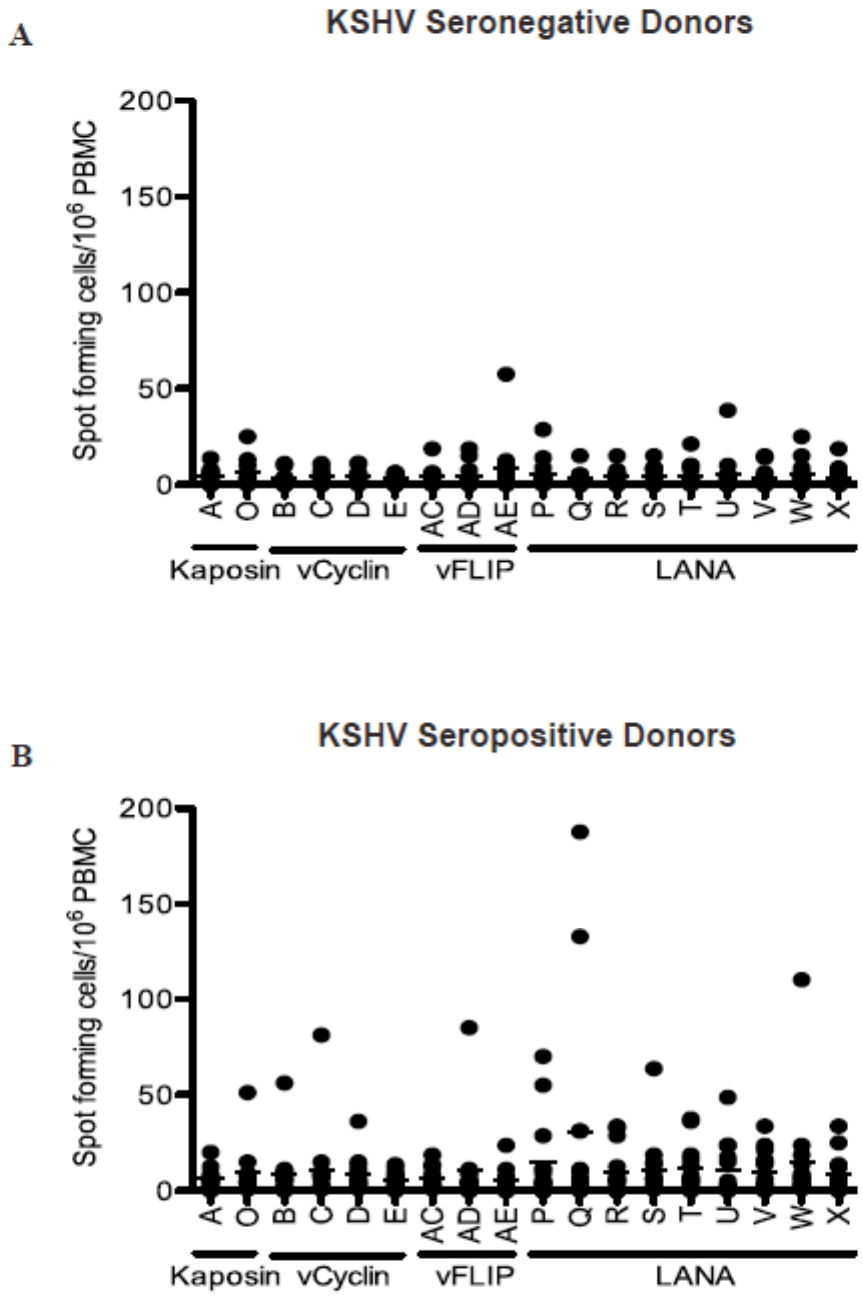


Figure 3.2 Summary of Gambian donors KSHV ELISpot responses to KSHV latent proteins. The KSHV latent peptide pools spanning the amino acid sequences of Kaposin, vCyclin, vFLIP and LANA were used to stimulate responses from all of the Gambian donor's PBMCs by IFN-γ ELISpot. The magnitude of the response calculated from the mean of duplicate wells and the result adjusted to no. of spots/10^6 cells. Each point on the graph represents an individual donor's response against each peptide pool and the mean of total responses against each peptide pool (A) from sixteen KSHV seronegative donors and (B) fourteen KSHV seropositive donors.

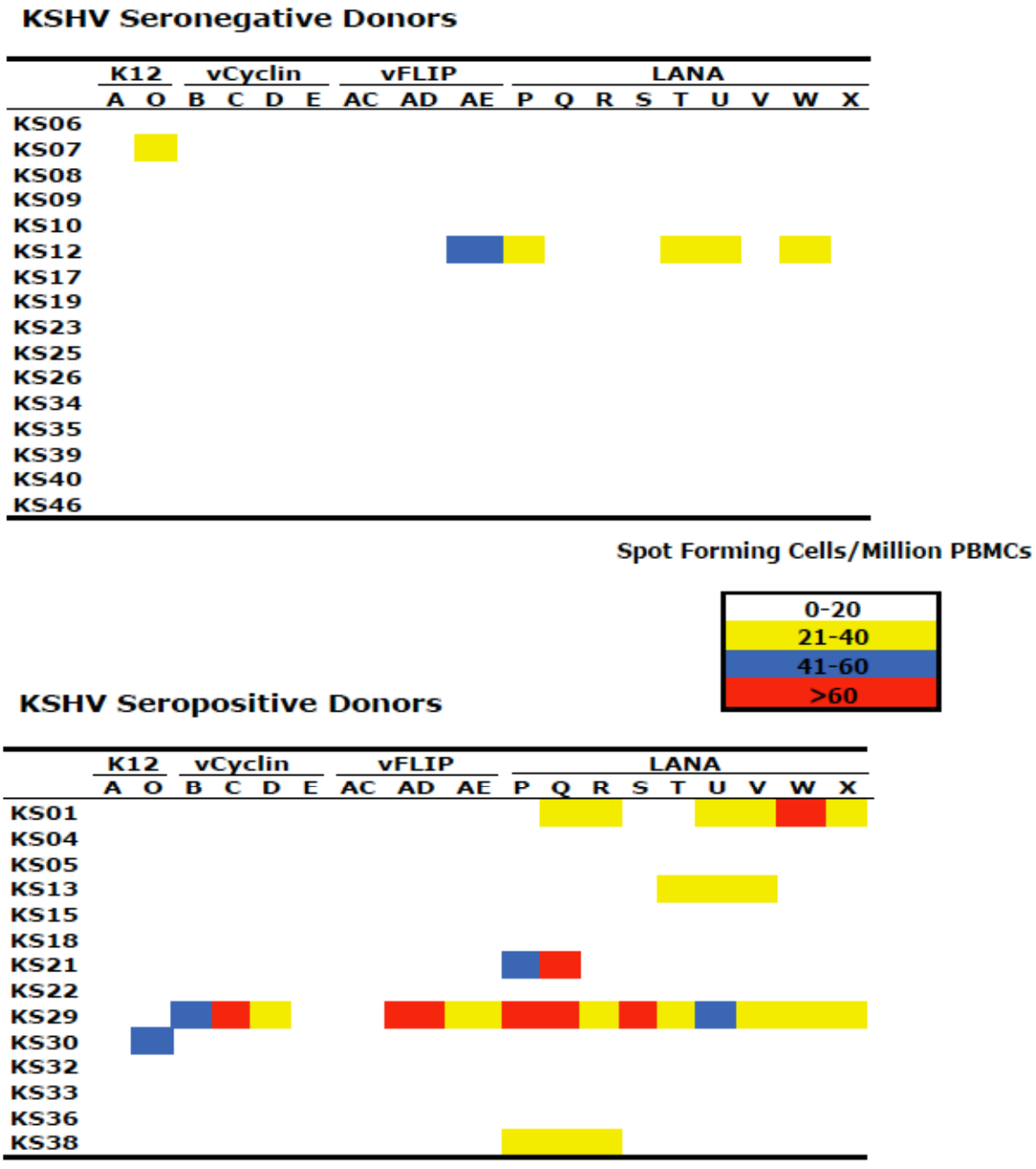


Figure 3.3 Quantitative summary of Gambian donors individual ELISpot responses to KSHV latent proteins. Each coloured box on the chart represents an individual donor's response against each of the latent KSHV proteins peptide pools in no. of spot forming cells (sfc)/10^6 PBMCs. White boxes represent 0-20, yellow 21-40, blue 41-60 and finally red over 60 sfc/10^6 PBMCs. (A) Responses from sixteen KSHV seronegative donors and (B) responses from fourteen KSHV seropositive donors.

seronegative donors. Of these six donors, four donors KS01, KS13, KS21 and KS38 targeted LANA peptides only, donor KS29 had the largest response overall targeting peptides from three latent proteins LANA, vFLIP and vCyclin, while donor KS30 responded only to one pool of the Kaposin peptides.

These results indicate that detecting KSHV-specific responses by PBMC ELISpot screening will be difficult as the responses appear very weak. However the LANA peptides appeared to be more frequently recognised by donors and induced the numerically dominant responses. Furthermore, as LANA plays a crucial role in KSHV biology, epitope mapping studies were focused on this protein using an alternative strategy, namely through the generation of LANA-specific T cell clones.

3.2 Identification and characterisation of LANA-specific clones

A panel of LANA-specific T cell clones was generated for use in epitope mapping and antigen recognition experiments. Clones were derived from the PBMCs of KSHV seropositive donors by initially expanding antigen-specific T cells and then selecting these and subjecting them to single cell cloning. PBMCs from eight KSHV sero-positive donors': KSB1, KS01, KS13, KS18, KS21, KS29, KS30 and KS48, were stimulated for one week with pools of overlapping peptides that spanned the LANA sequence (excluding the acidic repeat region) at a concentration of 0.5μg/ml to establish polyclonal cultures enriched in peptide-specific T cells. On day 8, cells were restimulated with peptide and those secreting IFN-γ were immunomagnetically sorted using an IFN-γ secretion assay cell enrichment and detection kit (Miltenyi Biotech). These cells were then seeded at both 0.3 and 3 cells per well in microcultures with irradiated feeder cells to derive clonal populations. Within approximately 2-3 weeks,

growth of the microcultures could be observed. Proliferating microcultures were expanded using allogeneic γ-irradiated PBMC feeders for two weeks prior to screening for LANA-specific T cells.

Figures 3.4, 3.5 and 3.6 show the characterisation of three representative LANA-specific clones from three different donors using this procedure. Aliquots of the clones were initially screened for specificity by stimulation with pools of the LANA peptides, followed by assaying for T cell function by measuring IFN-γ production using an ELISA. This method was repeated against individual peptides of reactive pools to identify the clone's cognate epitope-peptide within that pool. Clones were then confirmed to be CD4+ or CD8+ T cells by staining with monoclonal antibodies specific to these markers and analysing by flow cytometry. The HLA restriction of the clones was then identified by incubating these with peptide-sensitised LCLs which shared with the T cell donor one or more HLA class II or HLA class I alleles, for CD4+ or CD8+ clones respectively, and determining which could induce IFN-γ release. The functional avidity for each clone was then determined by sensitising autologous or HLA-matched LCLs with 10-fold dilutions of peptide, incubating these with the cognate T cells and responses assayed by IFN-γ production. The concentration of peptide which gave 50% of maximal IFN-γ production was then determined to allow comparisons between the clones.

Figure 3.4 shows representative results of the characterisation experiments carried out on donor KSB1 clone 33 showing specificity in the initial IFN-γ screen by ELISA to one of the nine LANA pools, pool S, which contains thirteen peptides. The peptide

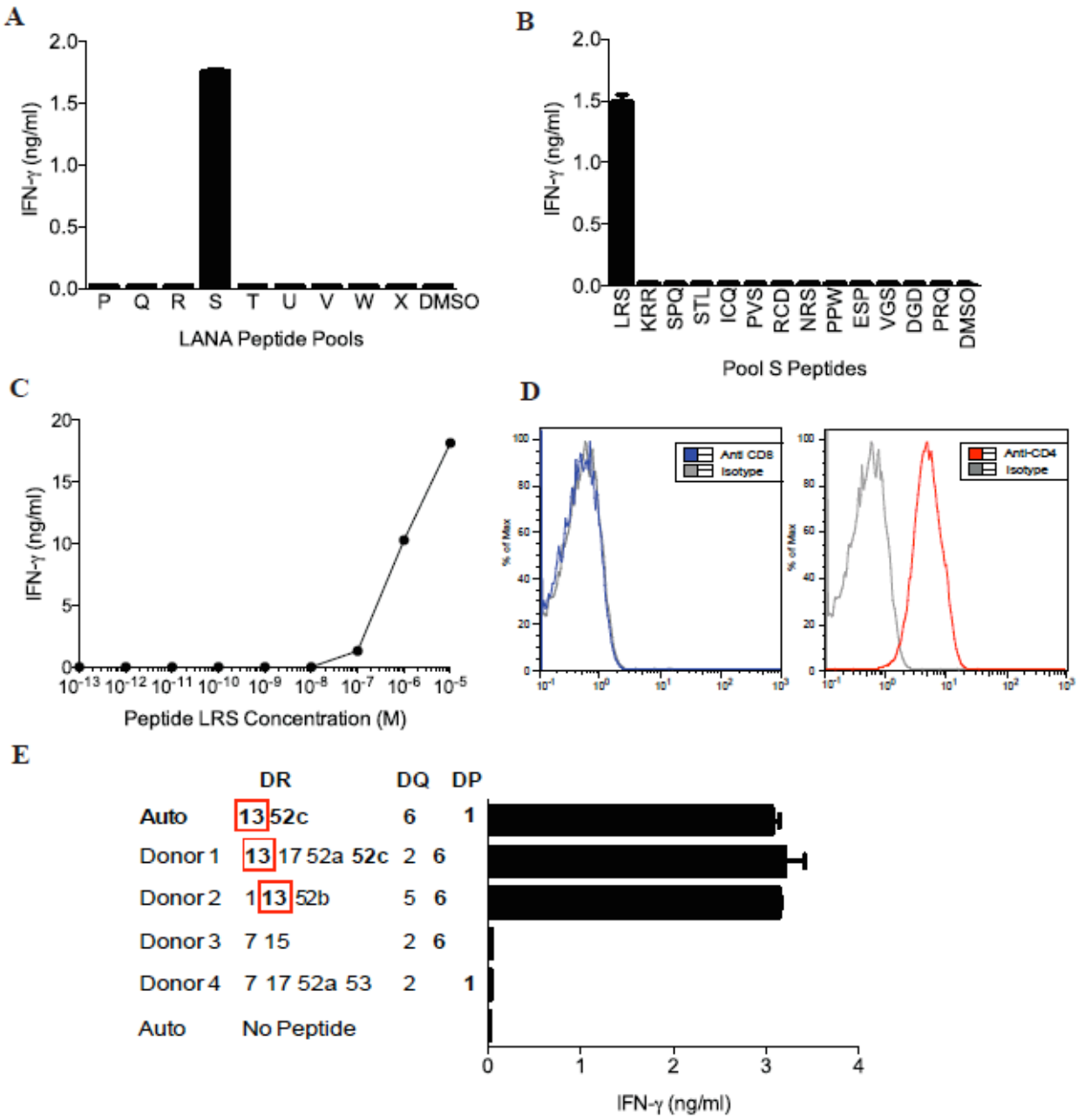


Figure 3.4 Identification of LANA epitope LRS and characterisation of LRS-specific clone. LANA-specific clone 33 from donor KSB1 was screened for IFN-γ release by ELISA after stimulation with (A) the LANA-peptide pools and (B) individual peptides from the reactive pool S. (C) The clone was assessed for its functional avidity by incubating the T cells with their autologous LCL which had been pre-sensitised with 10 fold dilutions of cognate peptide LRS and the concentration of peptide which elicited 50% of the maximal IFN-γ release as the end point. (D) Flow cytometric analysis of CD8 or CD4 expession of the LRS clone. (E) HLA restriction of the clone was assessed by incubating LRS-sensitised partially HLA matched LCLs with the LRS-specific T cell clone and IFN-γ release assayed.

solvent DMSO was used as a negative control (Figure 3.4A). The T cell response to pool S was mapped to peptide LRS on subsequent screening against the individual peptides (Figure 3.4B). This clone had a functional avidity of 10^{-6} M, determined as the concentration of peptide which elicited 50% of the maximal IFN-γ release (Figure 3.4C). The clone was confirmed as a CD4+ clone by expression of CD4 by flow cytometry analysis (Figure 3.4D). HLA class II restriction mapping indicated that the clone secreted IFN-γ in response to peptide-loaded autologous LCL and also peptide-loaded LCLs that were HLA-DR13 matched (Figure 3.4E). Thus it can be deduced that the 15-mer peptide LRS is presented in the context of HLA-DR13.

Figure 3.5 shows representative results of the characterisation experiments on donor KS48 clone 10 showing specificity to pool Q in the initial screen (Figure 3.5A). Pool Q contains thirteen LANA peptides and the response was mapped to peptide GSP upon screening individual peptides (Figure 3.5B), with the clone having a functional avidity of 10^{-7} M (Figure 3.5C). This clone was confirmed to express CD4 by flow cytometric analysis (Figure 3.5D). HLA class II restriction mapping indicated the clone secreted IFN-γ in response to peptide-loaded autologous LCL and also peptide-loaded LCLs that were HLA-DQ7 matched (Figure 3.5E). Thus it can be deduced that the 15-mer peptide GSP is presented in the context of HLA-DQ7.

Figure 3.6 shows representative results of the characterisation experiments for the only CD8+ LANA-specificity that was found in the T cell cloning screens. The LANA-specific clone 12 from donor KS021 showed specificity to LANA pool S in the initial screen (Figure 3.6A). Subsequent screening against the individual LANA peptides within pool S revealed the two LANA peptides, NRS and PPW, induced

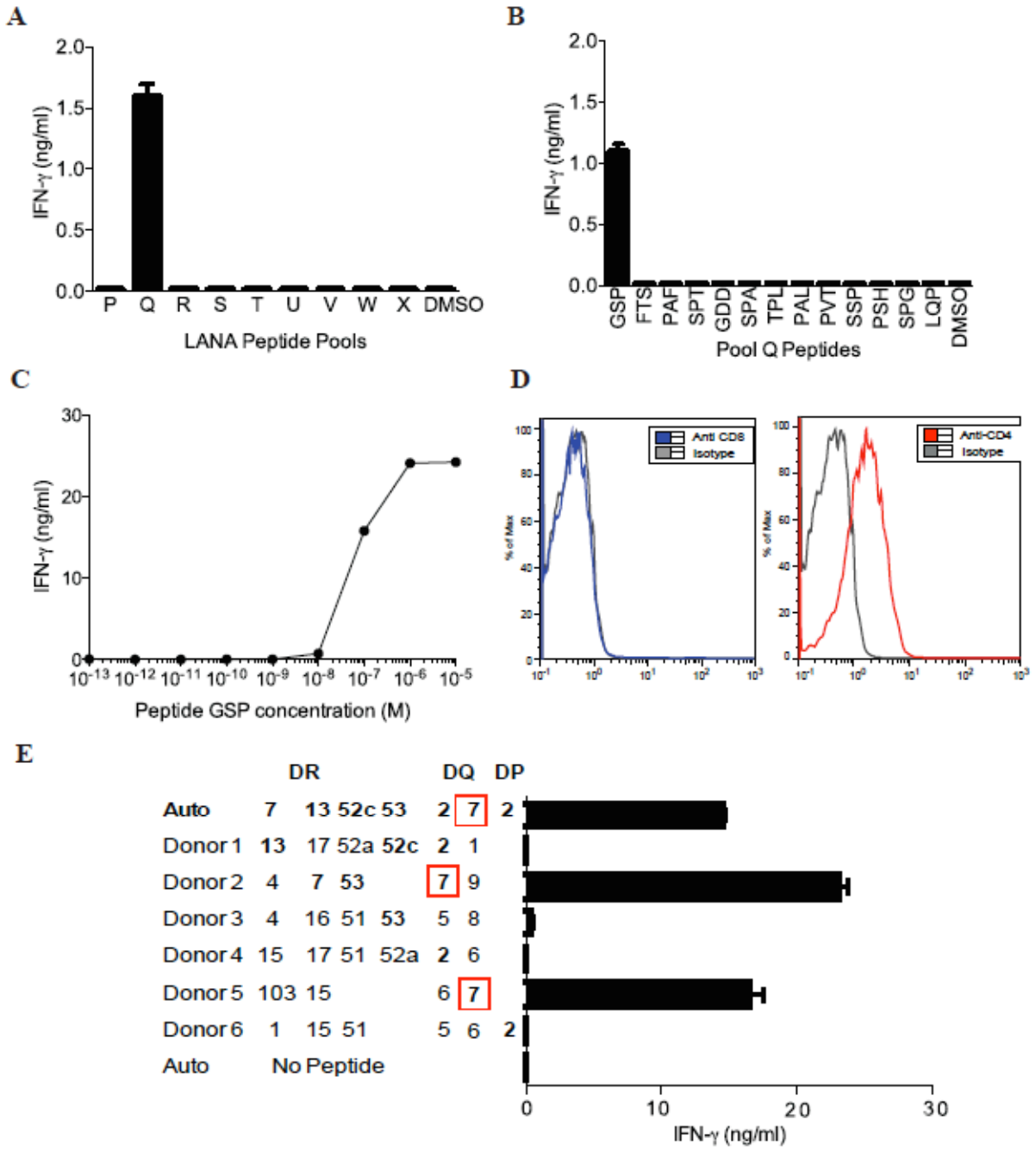


Figure 3.5 Identification of LANA epitope GSP and characterisation of GSP-specific clone. LANA-specific clone 10 from donor KS48 was screened for IFN-γ release by ELISA after stimulation with (A) the LANA-peptide pools and (B) individual peptides from the reactive pool Q. (C) The clone was assessed for its functional avidity by incubating the T cells with their autologous LCL which has been pre-sensitised with 10 fold dilutions of cognate peptide GSP and the concentration of peptide which elicited 50% of the maximal IFN-γ release as the end point. (D) Flow cytometric analysis of CD8 or CD4 expession of the GSP clone. (E) HLA restriction of the clone was assessed by incubating GSP-sensitised partially HLA matched LCLs with the GSP-specific T cell clone and IFN-γ release assayed.

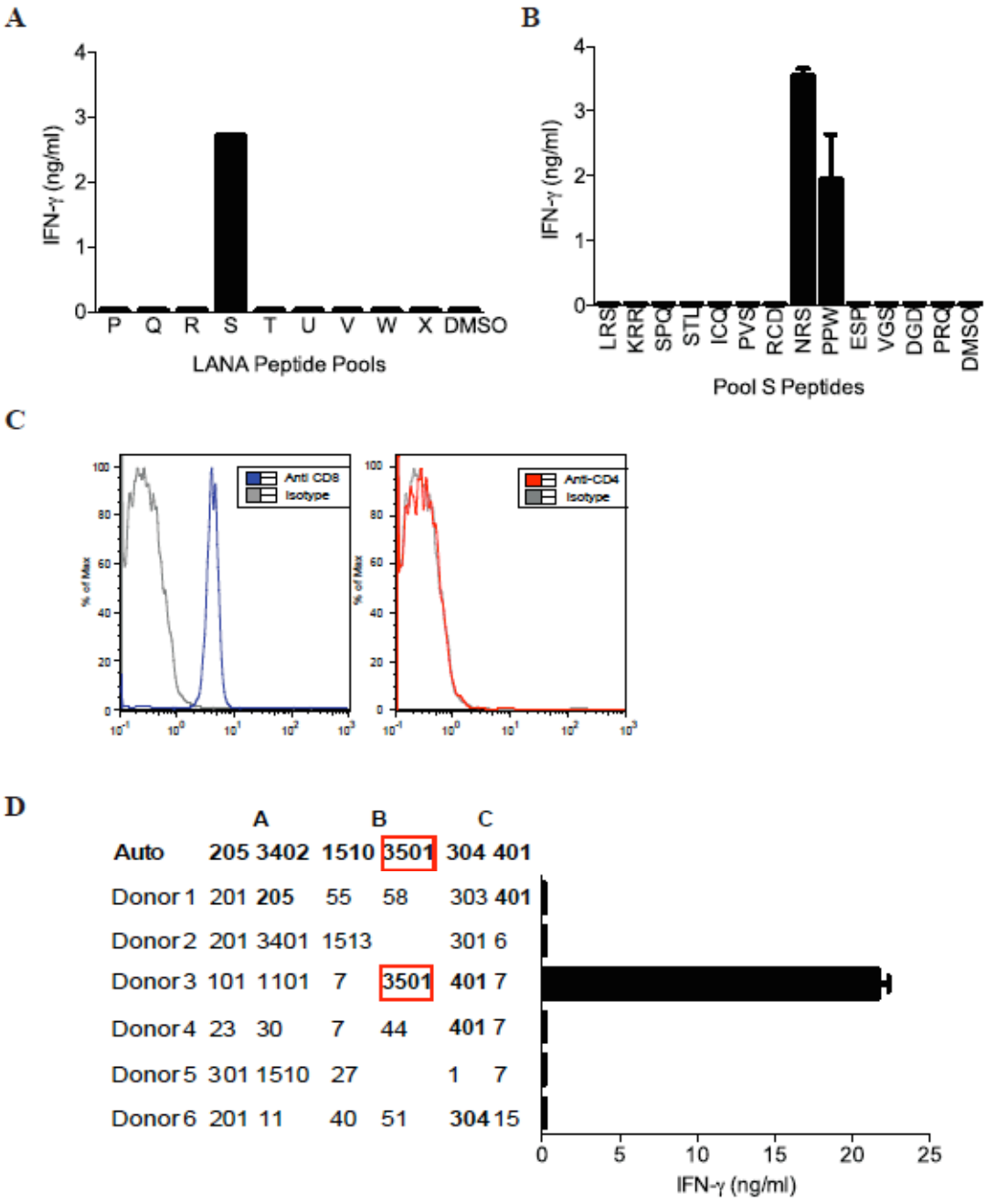


Figure 3.6 Identification of a LANA-specific CD8 T cell clone and its characterisation. LANA-specific clone 12 from donor KS21 was screened for IFN-γ release by ELISA after stimulation with (A) the LANA-peptide pools and (B) individual peptides from the reactive pool S. (C) Flow cytometric analysis of CD8 or CD4 expression of the clone. (D) HLA restriction of the clone was assessed by incubating peptide-sensitised partially HLA matched LCLs with the LANA-specific CD8 T cell clone and IFN-γ release assayed.

IFN-γ secretion from the clone (Figure 3.6B). The clone was confirmed to be CD8+ by flow cytometric analysis (Figure 3.6C). Upon HLA class I restriction analysis, the clone secreted IFN-γ in response to peptide-loaded LCLs that were matched only at HLA-B*3501 (Figure 3.6D); in this case no autologous LCL was available for this clone. Thus it can be deduced that the cognate peptide is presented in the context of HLA-B*3501.

Unlike class II presented peptides, the peptide binding groove of the class I molecule anchors the ends of the peptide within the molecule, consequently restricting their length. As such, most class I epitope-peptides then have a well defined minimal sequence which is between 8-10 amino acids. As such the minimal peptide-epitope sequence was then determined. The identification of HLA-B*3501 as the class I molecule presenting the epitope contained within the two peptides, NRS and PPW, assisted in the prediction of the minimal CD8 epitope, since many MHC-alleles contain characteristic peptide-binding motifs. B*3501 preferentially binds peptides with a proline in the second anchor position and quite frequently with a tyrosine as the final residue (Parham et al., 2000). The peptides NRSVYPPWATESPIY and PPWATESPIYVGSSS, which are both recognised by the LANA CD8 clone, overlap by the following sequence PPWATESPIY. The ten amino acid overlap contains two prolines at the beginning of the sequence and a tyrosine at the end, which is consistent with HLA-B35 epitope predictions. Consequently, three peptides were synthesised, the 9mer sequence PWATESPIY, the 10mer sequence PPWATESPIY and the 11mer sequence YPPWATESPIY.

To identify the minimal epitope, the LANA-specific CD8 T cell clone was then tested against a HLA-B*3501 positive LCL sensitised with the synthesised peptides. In this case, chromium release cytotoxicity assays were used as these gave clearer responses, compared to the IFN-γ secretion assays (data not shown). LCLs were individually sensitised with the truncated peptides and NRS 15mer peptide for comparison at decreasing peptide concentrations from 10^{-7}M to 10^{-12}M. The peptide solvent DMSO was used as a negative control and is presented as point 0 on the graph in Figure 3.7. T cells were incubated with the targets at an effector/target ratio of 1:1. Figure 3.7 shows that the peptide which stimulated the highest % lysis at the lowest concentrations was the 10mer peptide PPWATESPIY, suggesting it is the minimal epitope sequence. Henceforth, we refer to this novel CD8 epitope as PPW.

In total, fourteen LANA-specific epitopes were identified from five different donors; thirteen of the epitopes were HLA class II restricted and one HLA class I. Table 3.1 summarises the characteristics of the LANA-specific CD4+ and CD8+ T cell clones identified from five different donors. Table 3.1 shows that three of the donors' clones have specificity to one particular class II presented peptide, peptide PAF, each in the context of a different HLA-type including DQ6, DQ7 and DR52b, suggesting the promiscuity of this peptide. Furthermore, the frequency of T cell clones specific to each of the isolated T cell targets was very low, with the exception of those isolated towards CD4+ T cell targets LAP and LRS, suggesting a potential immunodominance towards these peptides in donor KSB1.

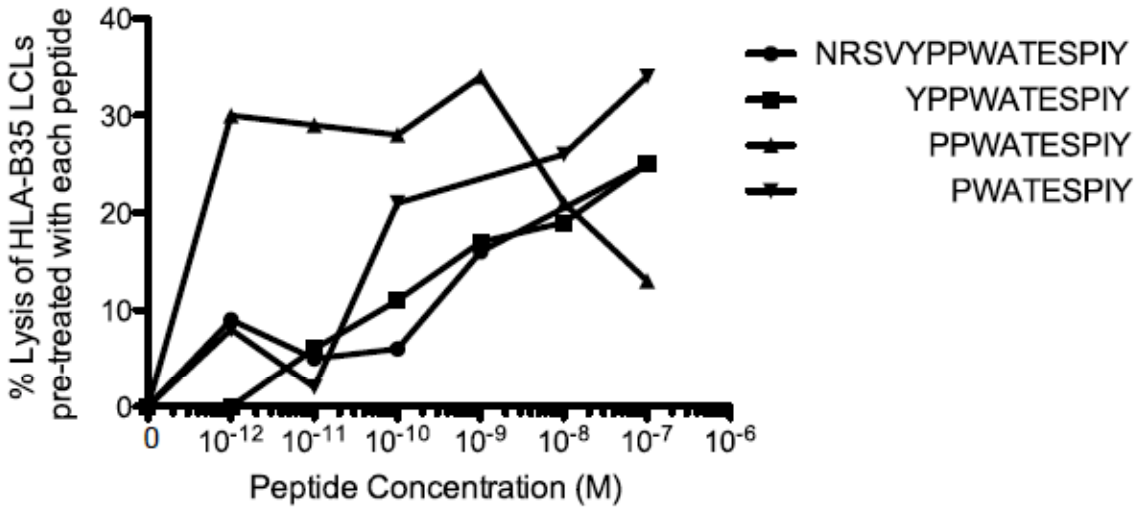


Figure 3.7 Characterisation of the minimal length of the LANA CD8 epitope. Truncated versions of the NRS 15mer peptide were tested in triplicate by chromium release assay over a wide range of concentrations for recognition by the LANA-specific CD8 clone. Target cells were HLA-matched B35 LCLs pulsed with peptide at concentrations of 10^{-7}M to 10^{-12}M with the peptide solvent DMSO as a negative control presented as point 0 on the graph. Targets cells were incubated with CD8 T cells at a target/effector cell ratio of 1:1.

Table 3.1 LANA-specific T cell clones generated

	Donor	Clone	Amino Acid position	Peptide Sequence	HLA Restriction	Functional Avidity	No. of T cell clones generated
CD4+	KSB1	C114	36-50	GDDLHLQPRRKHVAD	DR13	10-7	3
	KSB1	C71	76-90	PAFVSSPTLPVAPIP	DQ6	10-7	5
	KSB1	C86	191-205	LAPSTLRSLRKRRLS	DP1	10-8	17
	KSB1	C33	196-210	LRSLRKRRLSSPQGP	DR13	10-6	6
	KSB1	C110	191-205	LAPSTLRSLRKRRLS	DQ6	10-8	10
			196-210	LRSLRKRRLSSPQGP			
	KSB1	C204	276-290	WGDDTAMLVLLAEIA	DQ6	10-8	1
	KSB1	C63	982-996	EYRYVLRTSPPHRPG	DQ6	10-8	1
	KSB1	C55	1052-1066	CQWKFAVIFWGNDPY	DR52c	10-6	1
	KSB1	C39	1077-1091	FGGVKAGPVSCLPHP	DP11	nd	3
	KS29	C19	76-90	PAFVSSPTLPVAPIP	DR52b	10-8	1
	KS18	c15	1046-1071	RRDPKCQWKFAVIFW	nd	nd	1
	KS48	C10	66-80	GSPTVFTSGLPAFVS	DQ7	10-7	1
	KS48	C14	76-90	PAFVSSPTLPVAPIP	DQ7	10-6	1
CD8+	KS21	C12	236-245	PPWATESPIY	B3501	10-7	3

3.3 Identification and characterisation of vCyclin and vFLIP epitopes

In a further series of T cell cloning experiments, epitopes were sought in two other latent KSHV-proteins, namely vCyclin and vFLIP. Here the T cell clones were established using material from a local donor of Kenyan origin, KSB1, rather than the limited PBMC samples from the Gambian donors.

A series of 50 15mer peptides that covered the entire protein sequence of vCyclin were grouped into four pools, each containing 12-13 peptides and used to stimulate KSB1 PBMC cultures and establish clones similar to experiments conducted in section 3.2. Figure 3.8 shows representative results of the characterisation experiments carried out on clone 31. In the initial IFN-γ ELISA screen this clone showed specificity to the vCyclin pool E, which contains thirteen peptides (Figure 3.8A). Subsequently, the T cell response to pool E was mapped to peptide QIL (Figure 3.8B) and found to have a functional avidity of 10^{-6} M (Figure 3.8C). The clone was confirmed to express CD4 by flow cytometry staining analysis (Figure 3.8D). HLA class II restriction mapping indicated the clone secreted IFN-γ in response to peptide-sensitised autologous LCL and also peptide-sensitised LCLs that were HLA-DR52c matched (Figure 3.8E). Thus it can be deduced that the 15-mer peptide QIL is presented in the context of HLA-DR52c. A second vCyclin-specific CD4 clone was also identified in this manner which responded to the overlapping peptides TFQ and LTS presented by HLA-DR13, as summarised in Table 3.2.

A third vCyclin specificity was also identified in the cloning analysis. Figure 3.9 shows representative results of the characterisation experiments carried out on clone c69, showing specificity in the initial IFN-γ screen by ELISA to vCyclin pool B,

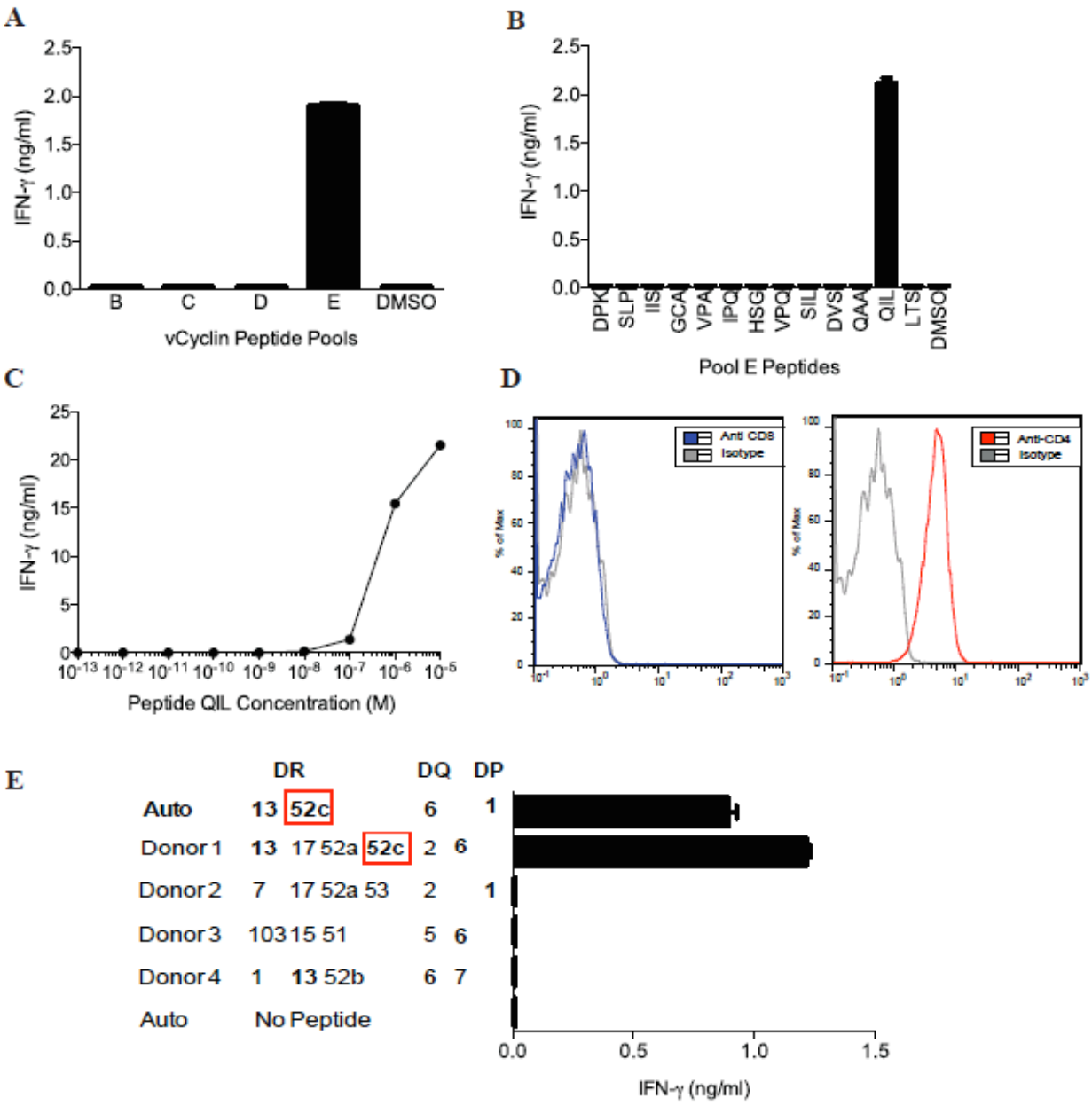


Figure 3.8 Identification of vCyclin-specific epitope QIL and characterisation of the QIL clone. vCyclin-specific clone 31 from donor KSB1 was screened for IFN-γ release by ELISA after stimulation with (A) the vCyclin-peptide pools and (B) individual peptides from the reactive pool E. (C) The clone was assessed for its functional avidity by incubating the T cells with their autologous LCL which had been pre-sensitised with 10 fold dilutions of the cognate peptide-epitope QIL and the concentration of peptide which elicited 50% of the maximal IFN-γ release as the end point. (D) Flow cytometric analysis of CD8 or CD4 expression of the QIL clone. (E) HLA restriction of the clone was assessed by incubating the QIL pre-sensitised partially HLA matched LCLs with the QIL-specific T cell clone and IFN-γ release assayed.

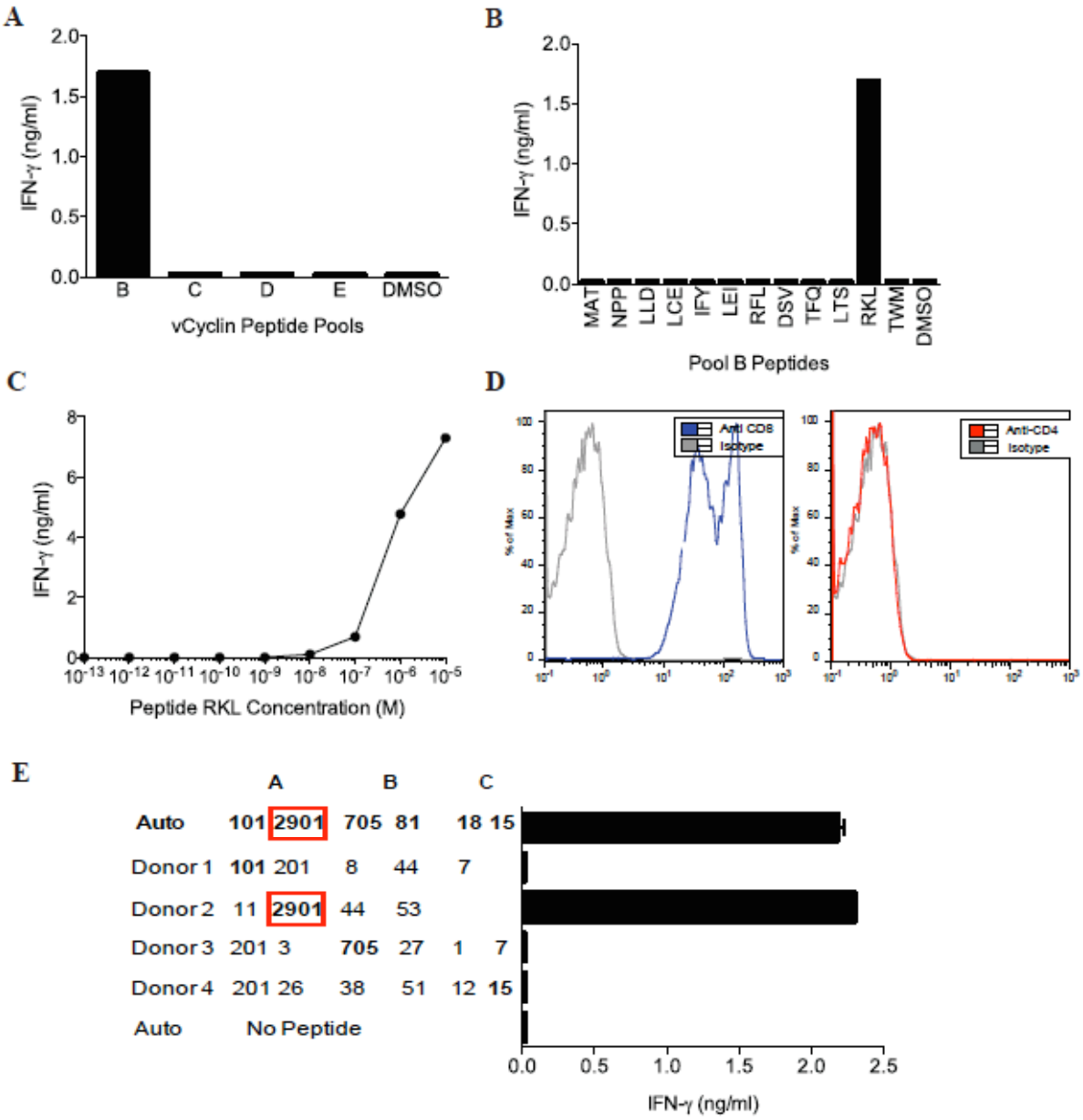


Figure 3.9 Identification of vCyclin-specific CD8 epitope and characterisation of the CD8 clone. vCyclin-specific clone 69 from donor KSB1 was screened for IFN-γ release by ELISA after stimulation with (A) the vCyclin-peptide pools and (B) individual peptides from the reactive pool B. (C) The clone was assessed for its functional avidity by incubating the T cells with their autologous LCL which had been pre-sensitised with 10 fold dilutions of the cognate peptide-epitope RKL and the concentration of peptide which elicited 50% of the maximal IFN-γ release as the end point. (D) Flow cytometric analysis of CD8 or CD4 expression of the RKL clone. (E) HLA restriction of the clone was assessed by incubating the RKL pre-sensitised partially HLA matched LCLs with the RKL-specific T cell clone and IFN-γ release assayed.

which contains twelve peptides (Figure 3.9A). Subsequently the T cell response to pool B was mapped to peptide RKL (Figure 3.9B) and this clone was found to have a functional avidity of 10^{-6} M (Figure 3.9C). The clone was confirmed to express CD8 by flow cytometry analysis (Figure 3.9D). HLA class I restriction mapping indicated the clone secreted IFN-γ in response to peptide-sensitised autologous LCL and also peptide-sensitised LCLs that were HLA-A*2901 matched. Note that we were unable to exclude HLA-B*81 or C*18 as being capable of presenting this peptide as these alleles are rare in Caucasian populations and we were unable to find partially matched LCLs which expressed them. Testing this clone against a second peptide sensitised HLA-A*2901 LCL demonstrated that it also could present this epitope suggesting HLA-A*2901 is responsible for presentation of peptide RKL (data not shown and Figure 3.9E). No clear peptide binding motif currently exists for HLA-A*2901 and so the minimal RKL CD8 epitope sequence was not determined.

Thus, the vCyclin T cell cloning identified two new CD4 target epitopes and one CD8 target epitope, the characterisation of which are summarised in Table 3.2. As can be seen the frequency of clones specific to the CD8 T cell target epitope was much higher than of that for both of the CD4 T cell target epitopes combined.

In the final cloning experiment epitopes from the KSHV latent protein vFLIP were identified using KSB1 PBMCs. In experiments similar to section 3.2 and 3.3. 36 15mer peptides that covered the entire protein sequence of vFLIP were grouped into three pools each containing 12 peptides and used to stimulate KSB1 PBMC cultures and establish clones. Figure 3.10 shows representative results of the characterisation experiments carried out on clone 17. In the initial IFN-γ ELISA screen this clone

Table 3.2 vCyclin-specific T cell clones generated

	Donor	Clone	Amino Acid position	Peptide Sequence	HLA Restriction	Functional Avidity	No. of T cell clones generated
CD4+	KSB1	C31	241-255	QILTSVSDFDLRILD	DR52c	10-6	1
CD4+	KSB1	C194	41-55 46-60	TFQQSLTSHMRKLLG LTSHMRKLLGTWMFS	DR13	10-7	1
CD8+	KSB1	C69	51-65	RKLLGTWMFSVCQEY	A2901	10-6	15

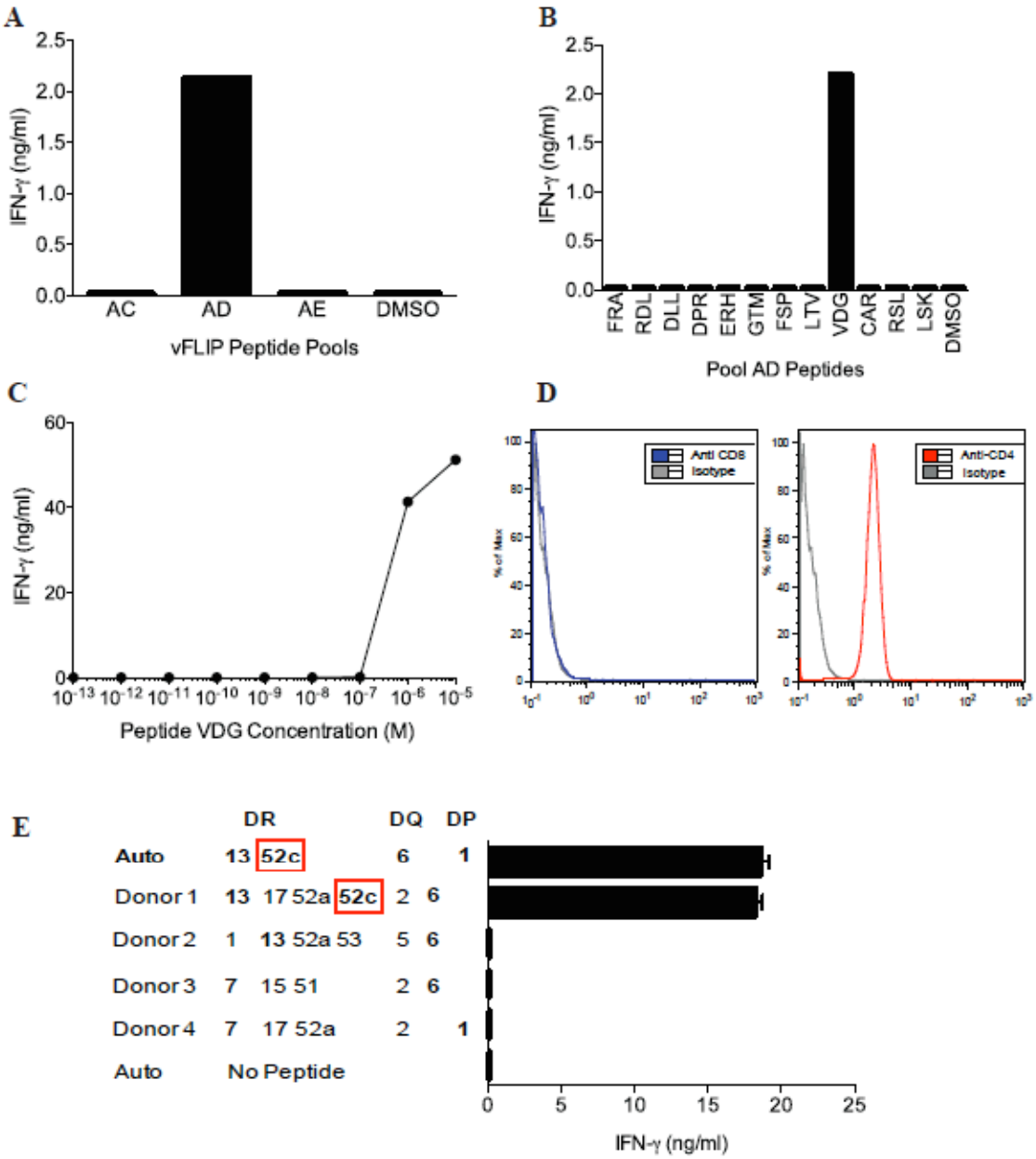


Figure 3.10 Identification of vFLIP-specific epitope VDG and characterisation of the VDG clone. vFLIP-specific clone 17 from donor KSB1 was screened for IFN-γ release by ELISA after stimulation with (A) the vFLIP-peptide pools and (B) individual peptides from the reactive pool AD. (C) The clone was assessed for its functional avidity by incubating the T cells with their autologous LCL which had been pre-sensitised with 10 fold dilutions of the cognate peptide-epitope VDG and the concentration of peptide which elicited 50% of the maximal IFN-γ release as the end point. (D) Flow cytometric analysis of CD8 or CD4 expression of the VDG clone. (E) HLA restriction of the clones was assessed by incubating the VDG pre-sensitised partially HLA matched LCLs with the VDG-specific T cell clone and IFN-γ release assayed.

showed specificity to the vFLIP pool AD, which contains twelve peptides (Figure 3.10A). Subsequently, the T cell response to pool AD was mapped to peptide VDG (Figure 3.10B) and found to have a functional avidity of 10^{-7} M (Figure 3.10C). The clone was confirmed as a CD4+ clone by expression of CD4 by flow cytometry staining (Figure 3.10D). HLA class II restriction mapping indicated the clone secreted IFN-γ in response to peptide-sensitised autologous LCL and also peptide-sensitised LCLs that were HLA- DR52c matched (Figure 3.10E). Thus it can be deduced that the 15-mer peptide VDG is presented in the context of HLA-DR52c. Using this strategy three other CD4+ T cell clones were identified and their characteristics are summarised in Table 3.3.

Within the vFLIP cloning, CD8+ T cell clones specific for one epitope were identified. Figure 3.11 shows representative results of the characterisation experiments for these clones. The vFLIP-specific clone 57 showed specificity to AD in the initial screen (Figure 3.11A), while subsequent screening against the individual vFLIP peptides within pool AD revealed the peptide-epitope was contained in the overlapping peptides GTM and FSP (Figure 3.11B). The T cell clone showed very similar functional avidity of 10^{-6}M for both peptides (Figure 3.11C). The clone was confirmed to be CD8+ by flow cytometric analysis (Figure 3.11D) and the minimal peptide-epitope sequence is most likely overlapping between the two 15mer peptides, however it was not determined.

Initial attempts to identify the HLA restriction of these GTM-specific clones were unsuccessful. Although the clone secreted IFN-γ in response to the peptide-sensitised autologous LCL, as shown in figure 3.12 (A), peptide-loaded LCLs that were matched

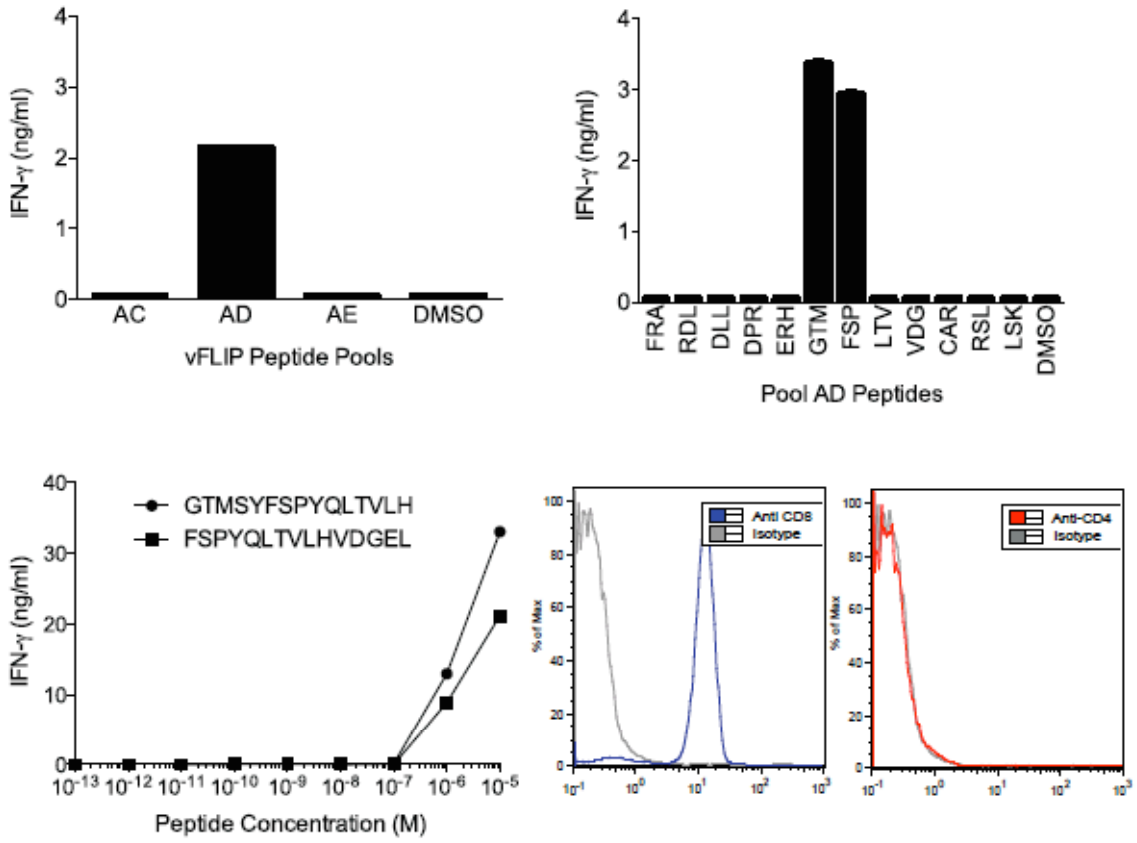
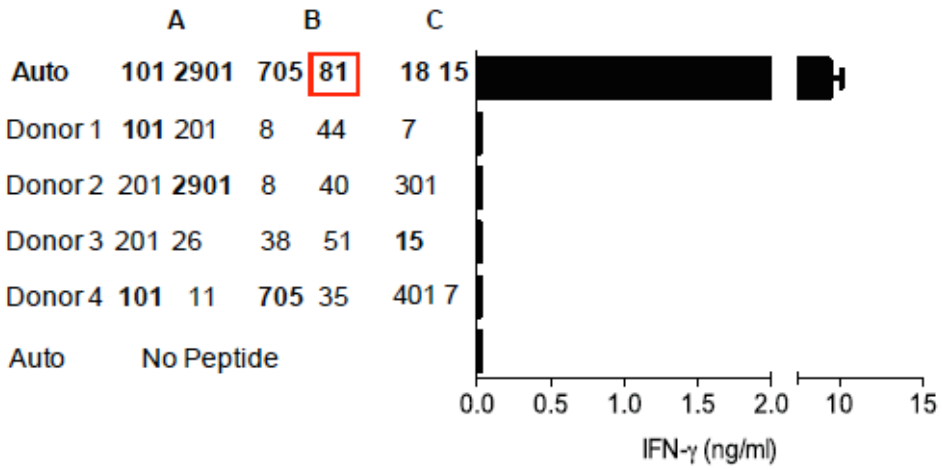


Figure 3.11 Identification of vFLIP-specific CD8 epitope and characterisation of the CD8 clone. vFLIP-specific clone 57 from donor KSB1 was screened for IFN-γ release by ELISA after stimulation with (A) the vFLIP-peptide pools and (B) individual peptides from the reactive pool AD. (C) The clone was assessed for its functional avidity by incubating the T cells with their autologous LCL which had been pre-sensitised with 10 fold dilutions of the cognate peptide-epitope and the concentration of peptide which elicited 50% of the maximal IFN-γ release as the end point. (D) Flow cytometric analysis of CD8 or CD4 expression of the vFLIP clone.

A



B

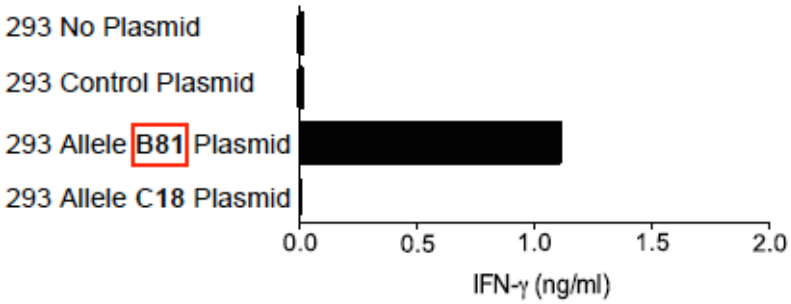


Figure 3.12 HLA-restriction of vFLIP-specific CD8 T cell clone. (A) HLA restriction of the clone was assessed by incubating peptide pre-sensitised autologous LCL and partially HLA-matched LCLs with the vFLIP-specific CD8 T ce ll clone and IFN-γ release assayed. (B) Non-transfected 293 cells and 293 cells that had been transfected with the control, B81 or C18 expression plasmids were pre-sensitised with peptide and incubated with the vFLIP-specific CD8 T cell clone and IFN-γ release assayed.

through HLA-A*0101, A*2901, B*705 and C*15 were not recognised by the clone. This implied that the HLA class I molecules B*81 or C*18 may be responsible for presentation of the peptide-epitope however other than the KSB1 LCL, no LCLs or other cell lines which expressed these molecules were available. Consequently, the cDNAs encoding HLA-B*81 and C*18 were cloned into the plasmid expression vector pLZRS-NGFR and these as well as an empty vector control plasmid transfected into the epithelial cell line 293. These transfected cells were then used as targets in recognition assays where transfected and non-transfected 293 cells were peptide-loaded and tested for recognition by the CD8 clone using the same method as described for LCLs. Figure 3.12 (B) shows that only 293 cells transfected with HLA-B*81 were capable of presenting the CD8 vFLIP peptides to the T cells and inducing recognition. Thus it can be deduced that the vFLIP CD8 epitope sequence is presented in the context of HLA-B*81.

Table 3.3 provides a summary of the vFLIP T cell responses identified and the characterisation of all the vFLIP clones from donor KSB1. Four novel CD4 responses were identified, three of which were restricted to the MHC Class II DR52c allele and one to the DR13 allele. However, the frequency of the clones isolated which were specific to these CD4 epitopes was low relative to the frequency of clones identified specific to the CD8 epitope.

Discussion

This study identified T cell responses to epitopes within the KSHV latency proteins made by a cohort of healthy KSHV-infected donors. T cell clones were successfully generated and used to identify target epitopes restricted across a range of HLA types

Table 3.3 vFLIP-specific T cell clones generated

	Donor	Clone	Amino Acid position	Peptide Sequence	HLA Restriction	Functional Avidity	No. of T cell clones generated
CD4+	KSB1	C17	159-173	VDGELCARDIRSLIF	DR52c	10-7	1
	KSB1	C230	65-80	RDLLRDLLHLDPRFL	DR52c	10-7	1
			71-85	DLLHLDPRFLERHLA			
	KSB1	C141	164-178	CARDIRSLIFLSKDT	DR13	10-6	3
	KSB1	C107	85-100	LAECLFRAGRRDLLR	DR52c	10-6	1
CD8+	KSB1	C57	91-105	GTMSYFSPYQLTVLH	B81	10-6	20
			96-110	FSPYQLTVLHVDGEL			

within the KSHV latent proteins LANA, vCyclin and vFLIP. LANA elicited the highest number of T cell targets with fourteen epitopes being identified within its sequence, followed by five in vFLIP and three in vCyclin. These responses were restricted through a range of HLA types. Furthermore, the frequency of CD4 epitopes identified appears to be much higher than those for the CD8's. All the epitope specificities and CD4 or CD8 phenotypes of the T cell clones produced to the KSHV latent proteins, together with their relative amino acid positions are summarised in figure 3.13.

IFN-γ ELISpot screens were initially used to examine the size of the ex-vivo response to the KSHV latent proteins and to map epitopes within these proteins. However the overall ex-vivo response in these screens was very weak and consequently it would have been difficult to identify epitopes using this strategy. Moreover, these ELISpot responses were substantially lower when compared to those made to the other human γ-herpesvirus, EBV, in a study conducted on a similar population (Njie et al., 2009). Furthermore, responses to LANA appear considerably weaker than those made to its EBV homologue, EBNA1, in donors from this (Njie et al., 2009) and other populations (Blake et al., 2000; Fogg et al., 2009).

Previous studies have used similar methods to screen for T cell responses within the KSHV lytic and latent proteins, the size of these T cell responses has been shown to vary within the different KSHV infected cohorts. The KSHV ELISpot screen responses detected in this study were lower than those reported in similar studies carried out on HIV co-infected individuals receiving HAART therapy (Bihl et al., 2007; Woodberry et al., 2005). However, the responses in the healthy carriers did appear more frequent than those described in HIV infected donors with KS before

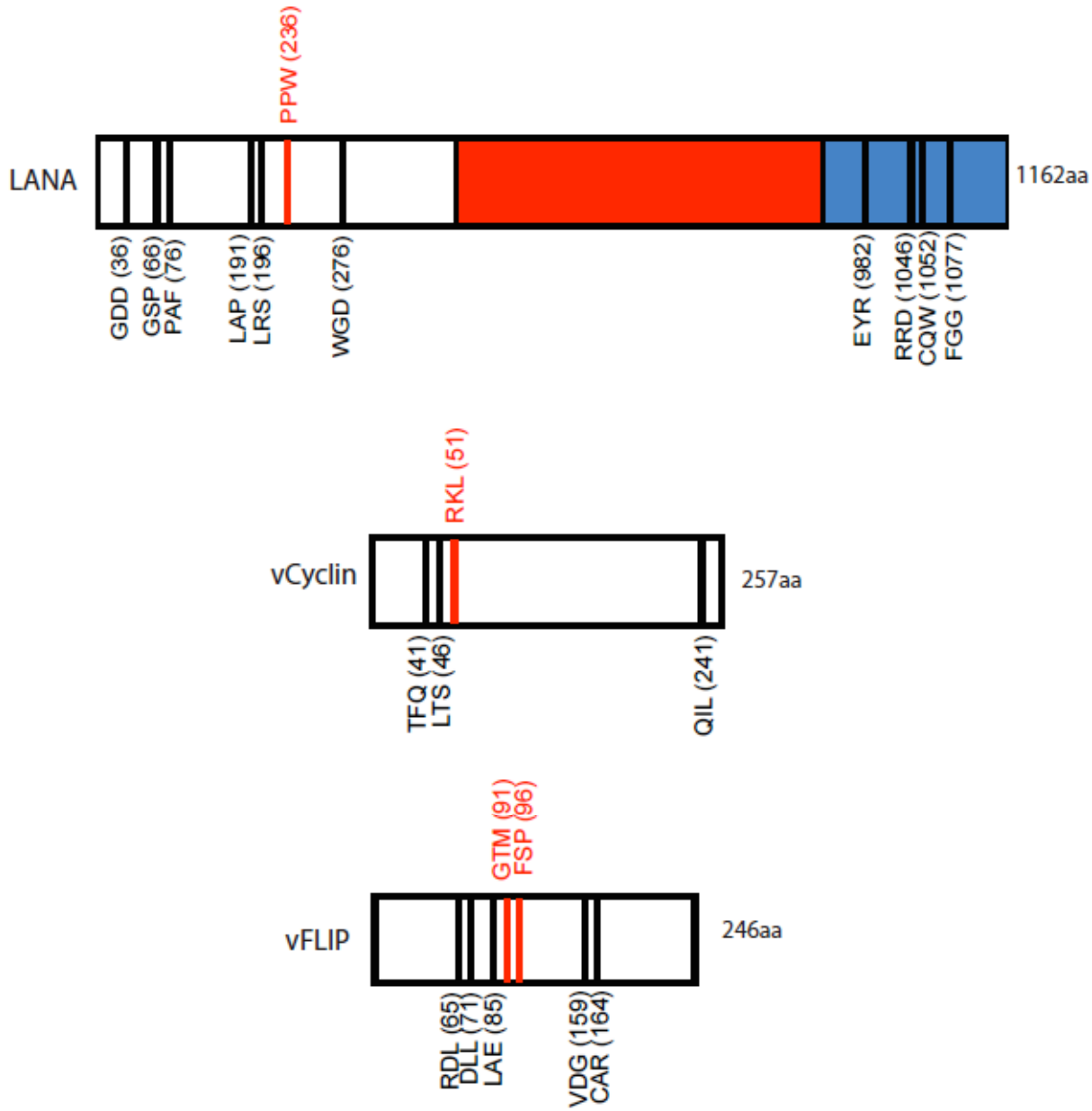


Figure 3.13 Location of T cell epitopes within the primary sequences of LANA, vCyclin and vFLIP. A diagrammatic representation of the LANA, vCyclin and vFLIP proteins, showing the location of the T cell epitopes used in this study. Positions for CD8+ epitopes are shown above by the red lines and for the CD4+ epitopes below by the black lines. Each epitope is represented by the first three amino acids of its sequence and the epitope start position is shown in brackets.

HAART treatment (Bihl et al., 2007) or transplant patients with KS prior to changing their immunosuppressive regime (Barozzi et al., 2008). An additional factor that may play a part in determining these variable T cell responses may be differences in KSHV virus loads present in these cohorts. Higher viral loads have been reported in HIV co-infected patients receiving HAART than in healthy KSHV infected individuals (Bourboulia et al., 2004; Qu et al., 2010), suggesting that higher viral loads may be driving the immune response. Although the viral loads were not tested in this study, based on published studies to date in KSHV infected healthy donors (Qu et al., 2010), it would be expected to be low.

Additional contributing factors to the weak IFN-γ ELISpot responses detected in this study may be the sequences of the peptides that were used to stimulate responses from these donors. The sequence of the manufactured peptides was derived from the BC-1 strain of the virus, which is classified as a North American A2 subtype virus. The donors used in this study however may be infected with a different subtype as the circulating strains in sub-Saharan Africa are predominantly B-type, based on sequence analysis of the hypervariable K1 and K15 genes present at each end of the genome, potentially resulting in amino acid differences in the sequences of the viral latent proteins (Zong et al., 2002). Few studies have examined sequence diversity of the latent proteins, however the available sequence analyses of LANA indicate that outside of the extensive repeat regions, this protein shows high levels of conservation between different strains (Piolot et al., 2001; Zhang et al., 2000). Furthermore, in this study a number of LANA epitopes were derived from a variety of donors, suggesting that the amino acid sequence of LANA is largely conserved.

As well as these variables, an additional factor that may have contributed to these weak responses, was the use of 15mer overlapping peptides to stimulate responses. These peptides were initially designed for the mapping of CD8+ T cell responses, consequently CD4+ T cell responses may have been missed as a result of the 10 amino acid overlap present between these 15mer peptides, as indeed others have found 15 mer peptides as used here tend to preferentially stimulate CD8+ T cells over CD4+ T cells (Draenert et al., 2003). However, the diversity of CD4+ responses identified to the latent proteins in the T cell clonings carried out were much greater than those of the CD8+ responses, especially for the genome maintenance protein LANA. This supports the observation that the CD4+ T cells may be the numerically dominant form of cellular immunity to LANA. This finding shows some contrast to the cellular immune responses made to the EBV homologue EBNA1 which, at least in some class I HLA contexts elicits relatively strong CD8+ responses (Fogg et al., 2009; Blake et al., 2000) and weaker but more diverse CD4+ responses (Leen et al., 2001).

It cannot, however, be formally excluded that the culture conditions used to establish the clones preferentially expanded CD4 over CD8 specificities. If there is a preferential outgrowth of CD4s as suggested by the larger diversity of CD4+ T cell specificities compared to the CD8s for each of the latent proteins in this study, the very high frequency of clones specific each of the CD8+ T cell epitopes identified within vFLIP and vCyclin highlights the magnitude of these responses. A previous study investigating the T cell response across a wide range of KSHV proteins, stimulated T cells from a range of KSHV infected cohorts for six days using monocyte-derived dendritic cells (moDCs) transduced with lentiviral expression

vectors, this revealed a higher magnitude of CD8+ T cell responses to the latent proteins LANA, vFLIP and vCylin (Robey et al., 2009). In an additional study to identify KSHV-specific T cell targets, LANA-specific HLA-A*0201 restricted CD8+ T cell responses were derived from healthy KSHV infected donors, following a 1 week stimulation peptide-loaded moDCs (Lepone et al., 2010). No such responses were identified in any of the HLA-A*0201 expressing donors used in this study, perhaps indicating that CD8+ T cell responses require an antigen-presenting cell stimulation from cells such as DCs in order to isolate these CD8 responses.

Chapter 4

CD8+ T cell recognition of cells expressing the KSHV-latent antigens LANA or vFLIP

Most viral genes are not expressed during latency, limiting the repertoire of viral antigens available for presentation to T cells by KSHV infected cells. However, the major latent viral proteins that are expressed during latent forms of infection include LANA, vFLIP, vCyclin and Kaposin. These proteins function in the establishment of latency and maintenance of the latent virus infection. They also play a role in viral oncogenesis, initiating growth and proliferative signals, evading apoptosis, inhibit pro-inflammatory signals and sustain replicative potential (reviewed in (Mesri et al., 2010)).

Similar to other herpesviruses such as EBV, the virus has evolved with humans and, having evolved in the presence of an immune system, is therefore likely to have developed strategies to minimise the impact of the T cells and natural killer (NK) cells responses. A number of immune evasion proteins have been described but these are mostly expressed during the lytic cycle, where the majority of KSHV proteins are expressed, increasing the range of potential immune targets. These immune evasion proteins include the lytic proteins K3 and K5 (also known as MIR1 and MIR2). These induce downregulation of the surface expression of MHC class I on infected cells by triggering endocytosis and proteasomal degradation of the MHC molecule through the ubiquitination of its cytoplasmic tail (Ishido et al., 2000). A third protein,

the vIRF1 product, also targets MHC class I by preventing basal transcription of the MHC class I gene (Lagos et al., 2007). Furthermore, the host shutoff or exonuclease protein (SOX) encoded by ORF37, accelerates the degradation of host mRNA, inhibiting host cell gene expression and consequently MHC class I expression and antigen presentation (Glaunsinger et al., 2004; Zuo et al., 2008). Despite these mechanisms for the downregulation of surface MHC class I expression, in vitro infected cells usually show normal levels of surface MHC class I several days after infection (Adang et al., 2007). As these cells at least also express latent proteins, conceivably KSHV latent epitope-specific CD8+ T cells could target cells expressing these proteins.

To date a number of studies have been performed to investigate whether the KSHV latent genome maintenance protein LANA mimics the behaviour of the EBV genome maintenance protein EBNA1, as both proteins perform similar functions and show some similar biochemical features (Kwun et al., 2007; Zaldumbide et al., 2007). These two proteins both possess central repeat domains, however they do not contain amino acid similarities: LANA's central repeat sequence is acidic as a result of its amino acid repeats of glutamine (Q), glutamic acid (E) and aspartic acid (D), while the EBNA1 central repeat sequence is smaller and consists of glycine-alanine repeats (GAr). Interestingly, although the two proteins show no amino acid sequence homology, when alternate reading frames of EBNA1 are examined, they can share up to 65% homology with the acidic repeat of LANA which has led to the suggestion of a common origin of these two sequences (Zaldumbide et al., 2007). The EBNA1 GAr have been shown to inhibit target cell recognition by CD8+ T cells in two ways: by inhibiting its proteasomal degradation (Levitskaya et al., 1995) and by retarding its

own translation and subsequently protein synthesis (Yin et al., 2003). Indeed it was initially thought that EBNA1 derived epitopes could not be processed and presented using the classic MHC class I processing pathway (Khanna et al., 1992; Murray et al., 1992). However in recent years and using assays which measure single cell effector function, recognition of EBNA1 by EBNA1- specific CD8+ T cells has been shown. These studies have suggested that the major source of EBNA1 epitope-peptides are not from the stable protein but rather an alternative source (Tellam et al., 2004; Mackay et al., 2009). This alternative source has been proposed to be derived from newly synthesised protein which has been rapidly degraded due to the error-prone nature of protein synthesis: up to 20% of newly formed proteins are misfolded. These are referred to as defective ribosomal products (DRiPs) and undergo rapid degradation, providing an efficiently generated source of peptide-epitopes (Schubert et al., 2000; Tellam et al., 2004; Mackay et al., 2009). Consequently, the effects of EBNA1 on retarding protein synthesis and proteasomal degradation will greatly impact the supply of DRiPs available for processing and presentation as peptide-epitopes.

Studies examining the CD8+ T cell recognition of LANA have shown that the central acidic repeat sequence has mechanisms similar to EBNA1 of evading CD8+ T cell recognition. This is mainly by inhibiting proteasomal degradation and inhibition of translation of the protein (Kwun et al., 2007; Zaldumbide et al., 2007). However, more recent work has suggested that LANA evades CTL recognition through an additional mechanism that is different to EBNA1. Here the initial part of the acidic repeat has been shown to reduce the translocation of LANA peptides from the cytosol into the endoplasmic reticulum (ER) for loading onto MHC I molecules (Kwun et al.,

2011). While these studies have addressed CD8+ T cell recognition of LANA, they have been undertaken using artificial model systems. Most frequently this has involved using non-pathogen derived model antigens, most commonly the ovalbumin peptide SIINFEKL presented by murine H-2K^b molecule, due to the availability of a unique MHC/epitope-specific antibody to assay epitope display by cell surface staining or by testing for recognition using the well characterised cytotoxic T lymphocyte hybridoma B3Z which has the same epitope specificity (Shastri et al., 1993; Zaldumbide et al., 2007). Most importantly, similar to initial CD8+ T cell recognition studies carried out on EBNA1, there have been no studies testing recognition of LANA expressing cells by LANA-specific CD8+ T cells. Therefore, assaying LANA-specific CD8+ T cells against cells expressing this KSHV antigen would give some indication of the ability of the cell to process and present antigen to T cells in a more physiologically relevant setting.

A second gene expressed in latently KSHV-infected cells such as those seen in KS and PEL biopsies is vFLIP or K13. This gene codes for the viral homologue of the Fas-associated death domain-like interleukin-1β converting enzyme (FLICE) inhibitor protein (FLIP). Proteins from this class have been shown to inhibit Fas-mediated apoptosis by interfering with caspase 8 recruitment to the death inducing signaling complex (DISC) (Chaudhary et al., 2000). Furthermore, vFLIP is a potent activator of the NF-κB pathway, promoting viral cell survival (Matta et al., 2004), transformation, inflammatory activation and morphological changes (Grossmann et al., 2006; Sun et al., 2006), all of which directly contribute to the pathogenesis of KSHV. However unlike LANA, the levels of vFLIP protein detected in infected or transfected cells is low (Alkharsah et al., 2011; Low et al., 2001; Guasparri et al., 2004). In the case of

cells transfected with vFLIP expression constructs, it has been shown that the low levels of vFLIP protein can be attributed to its sub-optimal codon usage. Thus expression of a re-engineered vFLIP gene with human-optimised codon usage generated abundant vFLIP protein and readily detectable levels of mRNA (Priya Bellare, data not published). As the vFLIP wildtype codon usage results in very low levels of vFLIP protein being expressed in comparison to the human-optimised vFLIP, it raises the question as to whether the inefficient protein synthesis would be reflected in reduced CD8+ T cell recognition of the vFLIP antigen. Assuming the DRiPs hypothesis also applies to the presentation of other antigens expressed within virally infected cells, this lower level of protein expression could potentially reflect a strategy employed by the virus to reduce peptide supply and consequently T cell recognition.

Previously, CD8+ T cell clones were established against the KSHV latent proteins vCyclin, vFLIP and LANA (Chapter 3). These clones were then to be tested for their ability to recognise cells expressing different forms of the latent-antigens. However in this series of experiments, the ability of the vCyclin-specific clone to recognise cells expressing vCyclin was not assessed. Ectopic expression of this protein in cells induces apoptosis or cell cycle arrest, likely through p53 dependent mechanisms, making these difficult targets to work with (Ojala et al., 1999; Ojala et al., 2000). Furthermore, no HLA-A*2901 matched cell lines permissive for KSHV infection were available to be used as target cells for the vCyclin-specific T cells. As such, experiments were focussed on the ability of the vFLIP- and LANA-specific CD8+ T cells to recognise cells expressing different forms of antigen and where possible, KSHV-infected cells.

4.1 CD8+ T cell recognition of cells expressing wildtype versus humanised vFLIP encoding a model T cell epitope.

vFLIP is a multifunctional protein, mostly known for its potent ability to activate NF-kB. Observations by Bellare have suggested that the codon usage of this gene is unusual in that rare codons are used throughout the entire sequence, suggesting that this protein is likely to be inefficiently synthesised in cells. Furthermore, experiments conducted by Bellare have shown that relative to a codon optimised version of this gene, vFLIP using the native viral sequence is poorly expressed (data not shown). Given that the DRiP hypothesis contends that newly synthesised proteins are likely to be the major source of epitope peptides for CD8+ T cells, restricting protein expression would limit the supply of peptides available for presentation to cognate CD8+ T cells, thereby reducing the chance of infected cells being killed by T cells. As such we examined whether CD8+ T cell epitopes are more efficiently presented to CD8+ T cells from a codon optimised vFLIP expression construct compared to the native viral sequence.

Plasmid constructs encoding the vFLIP sequence which used either the wildtype gene sequence or a codon optimised "humanised" sequence vFLIP were kindly provided by Dr Priya Bellare, University of California San Francisco, USA. Both constructs contained the Flag antibody epitope tag at the amino terminus of vFLIP to allow detection. These constructs were further modified to encode a reporter CD8 epitope, the HLA-B*0801 restricted EBV CD8+ T cell epitope FLRGRAYGL (FLR), derived from the EBV latent antigen EBNA3A, at the carboxy terminus of the vFLIP sequences. These genes were cloned into a modified pcDNA3.1 vector (Invitrogen) (Figure 4.1), downstream from a CMV promoter (PCMV) and upstream from an

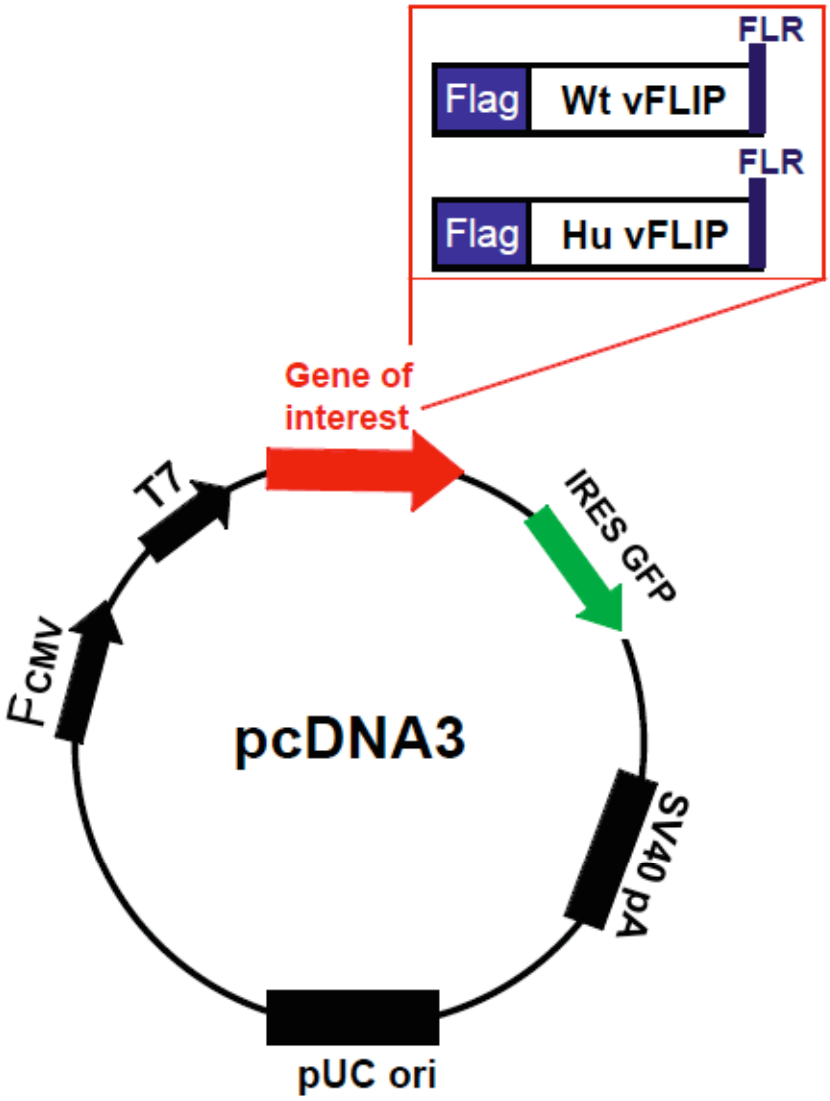


Figure 4.1 vFLIP expression plasmids. The vFLIP constructs encoding the wildtype vFLIP sequence (Wt vFLIP) and humanised vFLIP (Hu vFLIP) sequence. Both of which contain the flag tag sequence at the amino terminus and the EBV latent antigen EBNA3A CD8+ T cell FLRGRAYGL (FLR) at the carboxy terminus. The vFLIP constructs were expressed from the pcDNA3.1 plasmid with expression of the construct driven by the CMV promoter (P_{CMV}) and T7 promoter allowing in vitro transcription in the sense orientation. An internal ribosome entry site (IRES) GFP was also inserted for measuring transfection efficiency by measuring GFP fluorescence by flow cytometry.

internal ribosome entry site (IRES) and GFP gene, allowing measurement of transfection efficiency by flow cytometry. The empty pcDNA3.1 IRES GFP plasmid was used as a control.

Initially to investigate the efficiency of T cell recognition of cells expressing the different vFLIP constructs, the plasmids were transfected into the easily transfectable HLA-B*0801 melanoma target cell line MJS. These cells were incubated for 48hrs and the proportion of each transfected cell type expressing GFP was analysed by flow cytometry (data not shown). To allow meaningful comparisons between the transfected cells in recognition assays, the percentage of GFP expressing MJS cells was then equalised for the different transfectants by the addition of non-transfected MJS cells, such that all transfected cells were 50% GFP positive. These were then used as targets in recognition assays by incubation with FLR-specific CD8+ T cell clones. Additionally, MJS cells sensitised with the FLR-peptide or the peptide solvent DMSO were used as positive and negative controls respectively. Figure 4.2 shows the results of the recognition assay using two FLR-specific CD8+ T cell clones (A) c45 and (B) c147. Both clones showed recognition of the MJS FLR peptide-sensitised cells, with no recognition of the DMSO-sensitised MJS cells or MJS cells transfected with the control plasmid. More importantly, there was no recognition of the wildtype vFLIP FLR transfected MJS cells, however there was recognition of the cells expressing the humanised vFLIP FLR construct.

To interpret the results of this recognition assay, aliquots of the transfected cells were analysed for the levels of vFLIP protein expressed in each cell type by western blot analysis with monoclonal antibodies specific to Flag and calregulin as a loading

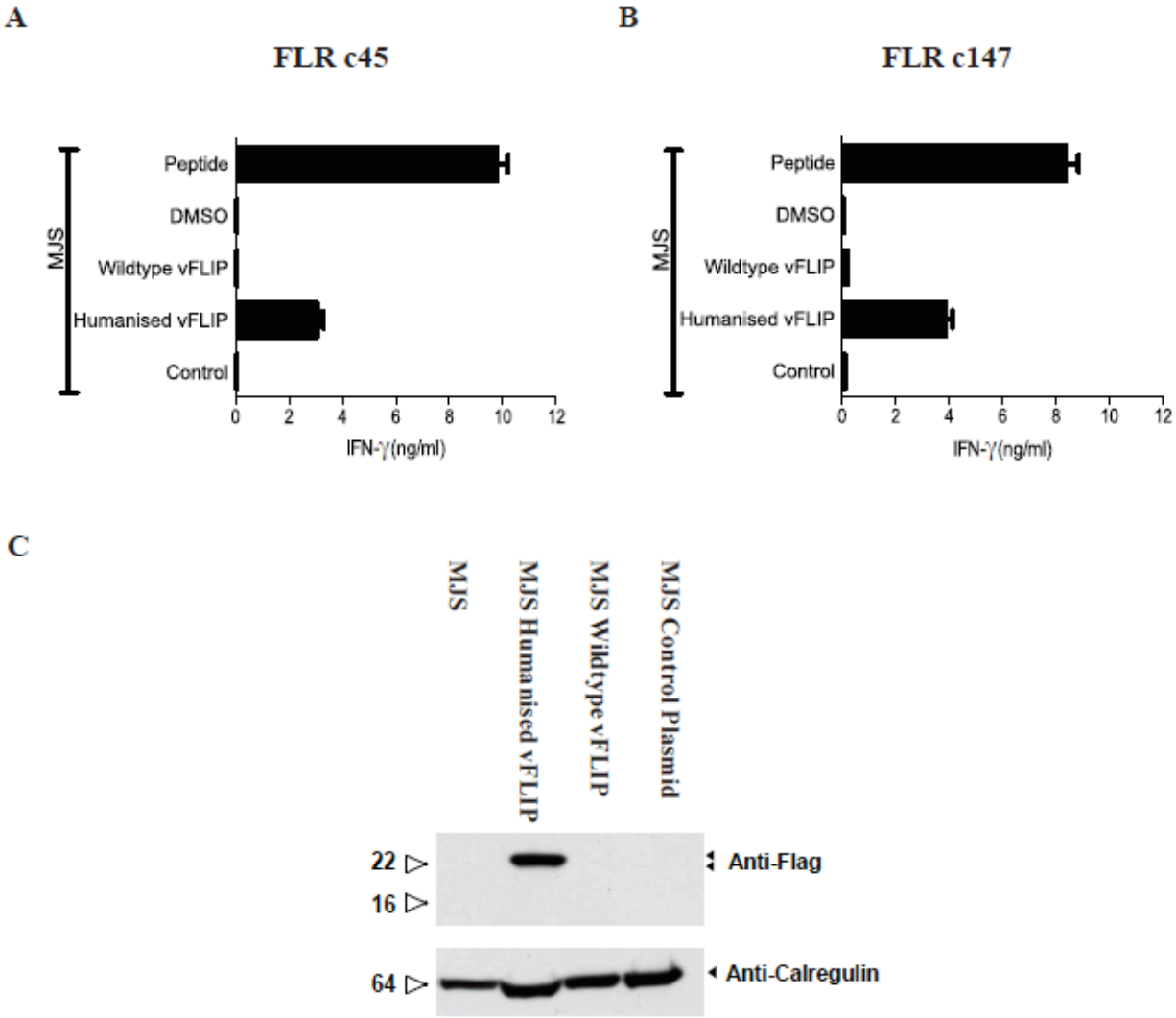


Figure 4.2 CD8+ FLR T cell recognition of MJS cells transfected with vFLIP expression plasmids and vFLIP protein expression. The HLA-matched B*0801 MJS expressing cells were transfected with the vFLIP expression plasmids, Wt vFLip and Hu vFLIP, as well as a control plasmid. The transfected MJS cells were incubated with the EBNA3A FLR-specific CD8+ T cell clones (A) c45 and (B) c147 and recognition quantified by IFN-γ ELISA. As a positive control, the MJS cells were peptide pulsed with the cognate epitope-peptide at 5μg/ml or as a negative control with the peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells. (C) Immunoblots showing vFLIP plasmid protein expression levels of the transfected MJS cells were detected using monoclonal flag antibody and calregulin levels served as a loading control detected using monoclonal calregulin antibody.

control. Figure 4.3 (C) shows that MJS cells transfected with the humanised vFLIP plasmid expressed this protein, while none was detected in lysates of non-transfected or control plasmid transfected cells. Importantly, no vFLIP protein was detected from cells transfected with the wildtype vFLIP plasmid despite these cells expressing the GFP reporter protein which is contained in the same messenger RNA molecule as vFLIP. This was repeated on three different occasions using two independently generated wildtype vFLIP expressions, all yielding the exact same result.

As one of the major cellular targets for KSHV infection are B lymphocytes, the next experiments determined whether the vFLIP plasmids expressing the FLR peptide could be recognised by the FLR-specific CD8+ T cells when expressed in an LCL background. The FLR plasmids were transfected by electroporation into HLA-B*0801 expressing LCLs from donors KSB6 and KSB7. LCLs used were made with the B95.8 strain of virus which, although it expresses EBNA3A, does not encode the FLR peptide. Transfection efficiency was determined by analysing GFP fluorescence by flow cytometry (data not shown) and the percentage of GFP expression equalised by addition of non-transfected LCLs such that all transfectants were 4% GFP positive. These equalised mixtures of cells were then incubated with the FLR-specific CD8+ T cells and IFN-γ release measured to quantify recognition. LCLs were sensitised with either the FLR peptide or the peptide solvent DMSO as positive and negative controls respectively. Figure 4.3 shows the results of this CD8 recognition assay for donors, KSB6 and KSB7, incubated with the FLR-specific clones c177 (A & C) and c27 (B & D), respectively. The clones recognised the FLR-sensitised LCLs, with no recognition of the DMSO sensitised LCLs. There was also no recognition of the LCLs transfected with the control plasmid or, more importantly, with the wildtype vFLIP FLR plasmid.

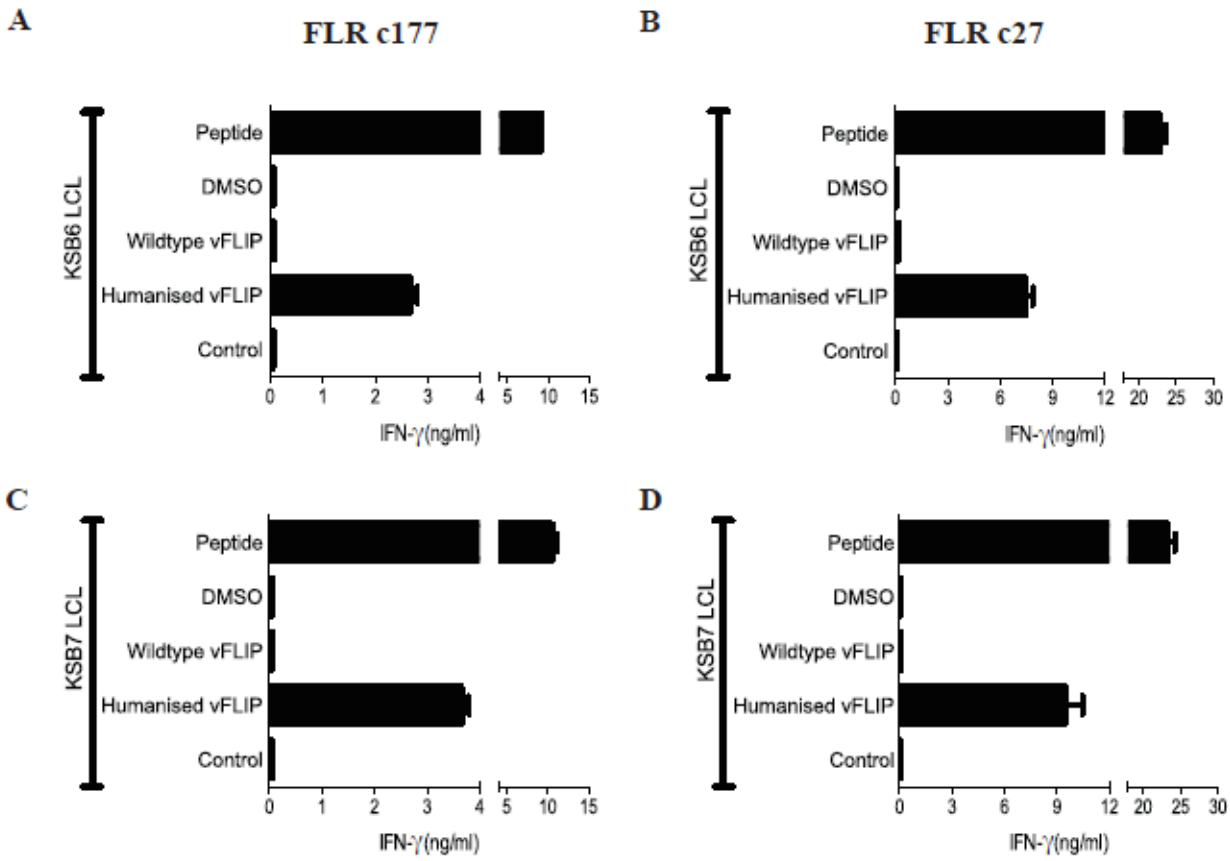


Figure 4.3 CD8+ FLR T cell recognition of KSB6 and KSB7 LCLs transfected with vFLIP expression plasmids. The HLA-matched B*0801 LCL targets were transfected with the vFLIP expression plasmids, Wt vFLip and Hu vFLIP, as well as a control plasmid. The transfected LCL targets, KSB6 and KSB7, were incubated with the EBNA3A FLR-specific CD8+ T cell clones (A and C) c177 and (B and D) c27 and recognition quantified by IFN-γ ELISA. As a positive control, LCL targets were peptide pulsed with the cognate epitope-peptide at 5μg/ml or as a negative control with the peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

However both LCLs transfected with codon optimised vFLIP FLR plasmid were recognised by the two FLR-specific T cell clones. This was repeated on three different occasions, each giving a similar result.

4.2 vFLIP-specific CD8+ T cell recognition of cells expressing wildtype versus humanised vFLIP.

The previous experiments established that in two different cell types, the model CD8+ epitope FLR is only presented from humanised vFLIP constructs which encode this epitope. We next asked whether a vFLIP-specific CD8+ T cell can recognise cells expressing either of these constructs. Initial clone screens carried out on donor KSB1 identified a HLA-B*81 restricted vFLIP CD8+ epitope located within the overlapping peptides GTM and FSP recognised by clone 57, described in Chapter 3. This clone was then used in recognition assays against MJS cells or LCLs expressing these constructs.

Initially the vFLIP-specific T cells were assayed against MJS cells transfected with the vFLIP and control plasmids. As MJS cells do not naturally express HLA-B*81, the vFLIP plasmids were co-transfected with a plasmid expressing the HLA-B*81 allele. The efficiency of the vFLIP and control plasmids transfection was determined by measuring GFP fluorescence by flow cytometry. Figure 4.4 (A) shows similar levels of transfection between the Wt vFLIP and Hu vFLIP plasmids in the MJS cells, with 25.2% and 26.7%, respectively, expressing GFP. The control plasmid transfected cells show slightly higher levels of transfection efficiency, with 30.6% of the cells expressing GFP. Previous studies have suggested that co-transfection of plasmids using lipid based reagents delivers both constructs to cells (Zuo et al., 2009). These

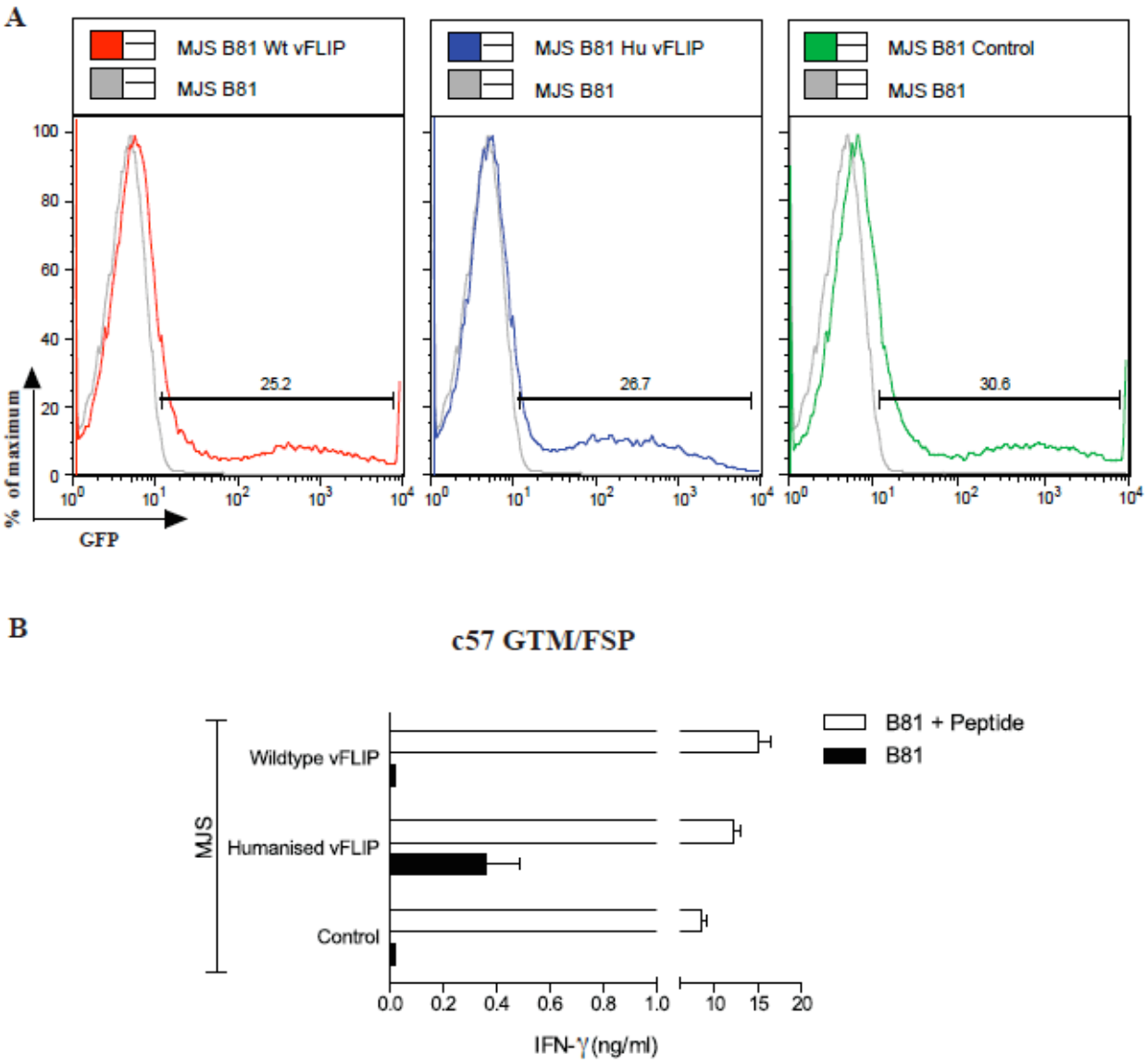


Figure 4.4 vFLIP CD8+ T cell recognition of MJS cells transfected with vFLIP expression plasmids and HLA B*81 allele. (A) MJS cells were transfected with the HLA B*81 expression allele, vFLIP expression plasmids, Wt vFLIP and Hu vFLIP, as well as a control plasmid. The transfection efficiency of the MJS cells was determined by measuring GFP fluorescence for each of the plasmid transfected MJS cells by flow cytometry. (B) The transfected MJS cells were incubated with the vFLIP CD8+ T cell clone 57 specific for the target peptides GTM and FSP and recognition quantified by IFN-γ ELISA. As a positive control, the transfected MJS targets were peptide pulsed with the cognate epitope-peptide at 5μg/ml. Results are expressed as the mean IFN-γ release from triplicate wells.

cells were then used in recognition assays and separate aliquots of each transfectant were sensitised with the cognate epitope-peptides before being incubated with the vFLIP-specific CD8+ T cell clone 57 and assaying for IFN-γ secretion. Figure 4.4 (B) shows that the MJS cells were successfully co-transfected with the HLA-B*81 allele, as each of the peptide-sensitised transfectants was recognised by the vFLIP-specific CD8+ T cell clones. These clones did not recognise the control plasmid transfected MJS cells or those transfected with the wildtype vFLIP construct. However the clones did recognise MJS cells transfected with the humanised vFLIP construct, despite similar levels of transfection efficiency in these cells.

To determine whether the lack of recognition of the wild type vFLIP transfected cells by the vFLIP-specific T cells was related to the cellular background in which the vFLIP genes were expressed, the vFLIP plasmids were transfected into a B cell background, namely LCLs, and these used as targets in recognition assays. Here the autologous donor KSB1 LCL was used as the target, as this expresses HLA-B*81. LCLs were transfected with the vFLIP and control plasmids 48 hours prior to being incubated with the vFLIP-specific CD8+ T cell clone 57 and assaying for IFN-γ secretion. LCLs were sensitised with either the cognate peptides or DMSO as positive or negative controls respectively. Peptide-sensitised LCLs were well recognised while those sensitised with DMSO were not (data not shown). However, there was no recognition of any of the vFLIP constructs (data not shown). Analysis of GFP fluorescence of transfected cells revealed a very low percentage of cells were transfected with the vFLIP plasmids, providing a potential explanation for the lack of recognition by the vFLIP-specific CD8+ T cell clone.

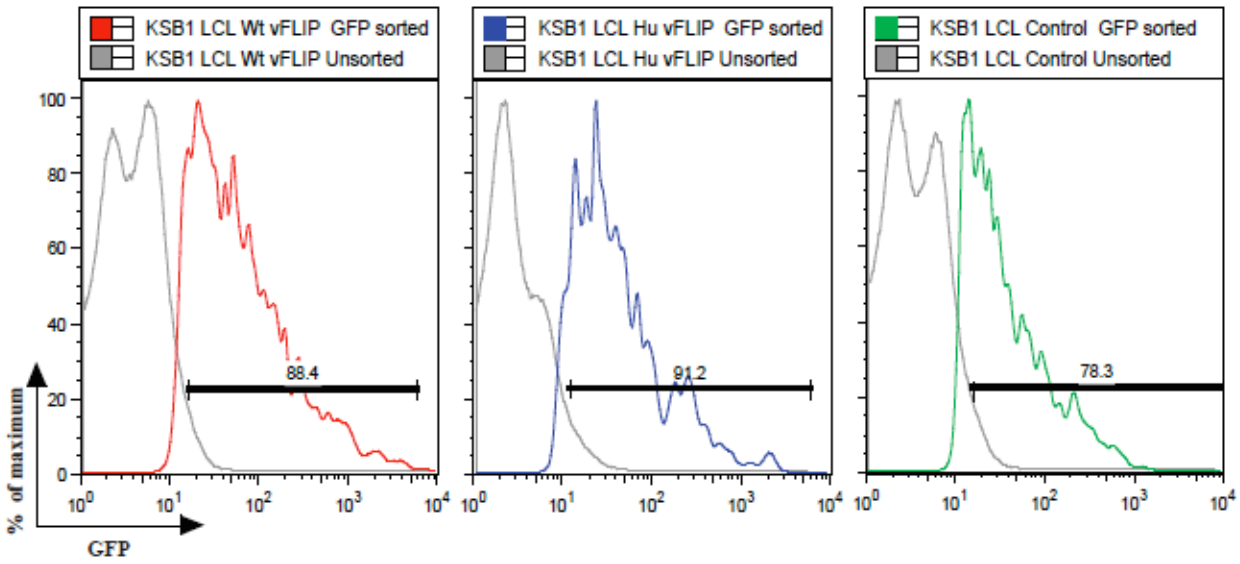
In order to increase the percentage of cells transfected with the expression constructs, KSB1 LCLs were transfected again with the vFLIP and control plasmids, and after 48 hours, the GFP-expressing transfected cell population enriched by FACS. Figure 4.5 (A) shows successful enrichment of the Wt vFLIP, Hu vFLIP and control plasmid transfected KSB1 LCLs, with 78-92% GFP expression. These GFP-sorted LCLs were subsequently incubated with the vFLIP CD8+ T cell clone 57 and assayed for recognition of the targets by measuring secreted IFN-γ by ELISA. As a positive control the LCLs were sensitised with cognate peptides and DMSO as a negative control. The assay revealed good levels of recognition of the peptide sensitised LCLs, with no recognition of the DMSO-sensitised LCLs. Furthermore, there was no recognition of the control plasmid in the autologous LCLs. Similarly there was no recognition of the wildtye vFLIP transfected LCLs but the humanised vFLIP LCLs were recognised by the vFLIP CD8+ T cell clone. This assay was repeated on two different occasions, yielding the same result.

4.3 LANA-specific CD8+ T cell recognition of LCLs expressing LANA

The previous experiments were unable to demonstrate T cell recognition of cells expressing the wild type vFLIP gene product. To determine whether CD8+ T cell clones specific for other latent antigens could recognise cognate antigen-expresing cells, the ability of a LANA-specific clone to recognise target cells was examined.

A model system was developed where LANA expression vectors were transfected into B cells to test LANA-specific CD8+ T cell recognition of antigen-expressing cells. LANA constructs were ectopically expressed using derivatives of the pRTS-CD2 plasmid shown in figure 4.6 (Bornkamm et al., 2005). This expression plasmid

A



B

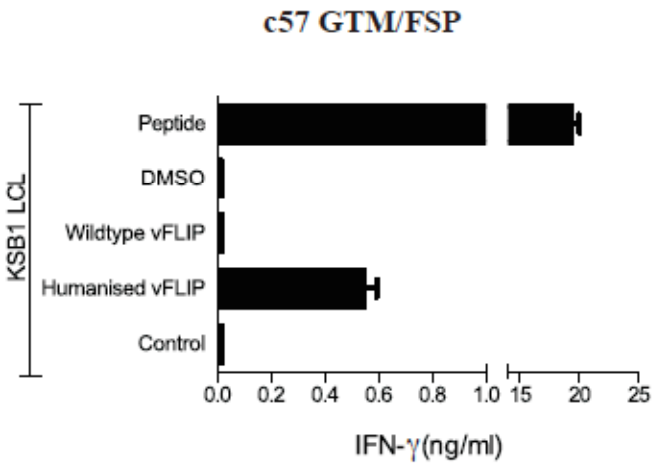


Figure 4.5 vFLIP CD8+ T cell recognition of GFP-sorted KSB1 LCLs transfected with vFLIP expression plasmids. (A) KSB1 LCLs were transfected with the vFLIP expression plasmids, Wt vFLIP and Hu vFLIP, as well as a control plasmid. The plasmid transfected LCLs were enriched by sorting for the GFP-expressing transfected LCL population by flow cytometry. (B) The GFP-sorted KSB1 transfected LCLs were incubated with the vFLIP CD8+ T cell clone 57 specific for the target peptides GTM and FSP and recognition quantified by IFN-γ ELISA. As a positive control, the LCL targets were peptide pulsed with the cognate epitope-peptide at 5μg/ml or as a negative control with the peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

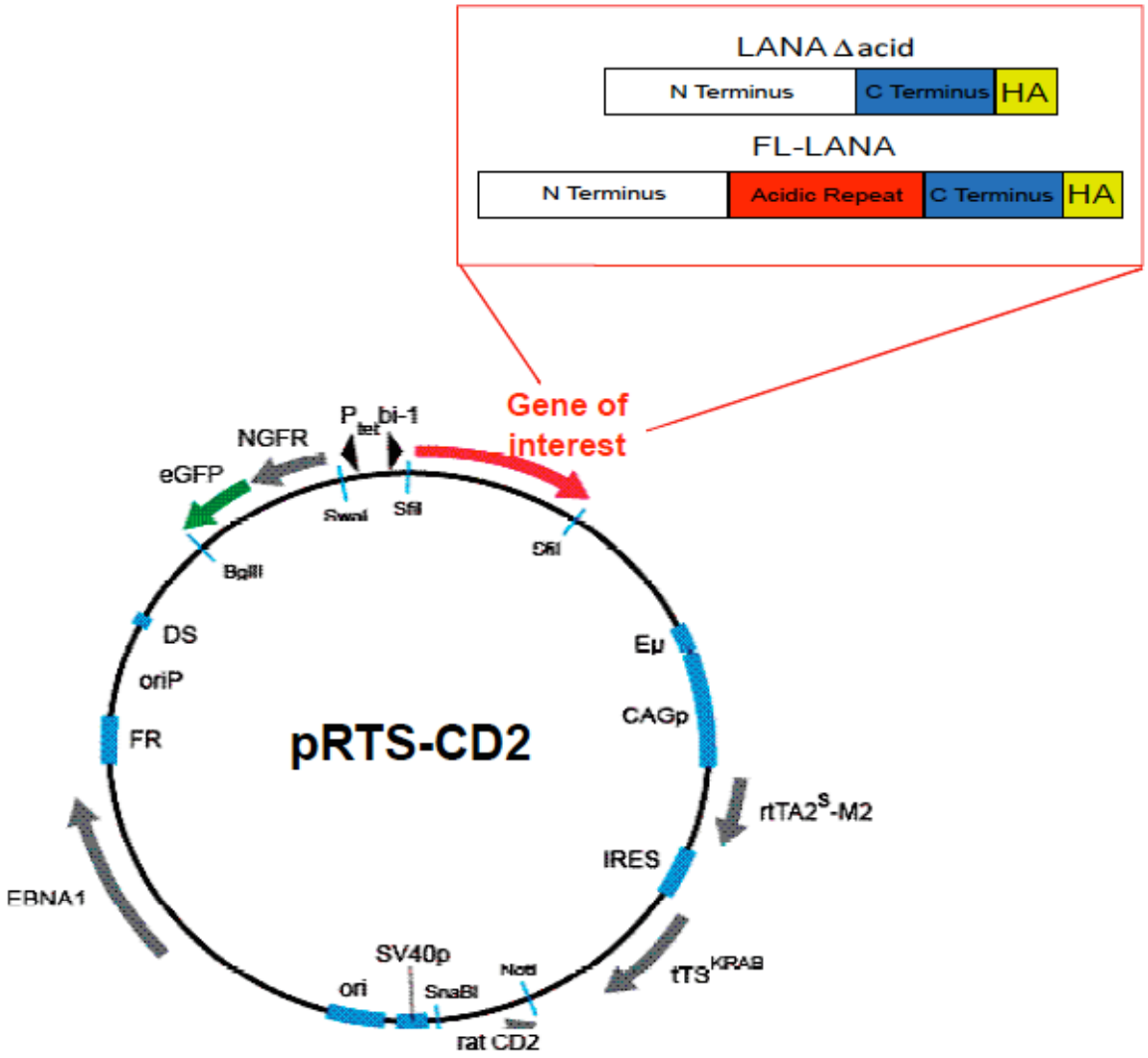


Figure 4.6 Schematic map of the pRTS-CD2 vector for the ectopic expression of LANAΔacid and FL-LANA. The vector contains a bidirectional tetracycline-regulated promoter, which activates expression of the LANAΔacid or FL-LANA genes of interest in one direction and eGFP and NGFR in the other. rtTAs-M2 is the tetracycline controlled transactivator, tTS^{KRAB} is the tetracycline repressor placed downstream of the internal ribosome entry site (IRES). The SV40 promoter (SV40p) drives expression of the constitutively expressed rat CD2, ori is the bacterial origin of replication and EBNA1 is the EBV genome maintenance protein. OriP denotes the EBV episomal origin of replication, this contains the family of repeats and the dyad symmetry element.

constitutively expresses the rat-CD2 molecule allowing selection of transfected cells. The vector also contains a bi-directional tetracycline responsive promoter which upon the addition of doxycyline (dox) allows inducible expression of the inserted LANA construct as well as a reporter cassette containing GFP and truncated nerve growth factor receptor (ΔLNGFR). The two LANA construct sequences inserted for these experiments were derived from the BCBL-1 strain of the virus. The first of which expresses the unmodified LANA sequence, the full length LANA (FL-LANA) sequence, while the second expresses the LANA sequence from which the acidic repeat region (amino acids 345-916) was removed (LANAΔacid). This allows the investigation of whether the acidic repeat sequence contained within LANA inhibits or interferes with CD8+ T cell recognition of LANA expressing cells, using for the first time, a LANA-specific CD8+ T cell clone. The CD8+ clone is specific to the LANA peptide PPW and is HLA-B*3501 restricted, the characterisation of which is described in Chapter 3 - Section 3.2. No autologous LCL was available from donor KS021, consequently B95.8 LCLs from two HLA B*3501 expressing donors, KSB4 and KSB5, were transfected with the LANA expression constructs for use as target cells.

Expression of the LANA constructs was induced in the LCLs by incubation with 2μg/ml dox for 24hrs. Prior to use in the CD8+ T cell recognition assay, the dox-induced vector transfected LCLs were further enriched by selecting for the vector expressed reporter protein NGFR by MACS NGFR-specific beads. Following NGFR selection, in order to determine the percentage of vector-expressing cells GFP expression was analysed by flow cytometry (data not shown). The perecentage of

GFP expressing LCLs was equalised to 70% GFP between FL-LANA and LANAΔacid expressing LCLs, by the addition of non-transfected LCLs. Figure 4.7 shows the results for donors, KSB4 and KSB5, NGFR enriched and GFP equalised expression in the LCLs. Initially, protein expression levels of the LANA constructs was checked by western blot analysis of lysates from the donor LCLs. Both donors (A) KSB4 and (C) KSB5 showed higher levels of LANA protein being ectopically expressed from LANAΔacid transfected LCLs than the FL-LANA transfected LCLs. This finding is consistent with previous studies, which have shown a higher rate of translation for LANAΔacid compared to the FL-LANA sequence (Kwun et al., 2007).

The FL-LANA and LANAΔacid expressing LCLs were incubated with the LANA PPW-specific CD8+ T cell clone 12, to test for recognition by measuring IFN-γ production by ELISA. As a positive control the donor LCLs were sensitised with the cognate epitope-peptide and LCLs sensitised with the peptide solvent DMSO as a negative control. Figure 4.7 shows the recognition assay results for both donors (B) KSB4 and (D) KSB5. The CD8+ LANA-specific T cell clone 12 recognised the positive control peptide sensitised LCLs but did not recognise LCLs sensitised with the peptide solvent DMSO. The LANAΔacid expressing LCLs were clearly recognised by the LANA-specific CD8+ T cell clone, importantly however the FL-LANA expressing LCLs induced some yet weak recognition by the CD8+ T cells, this experiment was repeated twice yielding the same result. This result also supports previous work using model systems that suggests the acidic repeat sequence within LANA has an immune evasion function that impairs CD8+ T cell recognition.

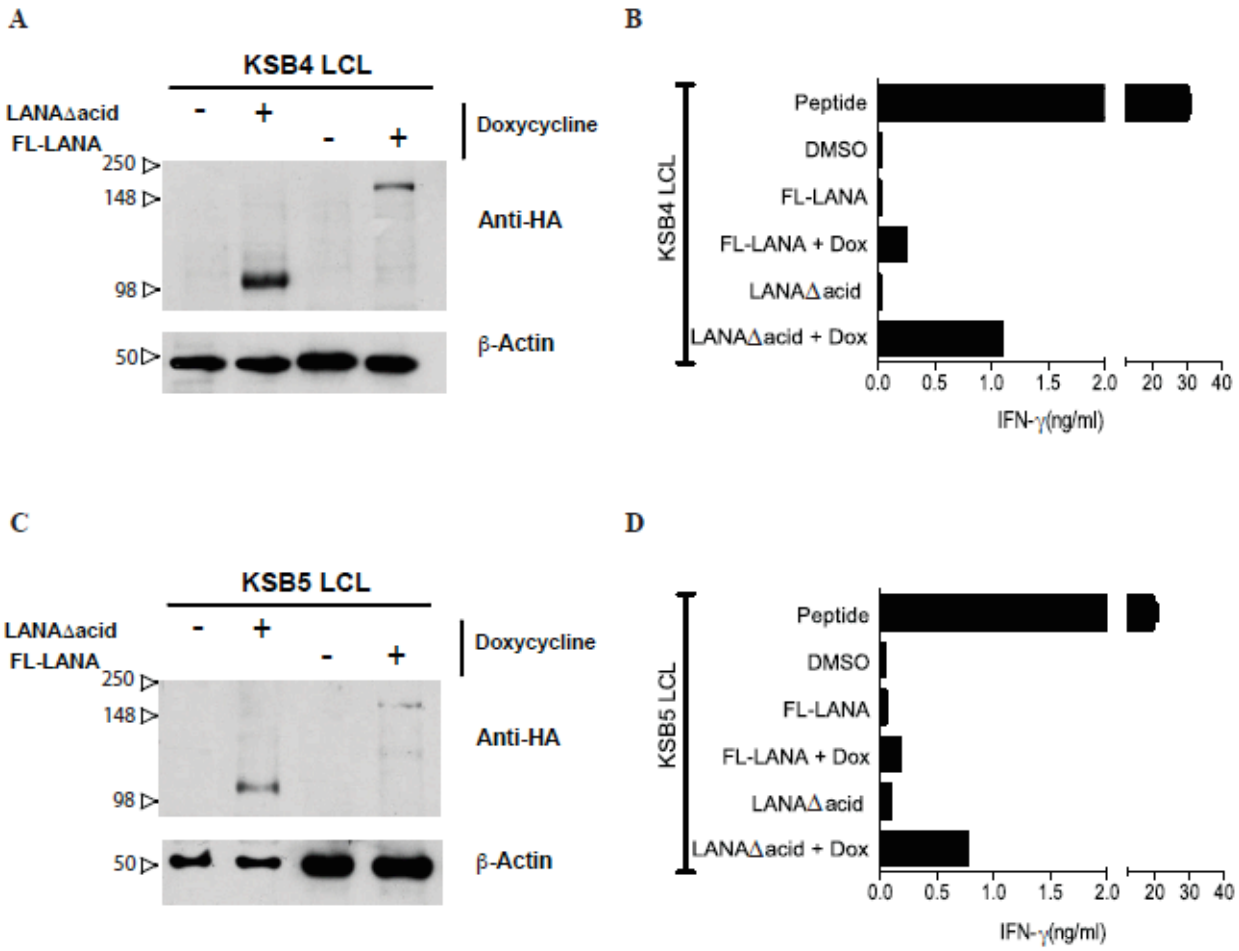


Figure 4.7 Recombinant LANA construct protein expression and CD8+ T cell recognition of transfected KSB4 and KSB5 LCL targets. Immunoblots showing recombinant LANA protein expression levels following dox induction of the HLA B*3501 matched (A) KSB4 and (C) KSB5 transfected LCL targets. LANA construct protein levels were detected using monoclonal HA antibody and actin levels served as a loading control detected using monoclonal β-actin antibody. The dox induced and uninduced (B) KSB4 and (D) KSB5 transfected LCL targets were incubated overnight with the HLA B*3501 donor KS21 LANA PPW-specific CD8+ T cell clone 12 and recognition quantified by IFN-γ ELISA. As a positive control, the HLA-matched (B) KSB4 and (D) KSB5 LCL targets were peptide pulsed with the cognate epitope-peptide at 5μg/ml or as a negative control with peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

4.4 KSHV-infection and CD8+ T cell recognition of HMEC-1 endothelial cells

The previous experiments established that the acidic repeat sequence within LANA interferes with CD8+ T cell recognition in an LCL background. Importantly the full length protein can be recognised to some degree by a cognate CD8+ T cell clone and when this acidic repeat sequence is removed recognition is increased. The next experiments investigated whether the LANA specific CD8+ T cell clone can recognise LANA antigen expressed in the context of a KSHV infected cell, not just LANA expressing cells. To address this question, HLA-B*3501 human microvascular endothelial cells (HMEC-1) were to be infected with KSHV and used as targets. These cells have been successfully immortalised with a plasmid expressing simian virus 40 gene product, large T antigen (Ades et al., 1992). The HMEC-1 cells were generously donated from Professor Gerard Nash, The University of Birmingham.

As HMEC-1 cells had not been previously used in CD8+ T cell recognition assays, their ability to process and present antigen needed to be confirmed. Here a model reporter antigen was expressed in these cells and their ability to process and present antigen to a cognate HLA-B*3501 restricted T cell examined. The HMEC-1 cells and, as a control, HLA-B*3501 LCLs known to be competent for antigen processing ability were infected with modified vaccinia Ankara (MVA) constructs expressing the EBV antigen EBNA1 lacking the glycine-alanine repeat (E1ΔGA). This construct was used as the repeat sequence has been previously shown to interfere with EBNA1 antigen processing and presentation for CD8+ T cell recognition (Tellam et al., 2004; Tellam et al., 2004; Voo et al., 2004; Tellam et al., 2007). Separate aliquots of the HMEC-1 cells and LCLs were infected with an MVA expressing an irrelevant antigen. As controls, cells were sensitised with specific cognate epitope-peptide HPV

or peptide solvent DMSO, followed by overnight incubation with the EBNA1-specific peptide HPV HLA-B*3501 restricted CD8+ T cell clone 41 and recognition assessed by measuring IFN-γ secretion. Figure 4.8 shows that the EBV EBNA1 HPV-specific CD8+ clone recognised the peptide sensitised HMEC-1 cells and LCLs, with little or no recognition of the DMSO sensitised cells. More importantly, both the MVA E1ΔGA infected HMEC-1 cells and LCLs were well recognised by the HPV CD8+ clone with no recognition of the cells infected with the control MVA. This result shows that HMEC-1 cells can process and present the EBV peptide-epitope HPV from EBNA1ΔGA for CD8+ T cell recognition, showing that HMEC-1 cells are capable of processing and presenting viral antigen for CD8+ T cell recognition.

To test whether the HMEC-1 cells were capable of processing and presenting LANA antigen for CD8+ T cell recognition. The HMEC-1 cells were infected with titrated recombinant KSHV virus (rKSHV.219) derived from the engineered JSC-1 strain (Vieira et al., 2004), kindly provided by Professor David Blackbourn, The University of Birmingham. The recombinant KSHV virus was engineered containing the red fluorescent protein (RFP) under the control of the lytic PAN RNA promoter and the GFP gene expressed by the human elongation factor 1-α promoter, used as an indicator of latent KSHV infection. HMEC-1 cells were infected with the virus at an MOI of 10 for 72 hours and then checked for KSHV latent infection efficiency through analysis of GFP expression levels by flow cytometry. Figure 4.9 shows a representative result of one such KSHV infection. In the absence of virus, mock infected cells show no GFP fluorescence. KSHV-infected HMEC-1 cells at 72 hours post-infection showed 40.2% of cells expressing GFP, suggesting successful latent KSHV infection of the HMEC-1 cells.

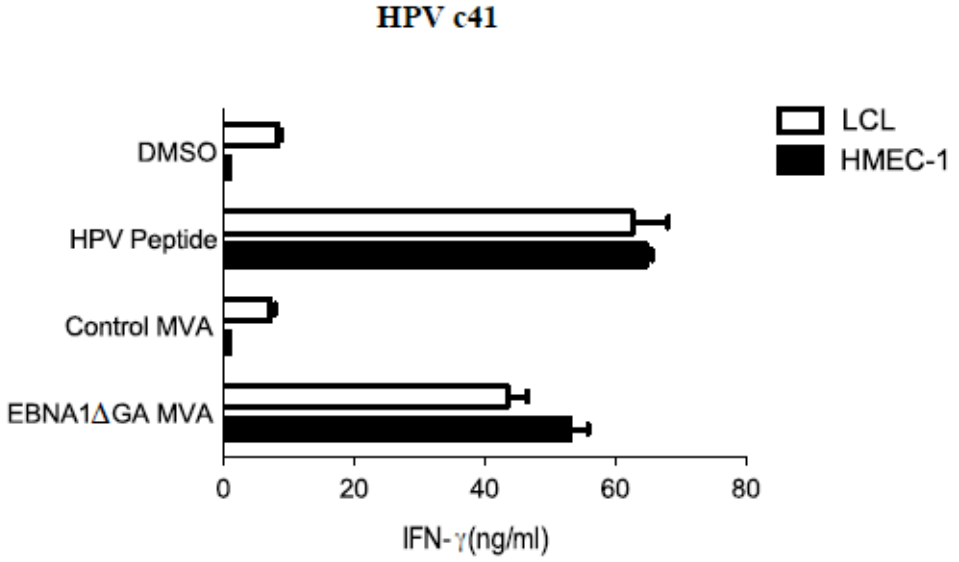


Figure 4.8 CD8+ T cell recognition of the HMEC-1 cells expressing EBV antigen EBNA1. The HLA B*3501-matched HMEC-1 cells and LCLs were infected with MVA- EBNA1ΔGA and with an irrelevant MVA as a control. The MVA-infected HMEC-1 cells and LCLs were then incubated with the EBNA1 HPV-specific CD8+ T cell clone 41 and recognition quantified by IFN-γ ELISA. The HMEC-1 cells and LCLs were pulsed with cognate epitope-peptide as a positive control at 5μg/ml and with peptide solvent DMSO as negative control. Results are expressed as the mean IFN-γ release from triplicate wells.

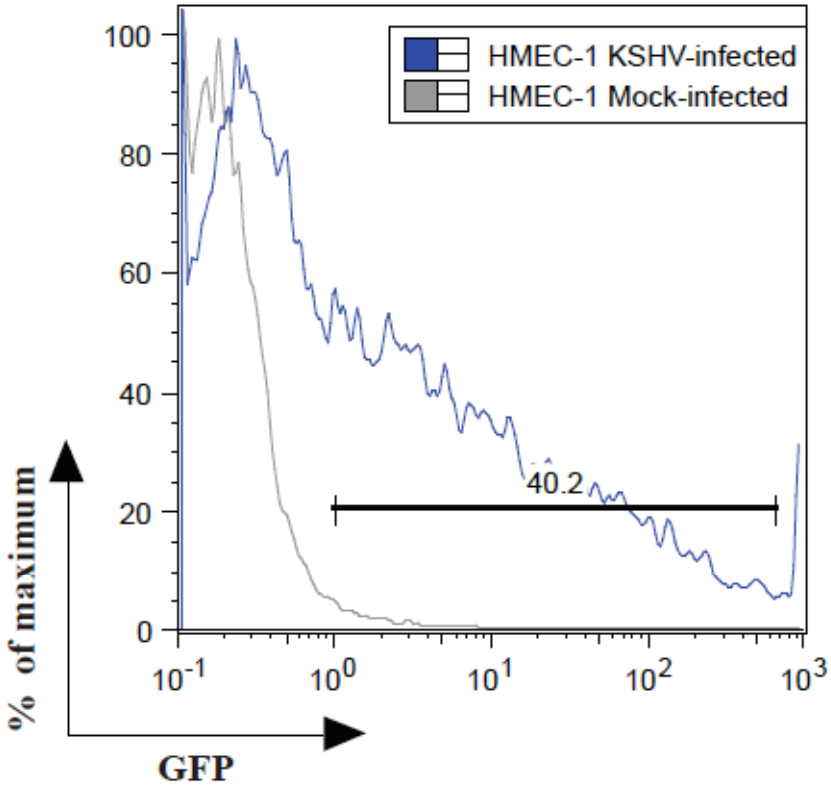


Figure 4.9 Infection of the HMEC-1 cells with rKSHV.219 virus. Human microvascular endothelial cells (HMEC-1) were infected with the recombinant KSHV virus (rKSHV.219) at an MOI of 10 for 72hrs. The virus possesses a GFP gene expressed by the human elongation factor 1-α promoter and an indicator of KSHV latently infected cells. GFP expression levels were analysed by flow cytometry.

The above experiment had suggested that the HMEC-1 cells had been successfully infected with KSHV, establishing latency 72 hours post-infection. These cells were then incubated with the LANA PPW-specific CD8+ T cell clone 12 and assayed for T cell recognition by IFN-γ ELISA. As a positive control the HMEC-1 cells were sensitised with the cognate peptide-epitope and as a negative control were mock infected. Figure 4.10 (A) shows the CD8+ LANA-specific clone recognised the peptide sensitised HMEC-1 cells but not the mock infected HMEC-1 cells. This suggests that the HMEC-1 cells are HLA-matched to the clone and that the T cells are functional and specific for their respective LANA epitope-peptide PPW. However, most importantly there was no recognition of the KSHV latently infected HMEC-1 cells. This was repeated on three occasions all giving the same result.

As no T cell recognition of the infected HMEC-1 cells was observed, the expression of LANA protein in the infected HMEC-1 cells was examined. Aliquots of KSHV infected HMEC-1 cells, HMEC-1 cells with no virus as a negative control and cells from the PEL JSC-1 as a positive control were lysed and analysed by western blot. The blots were probed with the rat monoclonal LANA antibody that binds to the acidic repeat sequence within LANA and β-actin was used as a loading control. Figure 4.10 (B) shows that the positive control PEL JSC-1 showed high levels of LANA protein expression, while no LANA protein expression was detected in the control HMEC-1 cells. LANA protein expression was detected in the KSHV infected HMEC-1 cells but at lower levels compared to the PEL JSC-1.

In the previous experiments it was unclear whether the lack of recognition of the KSHV-infected HMEC-1 cells was due to not all cells being infected, with 40.2% of

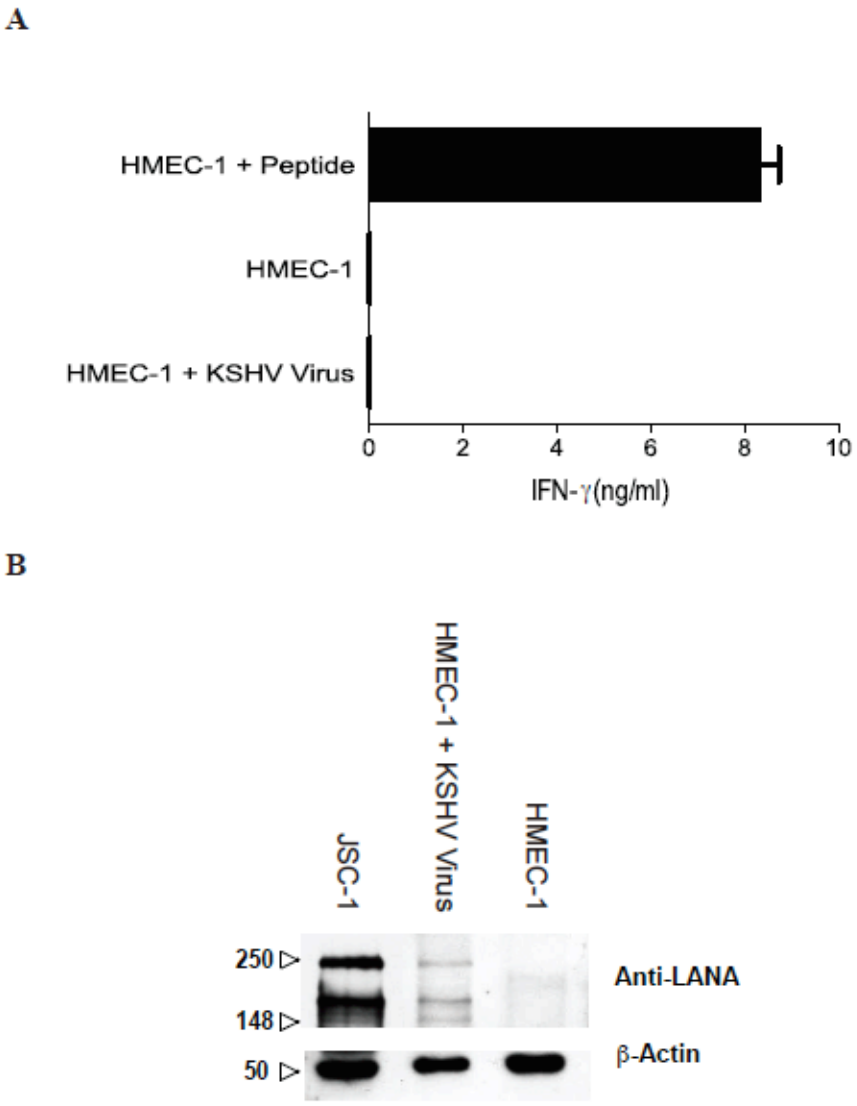


Figure 4.10 CD8+ T cell recognition of rKSHV.219-infected HMEC-1 cells and LANA protein expression. (A) The HLA B*3501-matched HMEC-1 cells latently infected with rKSHV.219 virus were incubated overnight with the HLA B*3501 donor KS21 LANA PPW-specific CD8+ T cell clone 12 and recognition quantified by IFN-γ ELISA. As a positive control, HMEC-1 targets were peptide pulsed with the cognate epitope-peptide at 5μg/ml or as a negative control with no peptide. Results are expressed as the mean IFN-γ release from triplicate wells. (B) Immunoblots showing recombinant LANA protein expression levels of the HMEC-1 cells, HMEC-1 rKSHV.219 infected cells and PEL line JSC-1 as a positive control. LANA construct protein levels were detected using monoclonal HA antibody and actin levels served as a loading control detected using monoclonal β-actin antibody.

cells showing GFP fluorescence. To increase the percentage of infected cells, HMEC-1 cells were infected with the recombinant KSHV virus as before and the GFP expressing cells enriched by FACS. Almost 80% of the sorted KSHV infected HMEC-1 cells were GFP positive as shown in figure 4.11. These enriched HMEC-1 cells were then used in a CD8+ T cell recognition using the same method described above, assaying IFN-γ secretion from the T cells by ELISA. Again there was no recognition of the KSHV infected HMEC-1 cells confirming that the numbers of KSHV infected cells was not preventing CD8+ T cell recognition in this assay. This experiment was repeated on two different occasions, both yielding a similar result.

4.5 HLA class I levels on KSHV-infected HMEC-1 cells

Previous and more recent work has highlighted the role of herpes virus proteins that interfere with the class I antigen processing pathway affecting the levels of MHC class I surface expression (Duncan et al., 2006; Ressing et al., 2008; Dugan et al., 2009; Zuo et al., 2010). Initially we confirmed that endothelial cells are known to express MHC class I on their surface by staining the cells with a PE-conjugated W6/32 MHC class I monoclonal antibody prior to infecting the cells with KSHV (data not shown).

To test whether proteins expressed during KSHV infection may have an effect on the surface MHC class I levels on HMEC-1 cells, a comparison of surface class I expression was carried out between the HMEC-1 cells and KSHV-infected HMEC-1 cells by staining for surface MHC class I. Figure 4.12 (A) shows the results from the HMEC-1 unstained cells, KSHV-infected HMEC-1 unstained cell and KSHV-infected HMEC-1 MHC class I w6/32-APC stained. The unstained KSHV infected

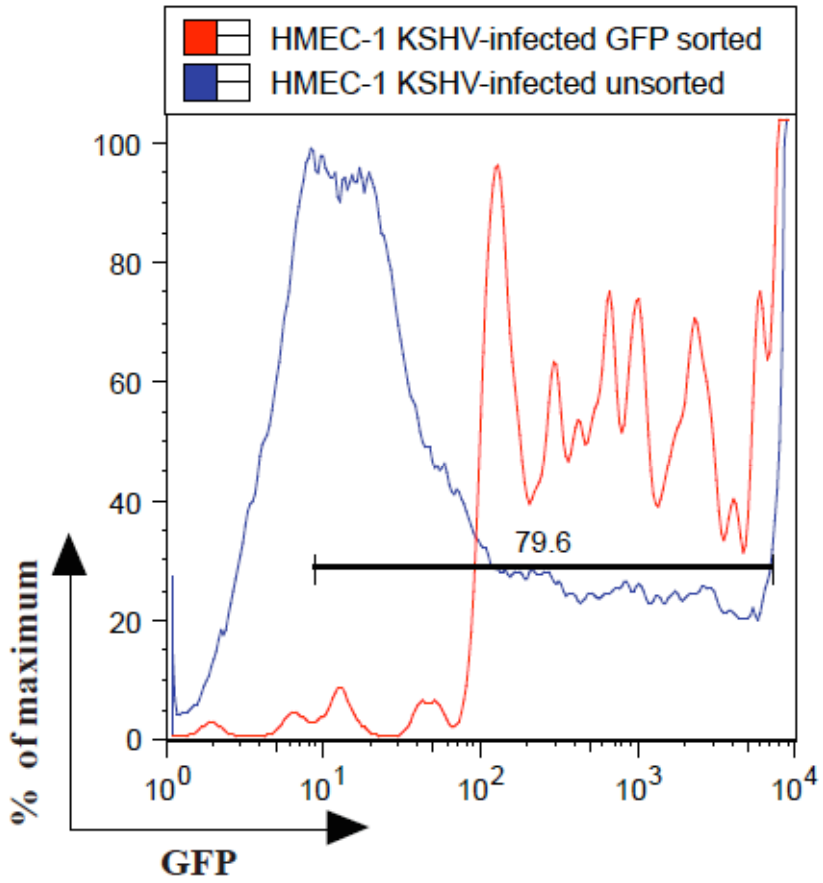


Figure 4.11 GFP-sorted rKSHV.219-infected HMEC-1 cells. HMEC-1 cells were infected with rKSHV.219 at an MOI of 10 for 72 hours (shown in blue). The KSHV latently infected cells expressing GFP were then sorted by flow cytometry to give an enriched population (shown in red).

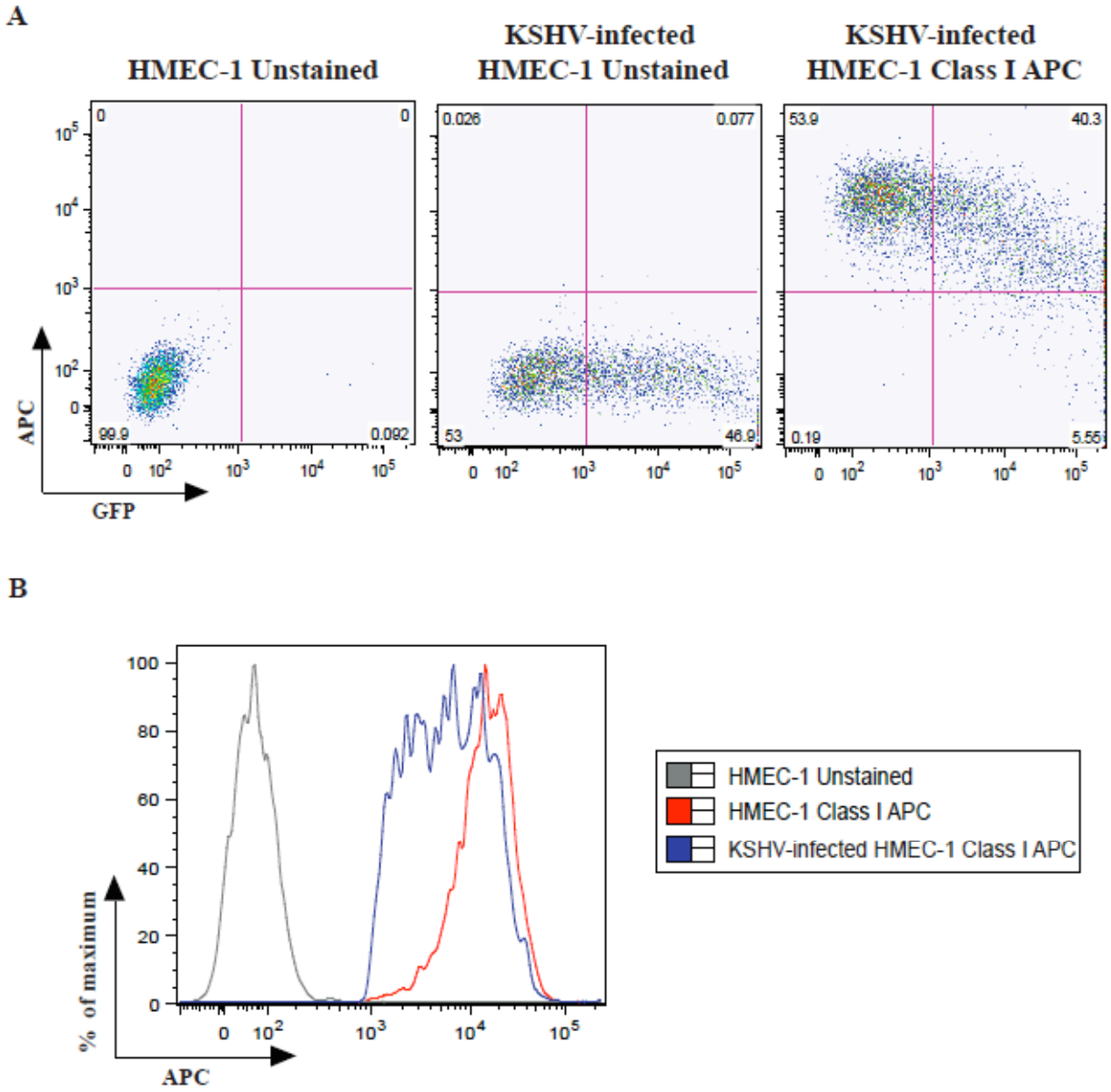


Figure 4.12 MHC Class I surface staining of HMEC-1 cells. (A) The HMEC-1 cells and KSHV latently infected HMEC-1 cells with the rKSHV.219 virus, were checked for GFP fluorescence (x-axis) and stained for surface expression of MHC class I using the APC conjugated w6/32 antibody (y-axis). The cells were analysed by flow cytometry. (B) Histogram comparing surface MHC Class I APC levels between unstained HMEC-1 cells (grey line), MHC Class I APC-stained HMEC-1 cells (red line) and KSHV-infected HMEC-1 cells (blue line).

HMEC-1 cells show approximately 47% GFP fluorescence, reflecting the percentage of the cells that had been latently infected with the rKSHV.219 virus. MHC class I surface levels were detected by staining with the APC-conjugated W6/32 antibody. The staining shows a clear reduction in the surface class I levels on the latently KSHV-infected HMEC-1 cells compared to the non-infected HMEC-1 cells, this result is further highlighted in the histogram in figure 4.12 (B) showing a clear reduction in class I W6/32-APC fluorescence for the KSHV-infected HMEC-1 cells. This result suggests that KSHV infection is potentially interfering with LANA-specific CD8+ T cell recognition of KSHV-infected HMEC-1 cells by reducing the levels of MHC class I expressed on the surface of the cells.

Discussion

These experiments examined native epitope recognition by CD8+ T cells specific for the LANA and vFLIP antigens expressed in various cellular backgrounds. In both cases, a role for each of these latent proteins in reducing or entirely inhibiting their own CD8+ T cell recognition was found. Initially, the influence of the sub-optimal codon usage identified within the wildtype vFLIP sequence and how this impacts its protein expression and subsequent CD8+ T cell recognition was investigated. The results showed that there was little or no protein expression or recognition of MJS or LCLs expressing the wildtype vFLIP sequence. However, using the human optimised codon sequence, protein expression was observed in MJS cells and CD8+ T cells recognised both MJS cells and LCLs expressing this construct. These results suggest that vFLIP uses sub-optimal codon sequences to reduce protein expression, a consequence of which is the limitation of the supply of viral peptides available for CD8+ T cell recognition.

Similar potential mechanisms to reduce CD8+ T cell targeting have been employed by a number of other viruses such as EBV, Human papillomavirus (HPV) and HIV. For EBV's genome maintenance protein EBNA1, the GAr domain is overloaded with purine containing codons. When the purine bias was reduced by substituting pyrmidine based codons and maintaining the amino acid sequence, this dramatically altered the predicted mRNA structure of EBNA1, reversed the cis-inhibitory effect on EBNA1 synthesis, consequently increasing the availability of viral peptides and CTL recognition (Tellam et al., 2008). While for HPV type 16, the E7 gene was reported to also be using a sub-optimal codon sequence inducing only moderate immune responses in murine DNA vaccination models. However, expression of an optimised E7 codon sequence in vitro resulted in increased levels of protein expression as a result of increased mRNA translation. When optimised vectors were used in vivo in vaccination studies, these induced much higher CTL responses than those elicited with the wildtype E7 sequence (Liu et al., 2002). In the case of HIV, optimising the codon usage of the gag protein increases protein expression, not through increased translation efficiency but rather through increased mRNA stability and export of nuclear mRNA (Ngumbela et al., 2008).

In the following vFLIP CD8+ recognition experiments, one issue that was repeatedly present in each of the recognition assays was the distinct lack of wildtype vFLIP protein expression being detected by western blot analysis, despite transfection being confirmed by GFP analysis by flow cytometry. This result conflicts with previous work in which wildtype vFLIP protein is detected using a vFLIP antibody after expression from a lentiviral vector in vitro (Rowe et al., 2009) but is consistent with other studies (Alkharsah et al., 2011). This could potentially be explained by the use

of different antibodies to detect expression, levels of transfection efficiency or the use of different expression vectors. An additional factor may be that the monocistronic vFLIP expression vectors used in these recognition studies do not recapitulate the expression of the vFLIP gene that occurs *in vivo* or in *in vitro* KSHV infected cells. While monocistronic vFLIP mRNA message can be detected in PELs, this is usually at a low abundance (Grundhoff et al., 2001). As vFLIP belongs to the latency transcript cluster that also encodes LANA and vCyclin, it is transcribed from a constitutively active promoter and through alternative splicing gives rise predominantly to an mRNA vFLIP encoding tricistonic or bicistronic transcript (Pearce et al., 2005). Ideally these CD8+ T cell recognitions experiments should be repeated using a different model system, in which the humanised and wildtype vFLIP sequences would be transcribed from a tricistronic or bicistronic transcript mimicking vFLIP expression in vivo.

The following study investigated CD8+ T cell recognition of the LANA antigen in a native setting using a LANA-specific CD8+ T cell clone. The role of the acidic repeat sequence present within LANA was investigated through a direct comparison of LANA CD8+ T cell recognition of both the native form of the protein (FL-LANA) and a form lacking the acidic repeat sequence (LANAΔacid) expressed in an LCL background. These experiments confirmed that similar to EBNA1, the repeat sequence within LANA reduces protein synthesis and consequently CD8+ T cell recognition. This retardation of LANA protein synthesis by the repeat sequence and consequent inhibition of its CD8+ T cell recognition has been highlighted using a model system (Kwun et al., 2007; Zaldumbide et al., 2007). However, like the EBNA1 studies using native EBNA1 clones to recognise EBV-infected or EBNA1

expressing cells, these experiments have demonstrated that the FL-LANA sequence can be recognised by LANA-specific CD8+ T cells, albeit to a lesser extent than LANA lacking the acidic repeat sequence.

In addition to this finding, the CD8+ T cell recognition of LANA was investigated in one of the most physiological settings possible by infecting an endothelial cell line, HMEC-1, with rKSHV.219. Latent KSHV infection was established and LANA protein expression detected. However there was no CD8+ T cell recognition of the LANA antigen presented by these cells. This is likely a result of the acidic repeat sequence present within LANA reducing the supply of epitope-peptides combined with the reduced class I surface expression upon KSHV infection of the cells. This effect on MHC class I expression may be attributed to the transient expression of the lytic gene K5 (MIR2) which has been shown to persist in KSHV infected endothelial cells *in vitro*, with K5 being detected up to 5 days post infection (Krishnan et al., 2004). Kedes et al. confirmed downregulation of surface MHC class I by K5 in latent KSHV infected endothelial cells, as well as identifying its effect on the reduction of surface expression of the leukocyte recruitment molecule ICAM-1. Furthermore, they showed that levels of K5 expression correlated with the MOI and degree of KSHV latent infection in endothelial cells and that after several days in culture, these cells would express normal levels of surface MHC class I (Adang et al., 2007). These studies have allowed us to hypothesise that K5 expression is responsible for the downregulation of surface MHC class I in the KSHV infected endothelial cells. Ideally K5 expression in these cells would have been confirmed in order to determine if it does play a role in the downregulation of surface MHC class I in the KSHV infected HMEC-1 cells. Alternatively, as the degree of downregulation of surface

class correlates with the multiplicity of infection used to infect the target cells (Adang et al., 2007), future experiments examining the recognition of endothelial cells infected with lower levels of virus would be useful to dissect the potential influence of K5 in this system. Alternatively, as the virus appears to stably maintain latency in these cells, infected cells could be cultured for several days and surface MHC class I levels allowed to be restored and then these cells used as targets in recognition assays. Given that LANA is expressed in all infected cells and malignancies, the observation that when full length LANA is expressed in target cells by itself and can elicit at least some degree of recognition from the T cells warrants future investigation of the ability of these T cell specificities to recognise LANA expressing cells.

Chapter 5

CD4+ T cell recognition of LANA-expressing cell lines and primary effusion lymphoma cell lines

Kaposi's sarcoma-associated herpesvirus (KSHV) and Epstein-Barr virus (EBV) are the two lymphotropic human herpesviruses with oncogenic potential. Similar to EBV, KSHV infects B cells and establishes latency in vivo. B cell infection is integral for the lifecycle of KSHV as latency is established in this cell type, preserving the potential for virion production upon reactivation and allowing the virus to spread infection. A number of studies have highlighted the role of CD4+ T cells in controlling herpes virus infections, such as mouse γ-herpesvirus 68 (MHV-68) and EBV. Mice infected with MHV-68 have shown the importance of CD4+ T cells in the control of chronic MHV-68 infection and its malignant consequences (Christensen et al., 1999; Robertson et al., 2001). Furthermore, evidence from the study of control of EBV infection in vivo and in vitro has also emphasised the importance of CD4+ T cells, in inhibiting outgrowth of EBV transformed B cells (Nikiforow et al., 2003; Omiya et al., 2002) and acting as CTL's in their own right, targeting LCLs (Haigh et al., 2008) and EBV-associated Burkitt's lymphoma (Paludan et al., 2002).

The role of KSHV-specific T cells in the control of KSHV-infected cells and associated malignancies has not been well defined. In contrast to EBV, the establishment of latent stably infected B cells *in vitro* for KSHV has proven very

difficult (Bechtel et al., 2003; Renne et al., 1998), greatly limiting the study of KSHV lymphoid infections. To date, two groups have identified systems for studying KSHV infection in primary B cells. The first of which showed that B cells can be infected *in vitro* but only when activated by IL-4 and CD40 Ligand (Rappocciolo et al., 2008; Hassman et al., 2011). More recently, tonsillar B cells have been successfully infected with recombinant KSHV virus (rKSHV.219), establishing lytic and latent infection. Activated CD4+ T cells were shown to inhibit viral replication in these cells, albeit in an MHC independent mechanism (Myoung et al., 2011). However, to date no published studies have tested the ability of KSHV-specific MHC-restricted T cells to recognise KSHV-infected cells from the endothelial neoplasm Kaposi's sarcoma (KS) or the B cell malignancy Multicentric Castleman's disease (MCD). Where this has been examined is in PEL cell lines expressing an HIV-reporter antigen and challenging these with their cognate CD8+ T cell. No recognition was seen as these cells were found to have a defective class I processing pathway; the PELs were found to express reduced levels of TAP-1 mRNA and low levels of surface MHC class I (Brander et al., 2000). Such cells are incapable of efficiently presenting intracellularly derived epitopes and are consequently likely to be poorly targeted by CD8+ T cells. Additionally, it has been shown that cell surface proteins involved in T cell activation and cell adhesion are expressed at significantly lower levels on PEL cells when compared to LCLs or primary B cells, potentially impairing the antigenicity of PEL cells *in vitro* (Suscovich et al., 2004). These findings question the ability of the immune response to effectively control these virus-associated pathologies.

From the previous chapter, evidence suggesting that the virus has developed mechanisms by which to reduce the supply of CD8 epitopes from two key latent

proteins, LANA and vFLIP, to T cells was found; thereby potentially limiting control exercised by these T cells. However these mechanisms may not be effective at constraining epitope presentation to CD4+ T cells. Currently a role for CD4+ T cell control of HLA class II targets such as infected B cells or PEL and MCD is unclear. This study was conducted to investigate whether LANA-specific CD4+ T cells can respond to LANA-expressing cells or infected cells in the form of PEL cell lines. The panel of LANA-specific CD4+ T cells identified and characterised in Chapter 3 were used to determine whether HLA-matched LANA-expressing EBV-transformed B lymphoblastoid cell lines (LCLs) or PEL cell lines can be targeted by CD4+ T cells.

5.1 Ectopic expression of LANA in lymphoblastoid cell lines (LCLs) for use as a model target cell line

To test recognition of LANA-expressing cells by LANA-specific CD4+ T cells, a model system was developed where LANA expression vectors were transfected into B cells to test LANA-specific CD4+ T cell recognition of antigen-expressing cells. LANA constructs were ectopically expressed using derivatives of the pRTS-CD2 plasmid shown in figure 5.1 and described in Chapter 4 (Kelly et al., 2009). The LANA-gene constructs used were derived from the BCBL-1 strain of virus and modified to remove the acidic repeat sequence (LANAΔacid) as this has been shown to increase protein expression (Kwun et al., 2007). These were further engineered to encode the haemagglutinin (HA) antibody epitope at the C-terminus of the protein to allow detection. A derivative of this construct was also engineered to incorporate the amino terminus of the MHC class II invariant chain sequence at the amino terminus of this protein (LANAΔacid li). This modification directs proteins into the endolysosomal compartment, giving efficient processing and presentation of epitopes

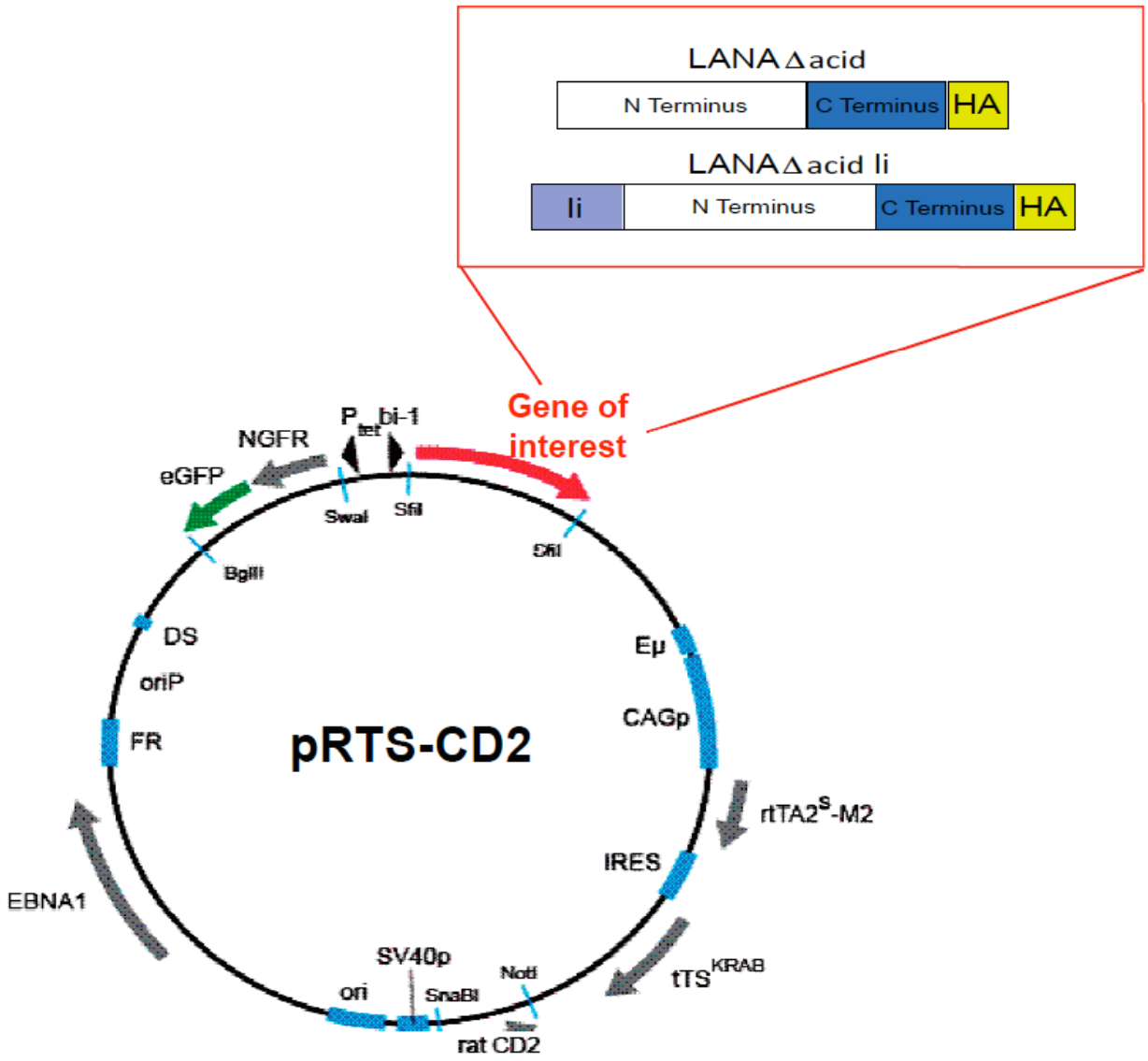


Figure 5.1 Schematic map of the pRTS-CD2 vector for the ectopic expression of LANAΔacid and LANAΔacid Ii. The vector contains a bi directional tetracycline-regulated promoter, which activates expression of the LANAΔacid or LANAΔacid Ii genes of interest in one direction and eGFP and NGFR in the other. rtTAs-M2 is the tetracycline controlled transactivator, tTS^{KRAB} is the tetracycline repressor placed downstream of the internal ribosome entry site (IRES). The SV40 promoter (SV40p) drives expression of the constitutively expressed rat CD2, ori is the bacterial origin of replication and EBNA1 is the EBV genome maintenance protein. OriP denotes the EBV episomal origin of replication, this contains the family of repeats and the dyad symmetry element.

from the fusion protein by class II MHC molecules (Sanderson et al., 1995), allowing cells transfected with this construct to act as positive controls in the LANA CD4+ T cell recognition experiments.

The LANAΔacid expression vectors were transfected into EBV-transformed B-lymphoblastoid cell lines (LCLs), selected for expression of rat-CD2 and expanded in the absence of dox. Once expanded, the transfected LCLs were stimulated with high levels of dox, 2μg/ml, for 24, 48, 72, and 96 hours to determine when maximal LANA construct expression occurs. Cells were lysed at each time point with 9M urea and kept at -70°C until all the time points were collected. Proteins in the lysates were separated on an 8% SDS gel, blotted onto nitrocellulose membranes which were probed for LANAΔacid expression using a monoclonal antibody specific for the HA epitope tag. LANAΔacid li and LANAΔacid expression in the dox-induced transfected LCLs from KSB1 and KS48 are shown in figures 5.2 and 5.3, respectively. LANAΔacid protein expression in each donor's LANAΔacid li and LANAΔacid transfected LCLs was sufficient at 72hrs post-induction so this time of induction was used in subsequent studies.

Figures 5.2 and 5.3 show that LANA protein was capable of being expressed in the transfected LCLs upon dox induction. However, the LANA constructs showed different levels of protein expression in the LCLs upon induction with the same concentration of dox. It was reproducibly found that the LANAΔacid li transfected LCLs expressed much higher levels of protein at each time point compared to the LANAΔacid transfected LCLs. So that meaningful comparisons between cells expressing the two constructs could be made in T cell recognition experiments, the

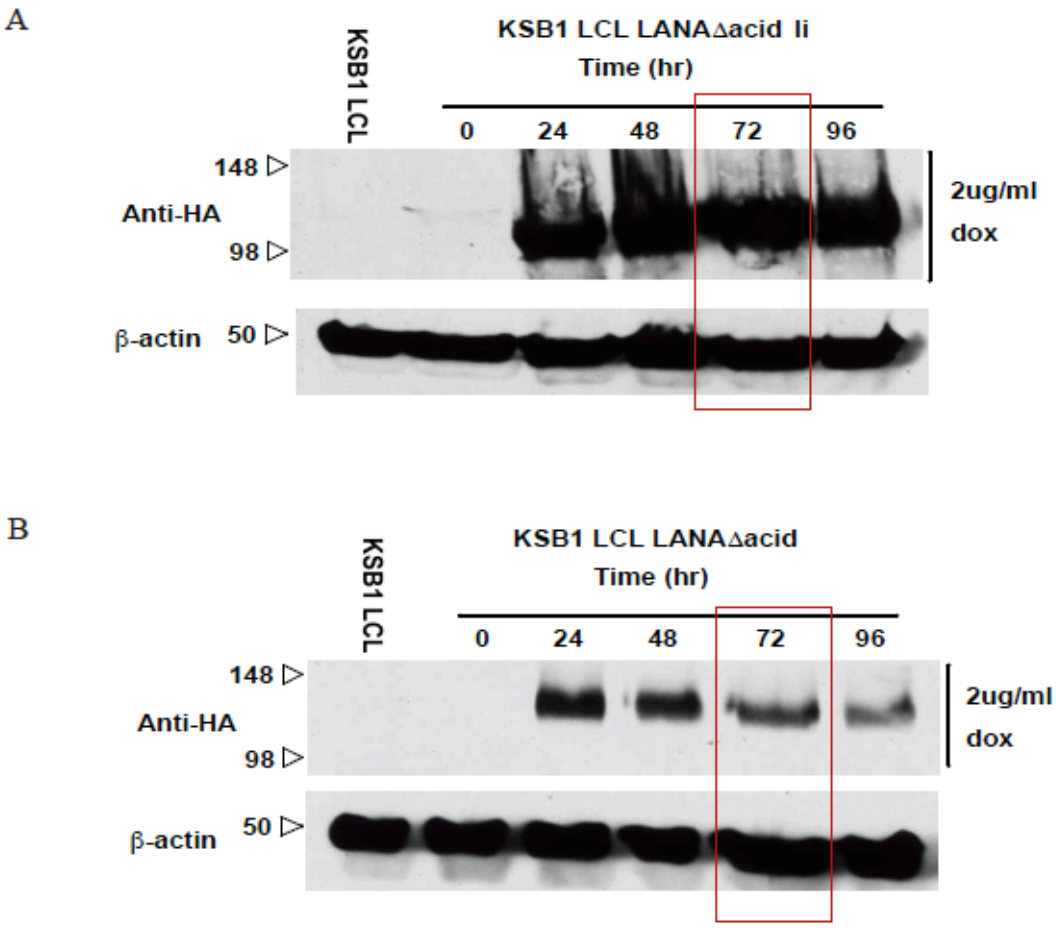


Figure 5.2 Time course of recombinant LANA construct protein expression of transfected KSB1 LCLs, following induction with 2 μg/ml dox. Immunoblot showing recombinant LANA protein levels in lysates from cells transfected with (A) LANAΔacid Ii or (B) LANAΔacid expression vectors after induction with dox at 2μg/ml. Non-transfected KSB1 LCL was used as a negative control. Transfected KSB1 LCLs were maintained in dox containing medium for up to 96 hours, with aliquots of cells lysed at 24hr time points. Recombinant LANA protein levels were detected using monoclonal HA antibody. Actin levels served as a loading control detected using monoclonal β-actin antibody.

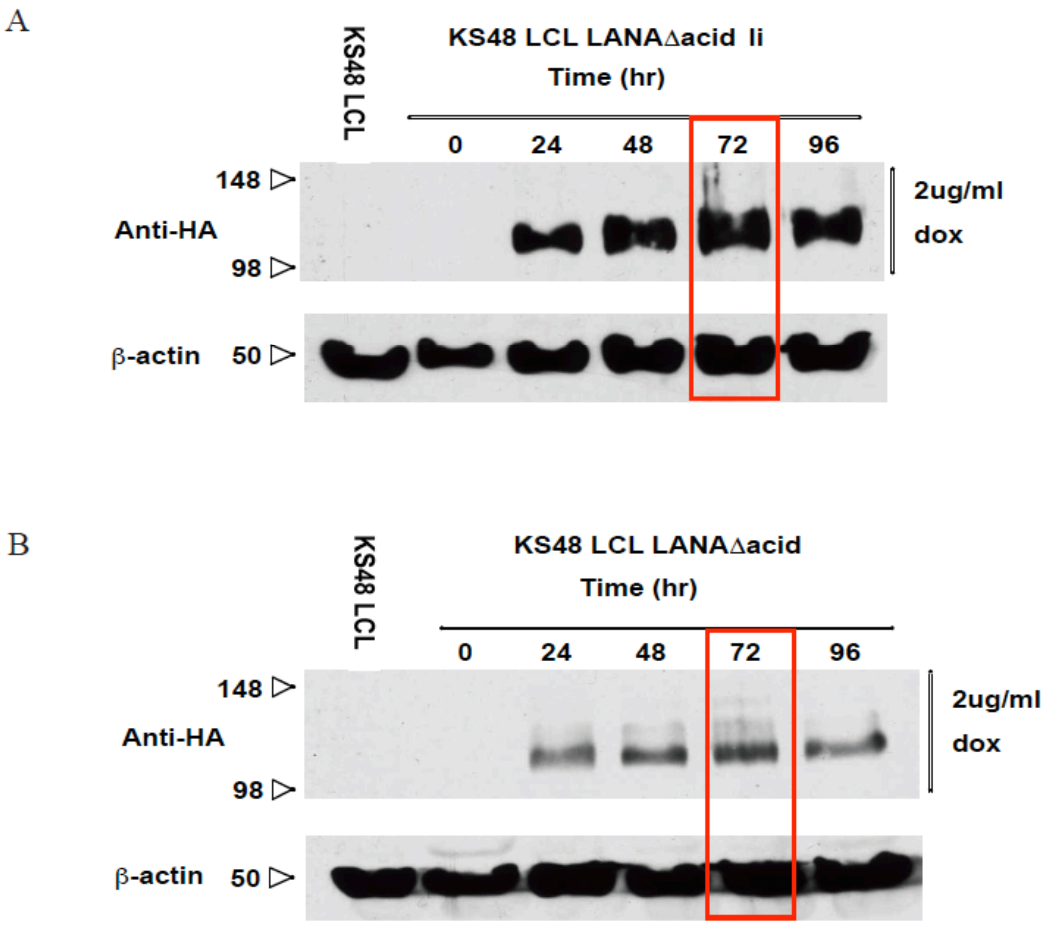


Figure 5.3 Time course of recombinant LANA construct protein expression of transfected KS48 LCLs, following induction with 2 μg/ml dox. Immunoblot showing recombinant LANA protein levels in lysates from cells transfected with (A) LANAΔacid Ii or (B) LANAΔacid expression vectors after induction with dox at 2μg/ml. Non-transfected KS48 LCL was used as a negative control. Transfected KS48 LCLs were maintained in dox containing medium for up to 96 hours, with aliquots of cells lysed at 24hr time points. Recombinant LANA protein levels were detected using monoclonal HA antibody. Actin levels served as a loading control detected using monoclonal β-actin antibody.

levels of protein expressed on a per cell basis were equalised by reducing the concentration of dox used to induce LANAΔacid li expression. LANAΔacid li transfected LCLs were induced with concentrations of dox titrated from 12ng/ml down to 0.75ng/ml and the levels of protein expressed in these cells were compared to LANAΔacid transfected LCLs induced with 2μg/ml dox by western blot analysis. As dox induction also induces expression of the GFP reporter molecule in this vector, the percentage of LANAΔacid and LANAΔacid li expressing LCLs can also be determined at each dox concentration as measured by GFP expression using flow cytometry analysis (data not shown). Figure 5.4 shows results of the western blot analysis probing for HA to detect LANA construct expression, or β-actin which was used as a loading control. The percentage of cells expressing GFP at each dox concentration is shown under the appropriate gel lane. The percentage of GFP expression and also LANAΔacid li expression in the LCLs decreased as the concentrations of dox was titrated out.

As LANAΔacid li is behaving as the positive control in the LANA CD4+ T cell recognition experiments, levels of dox used to induce LANAΔacid li protein expression were selected to ideally give equivalent or lower levels of expression relative to parallel induced LANAΔacid transfected LCLs used in the recognition assays. Taking these variables into account, the concentrations of dox selected to give LANAΔacid li expression levels similar to or lower than 2μg/ml dox induction of LANAΔacid was 6ng/ml for both KSB1 and KS48 respectively.

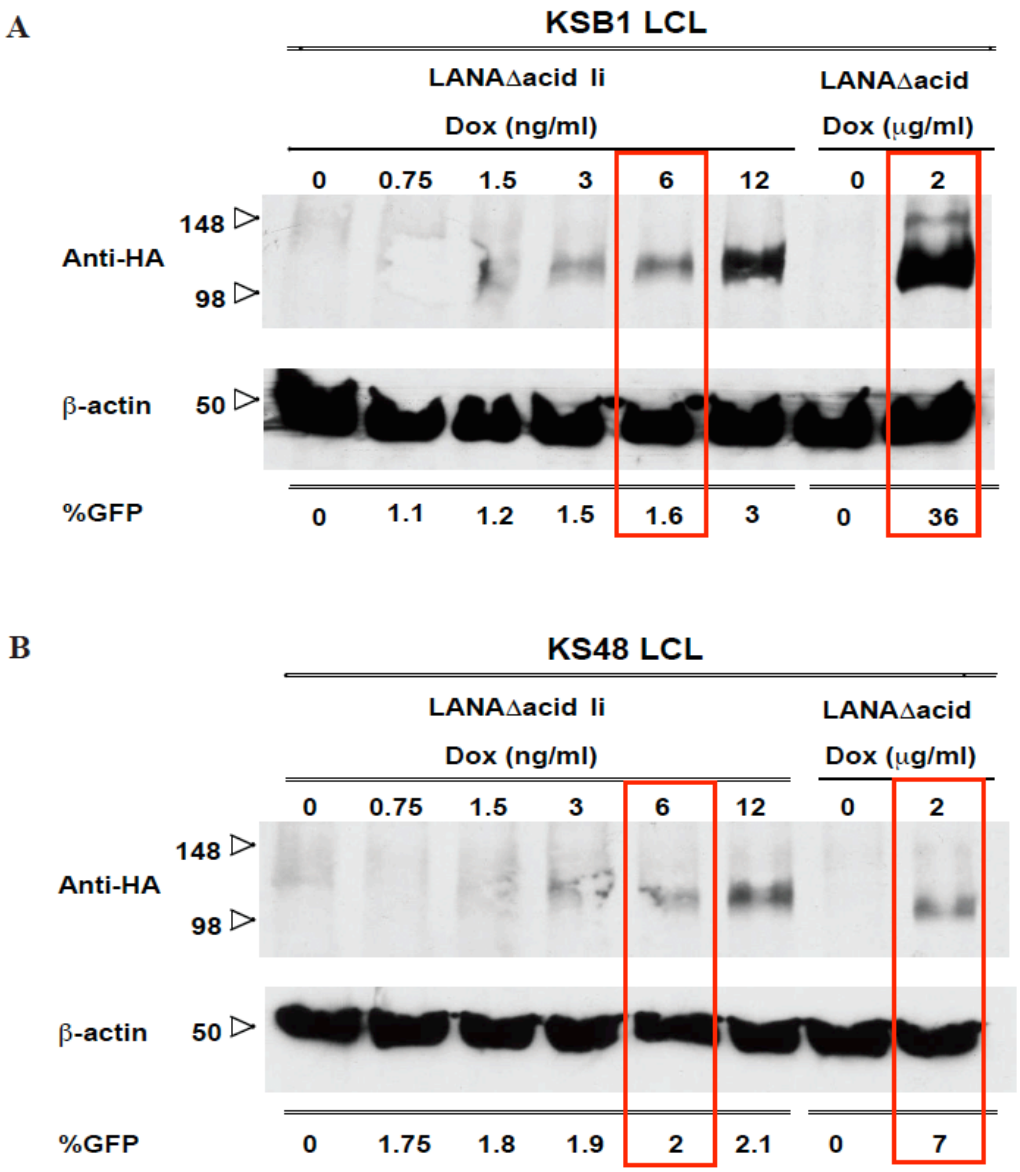


Figure 5.4 Recombinant LANA construct protein expression in transfected KSB1 and KS48 LCLs, following induction with different concentrations of dox. Immunoblot showing recombinant LANA protein expression following dox induction for 72hrs of LANAΔacid li and LANAΔacid transfected (A) KSB1 LCL and (B) KS48 LCL. LANAΔacid transfected LCLs were induced with dox at 2μg/ml. LANAΔacid li transfected LCLs were induced with titrated concentrations of dox from 12-0.75ng/ml. The percentage of cells expressing GFP at each dox concentration is shown under the appropriate gel lane. LANA construct protein levels were detected using monoclonal HA antibody. Actin levels served as a loading control detected using monoclonal β-actin antibody. The red box highlights the selected concentrations of dox to induce higher levels of LANA protein expression of the LANAΔacid transfected LCLs.

5.2 LANA-specific CD4+ T cell recognition of LCLs ectopically expressing LANAΔacid constructs

The next experiment sought to determine whether vector-derived LANAΔacid and LANAΔacid li antigen could be processed and presented by the MHC class II pathway of LCLs for recognition by LANA-specific CD4+ T cells. In this series of experiments, LANA-specific CD4+ T cells were incubated with their autologous LCLs expressing the LANAΔacid constructs and recognition assessed by measuring IFN-γ production by the T cells. LANAΔacid construct expression was induced in LCLs by incubation with the selected titrated dox concentration for 72hrs, as described above. The construct expressing LCLs were enriched by selecting for the induced co-expressed protein NGFR using MACS NGFR beads prior to their use in the CD4+ T cell recognition assay.

Figure 5.5 (A) shows representative results using transfected donor KSB1 LCLs assayed against a panel of LANA-specific KSB1-derived T cells. Initially expression levels of the LANAΔacid constructs was checked by western blot analysis of lysates from the KSB1 LCLs. Higher total levels of LANA protein were detected in lysates from LANAΔacid versus LANAΔacid li transfected LCLs. This correlated with 50% of the former LCLs expressing the reporter GFP, compared to the 2.6% GFP expression by the LANAΔacid li transfected LCLs. Representative results of recognition assays measuring IFN-γ production from 3 independent LANA-specific clones incubated with the dox induced autologous KSB1 transfected LCLs, or, as a positive control, cognate epitope-peptide sensitised autologous KSB1 LCLs are shown in figure 5.5 (B-D). Here the clones tested were the HLA-DQ6 restricted c63 specific for peptide EYR (B), the HLA-DR13 restricted c33 specific for peptide LRS

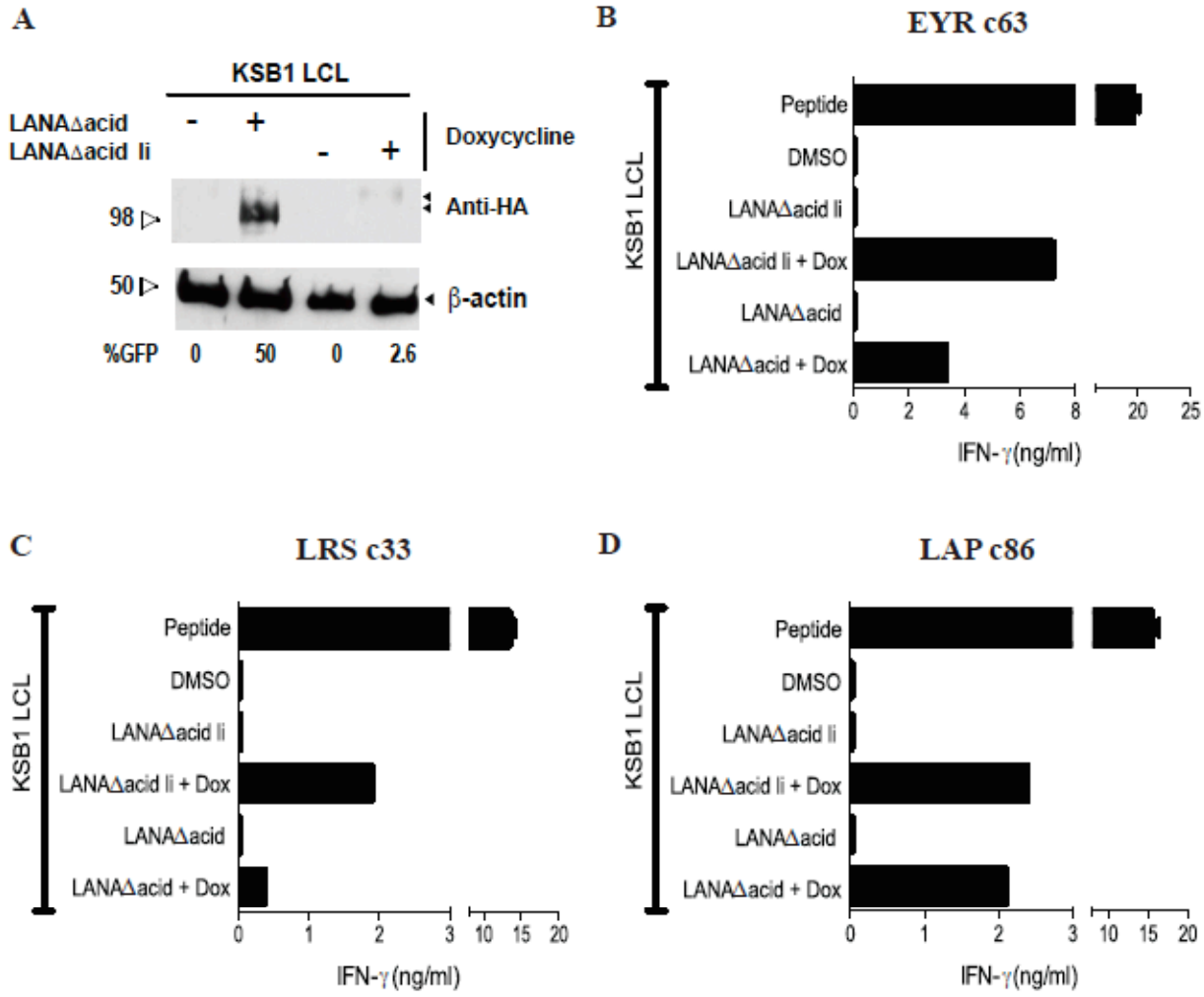


Figure 5.5 Recombinant LANA construct protein expression and T cell recognition of donor KSB1 transfected LCL targets. (A) Immunoblot showing recombinant LANA protein expression levels of KSB1 transfected LANAΔacid and LANAΔacid li LCL targets following 72hrs of dox induction at 2μg/ml and 6ng/ml, respectively. The percentage of cells expressing GFP is shown under the appropriate gel lane. LANA construct protein levels were detected using monoclonal HA antibody and actin levels served as a loading control detected using monoclonal β-actin antibody. Aliquots of the dox induced and uninduced KSB1 transfected LCL targets were incubated overnight with three autologous LANA specific CD4+ T cell clones with different peptide specificities and recognition quantified by IFN-γ ELISA. Results shown from (B) HLA DQ6-restricted c63 specific for peptide EYR, (C) HLA DR13-restricted c33 specific for peptide LRS and (D) HLA DP1-restricted c86 specific for peptide LAP. As a positive control the KSB1 LCL targets were peptide pulsed with the cognate epitope-peptide at 5μg/ml or as a negative control with peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

(C) and the HLA-DP1 restricted c86 specific for peptide LAP (D). For each of the LANA-specific clones, the positive control peptide pulsed LCLs were well recognised by the LANA-specific CD4+ T cells, but they did not recognise LCLs pulsed with peptide solvent DMSO showing that the T cells are functional and specific for their respective LANA epitope-peptide. The LANAΔacid li construct expressing LCLs were well recognised by each of the LANA-specific CD4+ T cell clones, showing that LANA protein directly routed into endo-lysosomal compartment in LCLs with the help of the MHC class II invariant chain can be processed and presented for CD4+ T cell recognition. More importantly, LANAΔacid construct expressing LCLs were recognised by the CD4+ T cells, showing that LCLs can process and present ectopically expressed LANAΔacid to LANA-specific CD4+ T cells. This experiment was repeated at least twice, using nine LANA-specific clones derived from donor KSB1 and gave similar results.

LANA CD4+ recognition experiments using the HLA-DQ7 restricted KS48 derived GSP-specific clone was assayed against two additional transfected LCLs; the autologous KS48 LCL and HLA-DQ7 matched KSB2 LCL. As described above, LANA expression was induced in LCLs by incubation with the selected titrated dox concentration for 72hrs and further enriched by selecting for the vector expressed protein NGFR. Figure 5.6 (A) and (C) show representative results of a western blot analysis for LANAΔacid construct expression from transfected LCLs from donors KS48 and KSB2. For both LCLs, higher levels of LANA protein were detected in lysates from LANAΔacid transfected LCLs as compared to LANAΔacid li lysates. These results correlate the percentage GFP expression in the transfected LCLs, with 44.6% and 44% GFP expression in the LANAΔacid transfected KS48 and KSB2

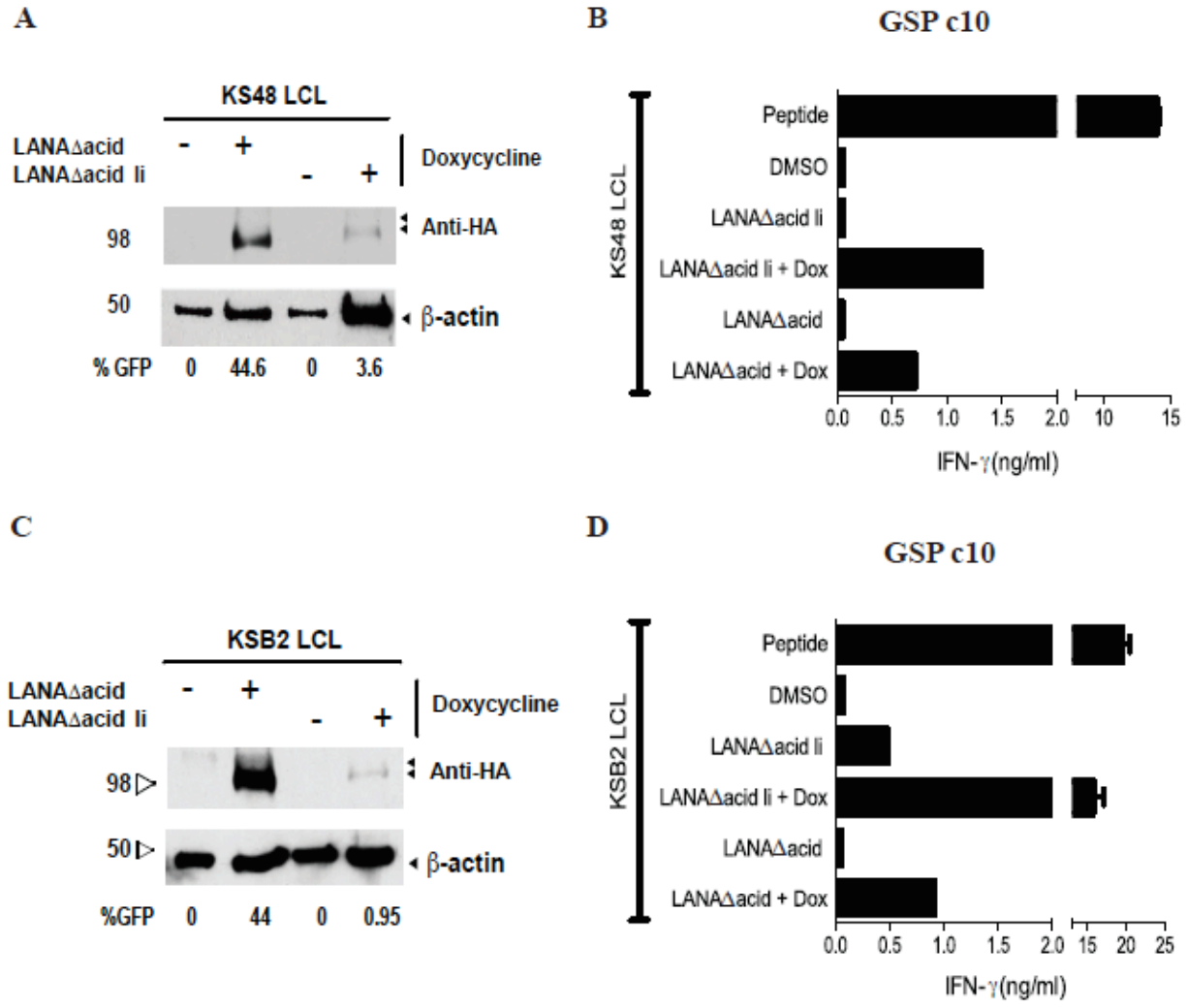


Figure 5.6 Recombinant LANA construct protein expression and CD4+ T cell recognition of donors KS48 and KSB2 transfected LCL targets. Immunoblots showing recombinant LANA protein expression levels of the (A) KS48 and (C) HLA DQ7 matched KSB2 transfected LANAΔacid and LANAΔacid li LCL targets, following 72hrs of dox induction at 2μg/ml and 6ng/ml, respectively. The percentage of cells expressing GFP is shown under the appropriate gel lane. LANA construct protein levels were detected using monoclonal HA antibody and actin levels served as a loading control detected using monoclonal β-actin antibody. The dox induced and uninduced (B) KS48 and (D) KSB2 transfected LCL targets were incubated overnight with the DQ7-restricted donor KS48 LANA GSP-specific CD4+ T cell clone and recognition quantified by IFN-γ ELISA. As a positive control, the (B) autologous KS48 and (D) HLA-matched KSB2 LCL targets were peptide pulsed with the cognate epitope-peptide at 5μg/ml or as a negative control with peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

LCLs, compared to the 3.6% and 0.95% GFP expression of the LANAΔacid li transfected KS48 and KSB2 LCLs, respectively.

These dox induced transfected LCLs were subsequently used in CD4+ LANA-specific recognition assays. Figure 5.6 (B) and (D) shows results with HLA-DQ7restricted KS48 C10 specific to LANA peptide GSP. The LANA-specific clone was incubated with the dox induced autologous KS48 (B) and HLA-DQ7 matched KSB2 (D) donor transfected LCLs. As a positive control, the LCLs were sensitised with cognate epitope-peptide GSP and peptide solvent DMSO as a negative control. The GSP-specific clone recognised the positive control peptide pulsed LCLs but not DMSO sensitised LCLs showing that the T cells are functional and specific. The LANAΔacid li construct expressing LCLs were also well recognised by the GSP-specific CD4+ clone, showing that LANAΔacid protein directly routed into endo-lysosomal compartment in LCLs can be processed and presented for CD4+ T cell recognition. More importantly, LANAΔacid construct expressing LCLs were recognised by the CD4+ T cells, showing that both autologous and HLA-matched LCLs can process and present ectopically expressed LANAΔacid to LANA-specific CD4+ T cells. For donor KS48 LANA expressing LCLs, this was tested on three different occasions and repeated twice for donor KSB2 LANA expressing LCLs with LANA-specific KS48 clone 10 derived. All experiments yielded a similar result.

5.3 CD4+ T cell recognition of LCLs exogenously fed LANA protein

These experiments have so far established that LCLs can process and present ectopically expressed LANAΔacid protein for LANA-specific CD4+ T cell recognition. Following on from this, the next question to address was whether the

LANA-specific CD4+ T cells were capable of responding to LCLs which had been fed exogenous LANA protein. In this case for successful recognition, the LCLs would have to take up the LANA protein, process and present the peptides from the protein using the classical MHC class II processing pathway. The processing and presentation of exogenous LANA protein was tested using enriched LANA protein preparations derived from KSHV LANA positive PEL cell line BCBL-1, or a control antigen derived from KSHV negative cell line DG-75 described in section 2.4 of the materials and methods. Autologous LCLs were incubated with the protein for 18 hours before being used in recognition assays with a range of LANA-specific CD4+ T cell clones. Figure 5.7 shows the results of this protein feeding experiment for four representative CD4+ LANA-specific clones using the two autologous donor's LCLs, KSB1 (A-C) and KS48 (D). For each of the LANA-specific CD4+ clones, the positive control peptide pulsed LCLs were recognised by the LANA-specific CD4+ T cells, but they did not recognise LCLs pulsed with peptide solvent DMSO. The three KSB1 clones tested were the HLA-DQ6 restricted c63 specific for peptide EYR (A), the HLA-DR13 restricted c33 specific for peptide LRS (B) and the HLA-DP1 restricted c86 specific for peptide LAP (C) and the donor KS48 HLA-DQ7 restricted c10 specific for peptide GSP (D). All recognised the autologous LCLs fed exogenous LANA protein at both 80μg/ml and 40μg/ml, with no recognition of cells fed the control DG75 protein. Similar results were obtained for a further five KSB1 clones specific for different target epitopes on two different occasions (data not shown). These results show that LANA antigen can be processed and presented for LANA-specific CD4+ T cell recognition using the classic MHC class II processing pathway and more importantly, the CD4+ LANA-specific T cell clones can recognise LANA antigen processed in this manner.

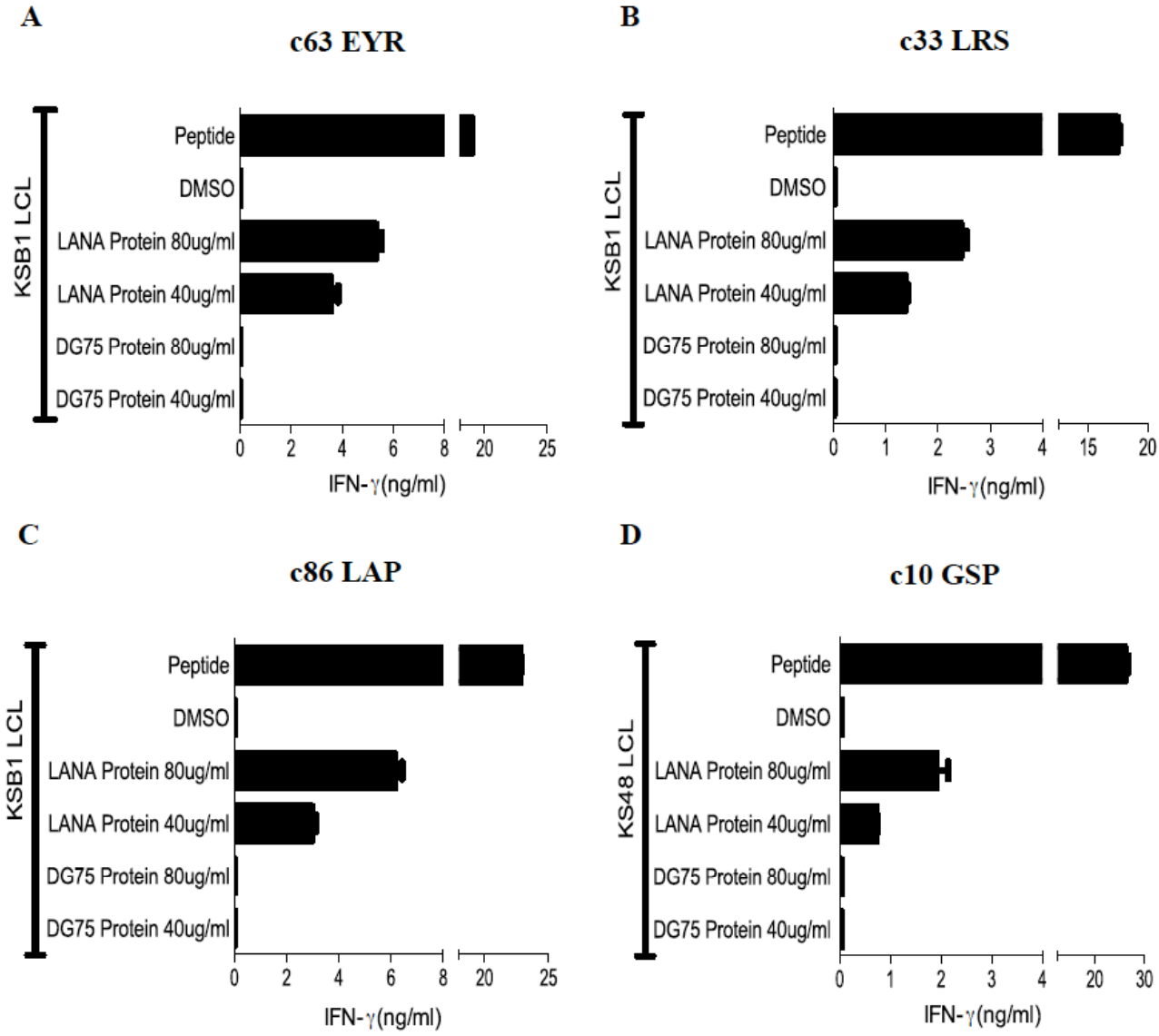


Figure 5.7 CD4+ T cell recognition of autologous KSB1 and KS48 LCL targets exogenously fed LANA protein. LANA protein derived from nuclear extracts of the PEL BCBL-1 or a control preparation derived from the KSHV negative cell line DG75 were incubated with the autologous LCL targets for 18 hours at 80μg/ml and 40μg/ml. These protein loaded cells were then used as targets in recognition assays by overnight incubation with the autologous LANA-specific CD4+ T cell clones and T cell recognition quantified by IFN-γ ELISA. Donor KSB1 LCL targets were incubated with three autologous LANA specific clones, (A) HLA DQ6-restricted c63 specific for peptide EYR, (B) HLA DR13-restricted c33 specific for peptide LRS and (C) HLA DP1 - restricted c86 specific for peptide LAP. (D) Donor KS48 LCL targets were incubated with the autologous HLA DQ7-restricted c10 specific for peptide GSP. As a positive control the autologous LCL targets were sensitised with cognate epitope-peptide or DMSO and also incubated for 18 hours with the T cell clone. Results are expressed as the mean IFN-γ release from triplicate wells.

5.4 CD4+ T cell recognition of KSHV associated primary effusion lymphoma cell lines using EBV proteins as model antigens

So far this work has established that LCLs can process and present LANA antigen for LANA-specific CD4+ T cell recognition. Following on from this finding, the next question to address was if KSHV infected B cells in the form of the KSHV malignancy primary effusion lymphoma (PEL) cell lines, are capable of processing and presenting CD4+ T cell epitopes for T cell recognition. PELs are B cells stably infected with KSHV, constitutively express LANA and are known to express MHC class II molecules on their surface (Cesarman et al., 1995). Moreover, PELs have previously been shown to have a defective CD8+ processing pathway as a result of reduced TAP-1 mRNA and decreased surface MHC class I (Brander et al., 2000). Initially then, surface MHC class I, total MHC class II, HLA-DQ and HLA-DR levels were measured by staining with mouse primary antibodies, these were detected with a secondary anti-mouse FITC conjugated antibody and intensity measured by flow cytometry analysis. Figure 5.8 shows representative results from four PELs BC-1, JSC-1, VG-1 and BCBL-1 that are HLA-matched (see Table 5.1) to the LANA-specific CD4+ epitope-peptides identified in chapter 3, while figure 5.9 shows staining from two LCLs for comparison. For PELs BC-1 and JSC-1, relatively low levels of total class II staining were observed using the pan class II antibody, with slightly lower levels of expression of class II molecules HLA-DR and HLA-DQ observed on these cells. The PEL line VG-1 displayed extremely low surface levels of overall class I, class II, and the class II molecules HLA-DR and HLA-DQ. BCBL-1 cells however differed from the other three PEL lines showing levels of surface class I and class II expression comparable to that of the donor LCLs KSB1 and KS48 (figure 5.9).

Table 5.1 PEL lines viral status and class II HLA types

Cell line	KSHV	EBV	DR	DQ	DP
BC-1	+	+	04 07 53	07 09	0301 0401
JSC-1	+	+	04 53	07	0401
VG-1	+	-	07 13 52	02 06	0101 0201
BCBL-1	+	-	14 15 51 52	05 06	0301 0401

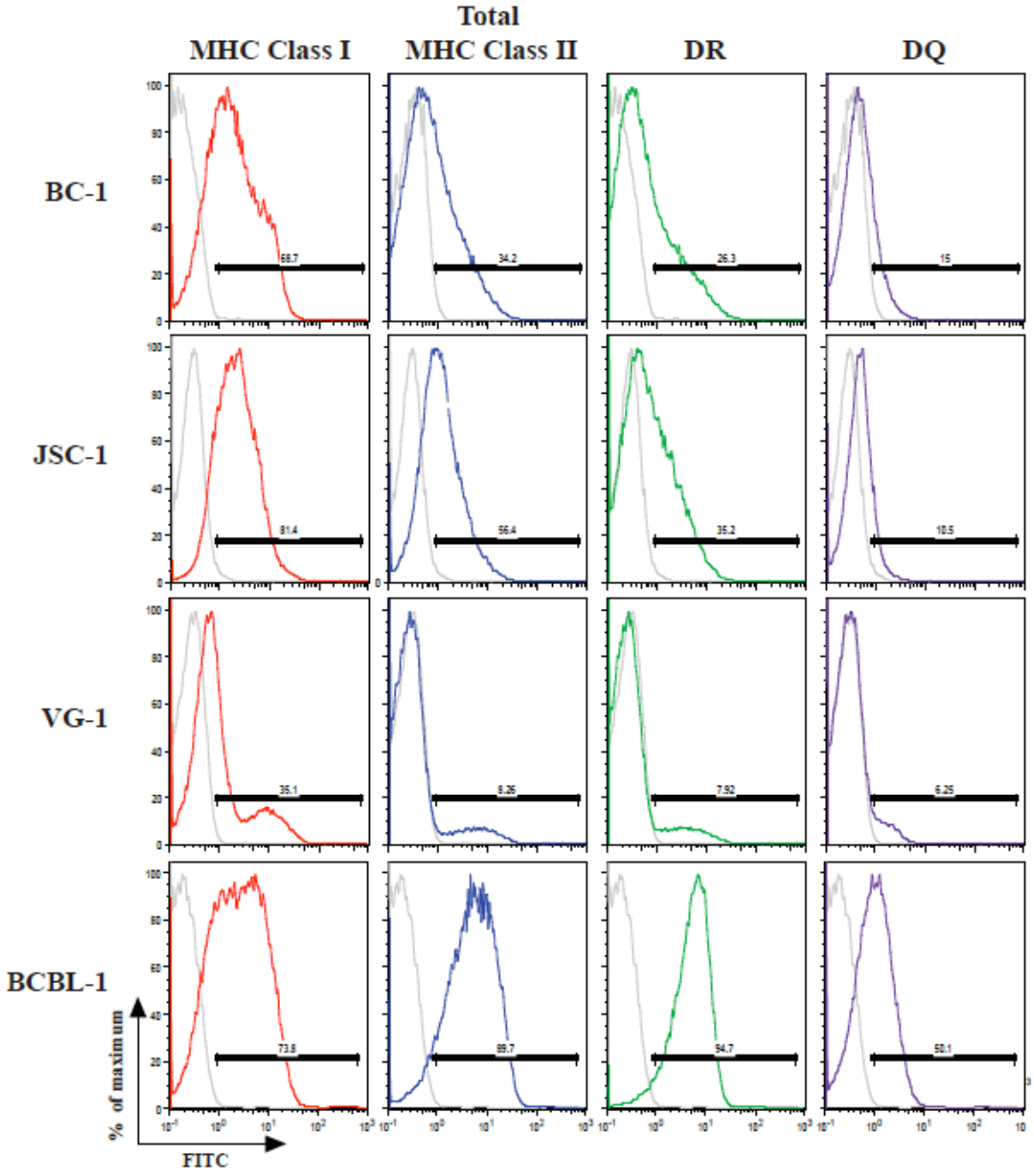


Figure 5.8 MHC surface staining of the PEL cell lines. The PELs BC-1, JSC-1, VG-1 and BCBL-1 were stained for surface expression of MHC class I (red line), total MHC class II (blue line), class II molecules DR (green line) and DQ (purple line) using mouse non-conjugated antibodies. A non-conjugated mouse IgG2a isotype antibody was used as a control for each staining (Grey line). A secondary anti-mouse FITC conjugated antibody was used to detect bound antibodies and the cells analysed by flow cytometry.

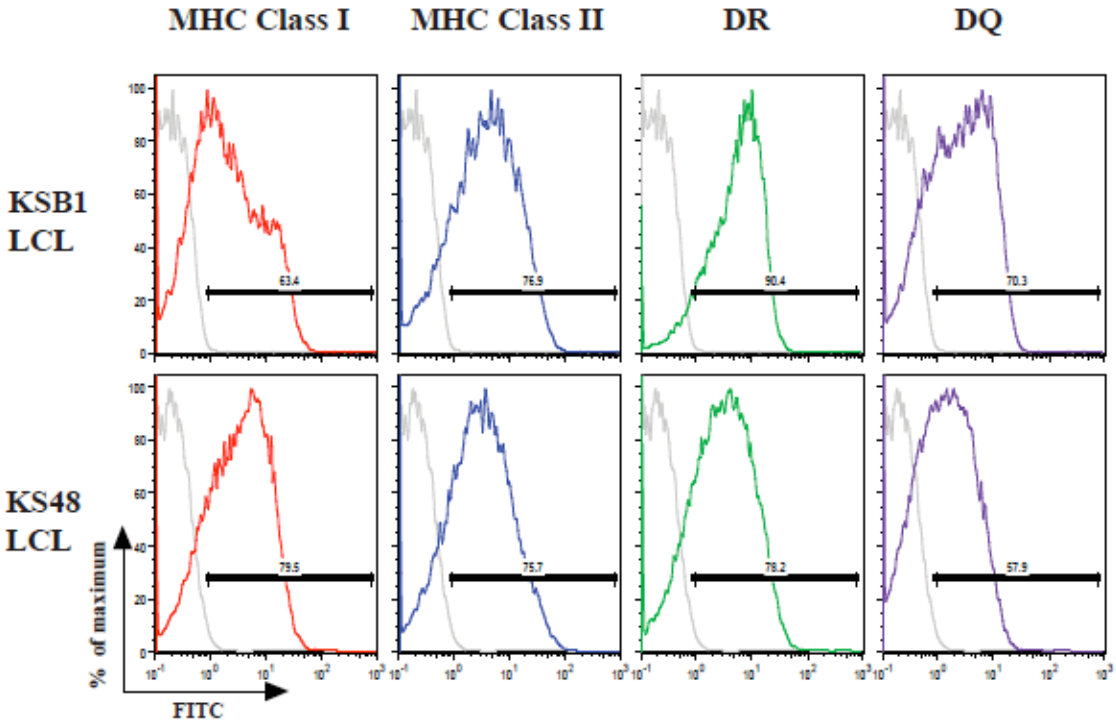


Figure 5.9 MHC surface staining of donor LCLs. The donor LCLs KSB1 and KS48 were stained for surface expression of MHC class I (red line), MHC class II (blue line), class II molecules DR (green line) and DQ (purple line) using mouse non-conjugated antibodies. A non-conjugated mouse IgG2a isotype antibody was used as a control for each staining (Grey line). A secondary anti-mouse FITC conjugated antibody was used to detect bound antibodies and the cells analysed by flow cytometry.

As little is known about the class II processing of antigens in PEL lines, a series of preliminary experiments were conducted using a model system to assess their ability to present epitopes to CD4+ T cells. Here a panel of EBV-specific CD4+ T cells matched with the HLA types of the PELs was used to probe recognition of PELs expressing invariant chain tagged EBV-antigens delivered by infection with recombinant modified vaccinia Ankara (MVA) constructs encoding the EBV-antigens. As a positive control, cells were pulsed with the cognate epitope-peptide or, as negative controls, infected with an MVA expressing an irrelevant protein or sensitised with DMSO. The EBV matched CD4+ T cells were incubated with the relevant target cells overnight and recognition assessed by measuring IFN-γ secretion. Figure 5.10 shows representative results for PELs BC-1, JSC-1, VG-1 and BCBL-1. PELs (A) BC-1, (B) JSC-1 and (C) VG-1 and HLA-matched LCLs were infected with an MVA-li expressing EBV protein EBNA2, and incubated with DR7 or DR4 restricted CD4+ T cell clones specific to EBNA2's PRS-peptide, as the PRS-peptide can be presented in more than one HLA context. Recognition of the EBNA2 MVA-li infected HLA-matched LCLs was detected for all three clones, confirming the specificity of the EBNA2-specific CD4+ T cell clones. In parallel, both the EBNA2 MVA-li infected BC-1 and JSC-1 PELs were also recognised by their respective EBNA2-specific CD4+ T cell clones. In the case of VG-1, shown in figure 5.10 (C), there was relatively poor recognition of the VG-1 PRS pulsed cells and the EBNA2 MVA-li infected VG-1 cells, consistent with the very low levels of MHC class II expressed on the surface of these cells (figure 5.8 (C)). Finally, BCBL-1 and HLA-matched LCLs were infected with the EBNA3C MVA-li and incubated with the DQ5 restricted CD4+ T cell clone specific to EBNA3C's SDD peptide. As illustrated in figure 5.10 (D), both the MVA-li HLA-matched DQ5 LCL and BCBL-1 were

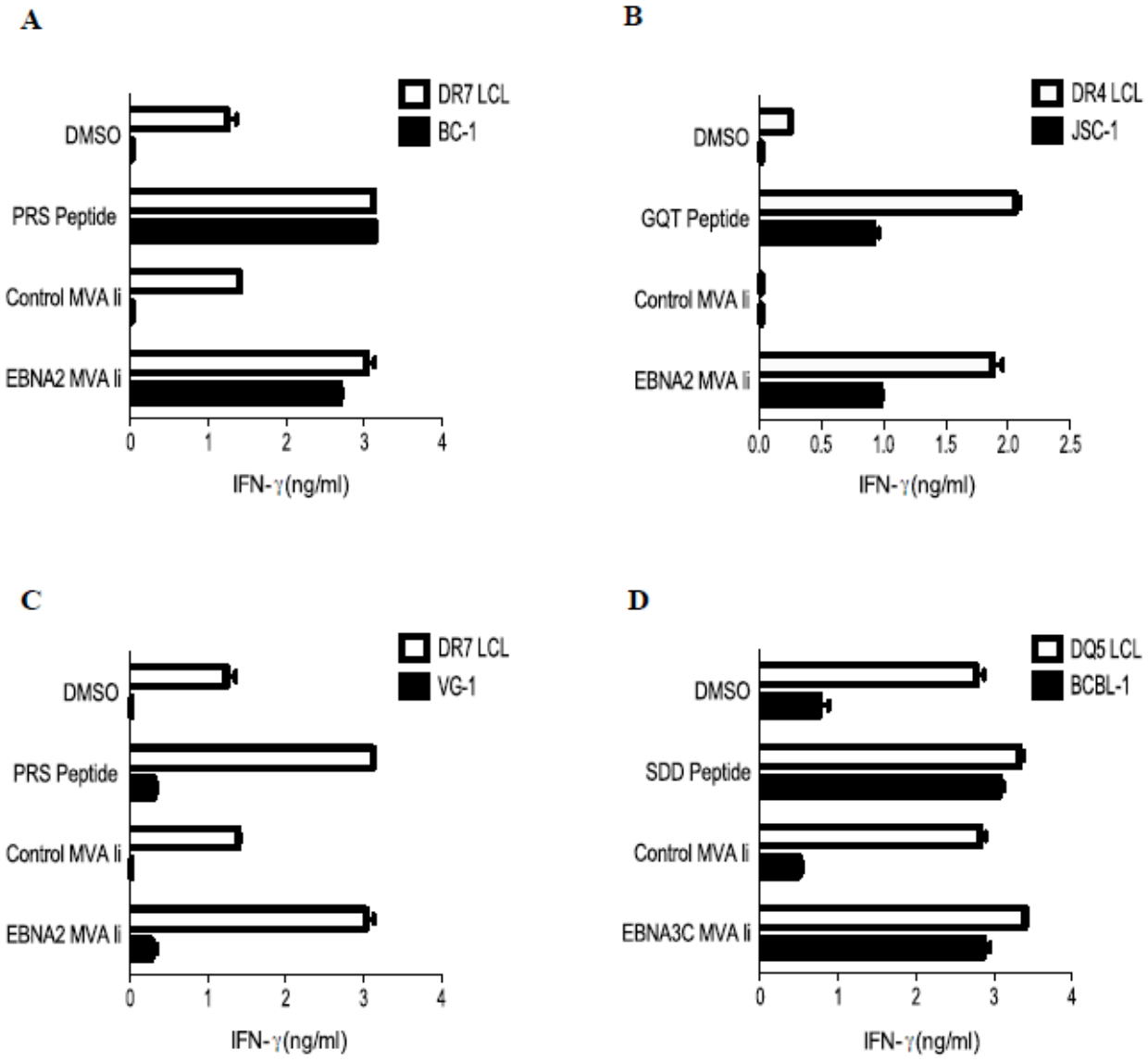


Figure 5.10 CD4+ T cell recognition of the KSHV PEL cell lines expressing EBV model antigens. PEL cell lines (A) BC-1 (B) JSC-1 (C) VG-1 and HLA-matched LCLs were infected with MVA-li EBNA2. The PEL cell line (D) BCBL-1 and HLA-DQ5 matched LCLs were infected with MVA-li EBNA3C. Each of the PELs and HLA-matched LCLs were infected with an irrelevant MVA as a control. The infected PELs and HLA-matched LCLs were then incubated with the EBV specific CD4+ T cell clones, EBNA2-PRS or EBNA3C–SDD and recognition quantified by IFN-γ ELISA. The PELs and HLA-matched LCLs were pulsed with cognate epitope-peptide as a positive control at 5μg/ml and with peptide solvent DMSO as negative control. Results are expressed as the mean IFN-γ release from triplicate wells.

recognised by the CD4+ SDD specific clone, the SDD clone also recognised endogenous EBNA3C antigen expressed in these LCLs; a pattern noted by others who used this clone (Long et al., 2005). Overall, the PELs were recognised by their respective CD4+ T cell clones when the antigen is directed to the endo-lysosomal pathway by the invariant chain. This suggests that at least part of the MHC class II processing pathway is intact in PELs and that they would be suitable targets for recognition by the LANA-specific CD4+ T cells.

5.5 Establishment of control PEL lines overexpressing LANAΔacid and LANAΔacid li

The previous experiments have established that LCLs ectopically expressing LANA-antigen can be recognised by LANA-specific CD4+ T cells and PELs are capable of processing and presenting EBV li tagged antigens. The subsequent experiments set out to investigate whether the HLA-matched PELs can process and present LANA-antigen for LANA-specific CD4+ T cell recognition. As well as addressing recognition of the PELs by the T cells, derivatives of the PELs were established which were transfected with the inducible LANAΔacid and LANAΔacid li plasmid constructs as used in the LCL studies. These lines were established as controls to allow overexpression of the LANAΔacid constructs in T cell recognition assays. PELs that are HLA-matched to the LANA-specific CD4+ T cell clones were transfected with the LANAΔacid and LANAΔacid li expression constructs using the same methods to transfect the LCLs described above in section 5.1. Initially the optimal time of induction of LANAΔacid construct expression was determined by inducing expression with 2μg/ml dox for 24, 48, 72, and 96 hours and monitoring protein expression levels by immunoblotting. LANAΔacid li and LANAΔacid

expression in the dox-induced transfected BC-1 cells are shown in figure 5.11. LANA protein expression in the LANAΔacid li was maximal at 96hrs post-induction, however LANAΔacid transfected BC-1 cells were maximal at 72hrs post-induction so this time of induction was used for the PELs in subsequent studies.

As shown above with the transfected LCLs, higher levels of the LANAΔacid li expression compared to LANAΔacid were detected in the transfected BC-1 cells. As before, the levels of LANAΔacid produced by the different constructs was equalised so that meaningful comparisons of T cell recognition of the PELs could be made. The concentration of dox used to induce expression of the LANAΔacid li construct was then titrated to decrease expression of the protein, using the same methods carried out on the transfected LCLs. Figures 5.12 and 5.13 shows results of the western blot analysis probing for HA to detect LANAΔacid construct expression at the different concentrations of dox in the HLA matched PELs. β-actin was used as a loading control. The percentage of cells expressing the GFP reporter at each dox concentration is shown under the appropriate gel lane. As LANAΔacid li is to be used as the positive control in the LANA CD4+ T cell recognition experiments, levels of LANAΔacid li protein expression were induced such that they were at a similar or lower level than that of LANAΔacid in the PELs. Taking these variables into account, figure 5.12 illustrates the concentrations of dox selected to give LANAΔacid li expression levels similar to 2μg/ml dox induction of LANAΔacid for BC-1 and JSC-1 was 0.75ng/ml and 1.5ng/ml, respectively. Figure 5.13 shows the selected dox concentration for LANAΔacid li expression in VG-1 and BCBL-1 of 3ng/ml gave lower levels of expression.

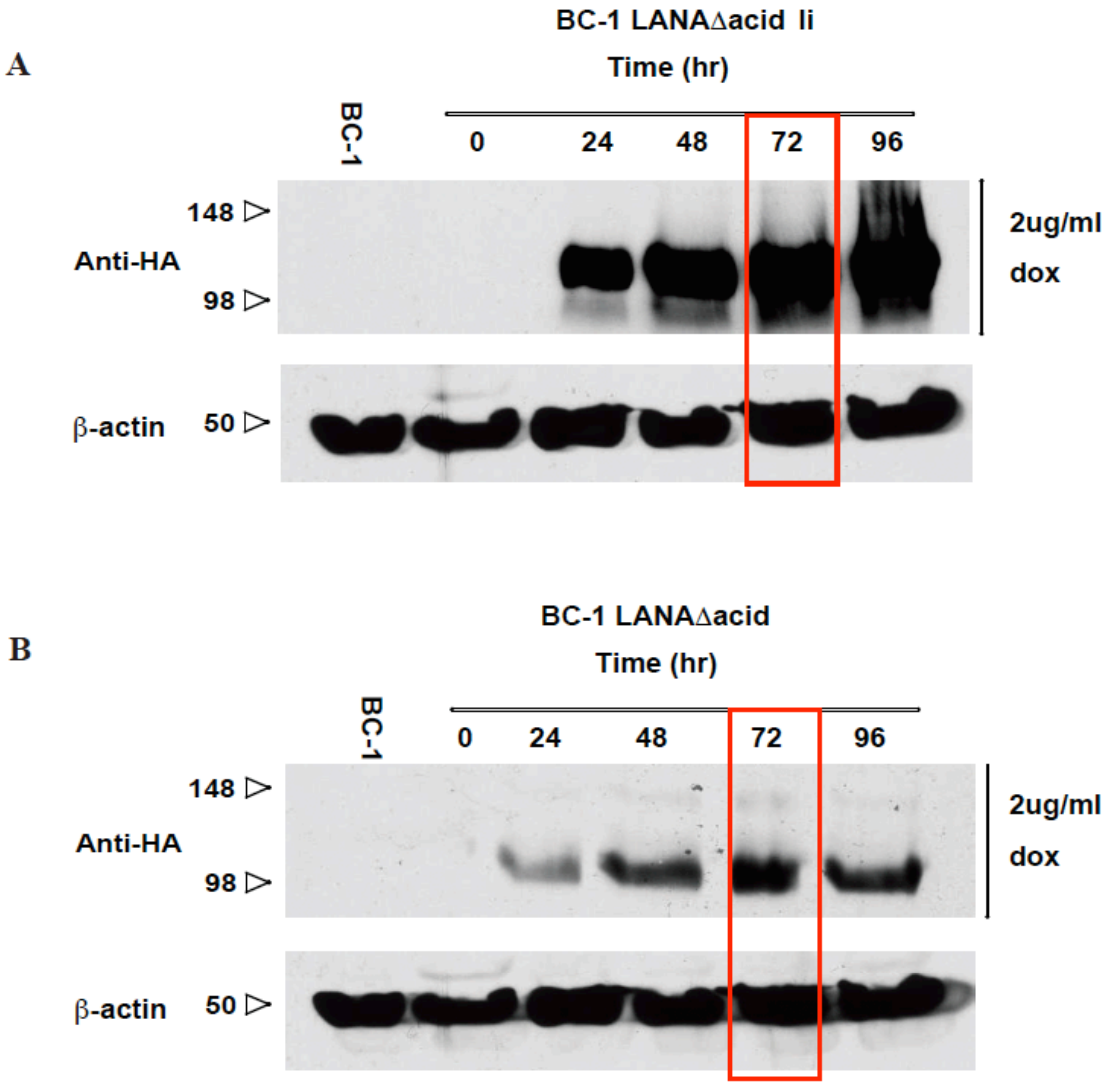


Figure 5.11 Time course of recombinant LANA construct protein expression of the transfected PEL BC-1, following induction by 2μg/ml dox. Immunoblot showing recombinant LANA protein levels in (A) LANAΔacid Ii or (B) LANAΔacid expression vectors after induction with dox at 2μg/ml. Non-transfected BC-1 cells were used as a negative control. Transfected BC-1 cells were maintained in dox containing medium for up to 96 hours, with aliquots of cells lysed at 24hr time points. LANA protein levels were detected using monoclonal HA antibody. Actin levels served as a loading control detected using monoclonal β-actin antibody.

5.6 LANA-specific CD4+ T cell recognition of LANA and LANAΔacid expressing PEL lines

The previous sets of experiments confirmed that PELs express MHC class II on their surface, albeit to variable levels, and at least some of the class II processing pathway is functional in these cells. It has been established that PELs are plasmablastoid B cells stably infected with KSHV and consequently express the genome maintenance protein LANA in every cell (Fakhari et al., 2006). Initially LANA expression was confirmed in the HLA matched PEL lines by immunoblotting with monoclonal LANA-antibody that is specific for the repeat sequence present in LANA (Figure 5.14). The next set of experiments sought to determine whether PELs can process and present endogenous and ectopically expressed LANA antigen for CD4+ T cell recognition.

LANA-specific CD4+ T cells were incubated with HLA-matched PELs expressing endogenous LANA or, as controls, the PELs transfected induced LANAΔacid constructs and recognition assessed by measuring IFN-γ production by the T cells. LANAΔacid construct expression was induced in PELs by incubation with the selected titrated dox concentration for 72hrs, as described above. Prior to use in the CD4+ T cell recognition assay, the dox-induced LANAΔacid vector transfected PELs were enriched by selecting for the vector expressed reporter protein NGFR using MACS NGFR selection beads. Initially expression levels of the LANAΔacid constructs was checked by immunoblotting with the rat monoclonal HA antibody. Figures 5.15 (A) and 5.16 (A) show representative results of the NGFR enriched BC-1 and JSC-1 transfected cells, showing expression of both the LANAΔacid and LANAΔacid li constructs. BC-1 LANAΔacid li transfected cells showed higher levels

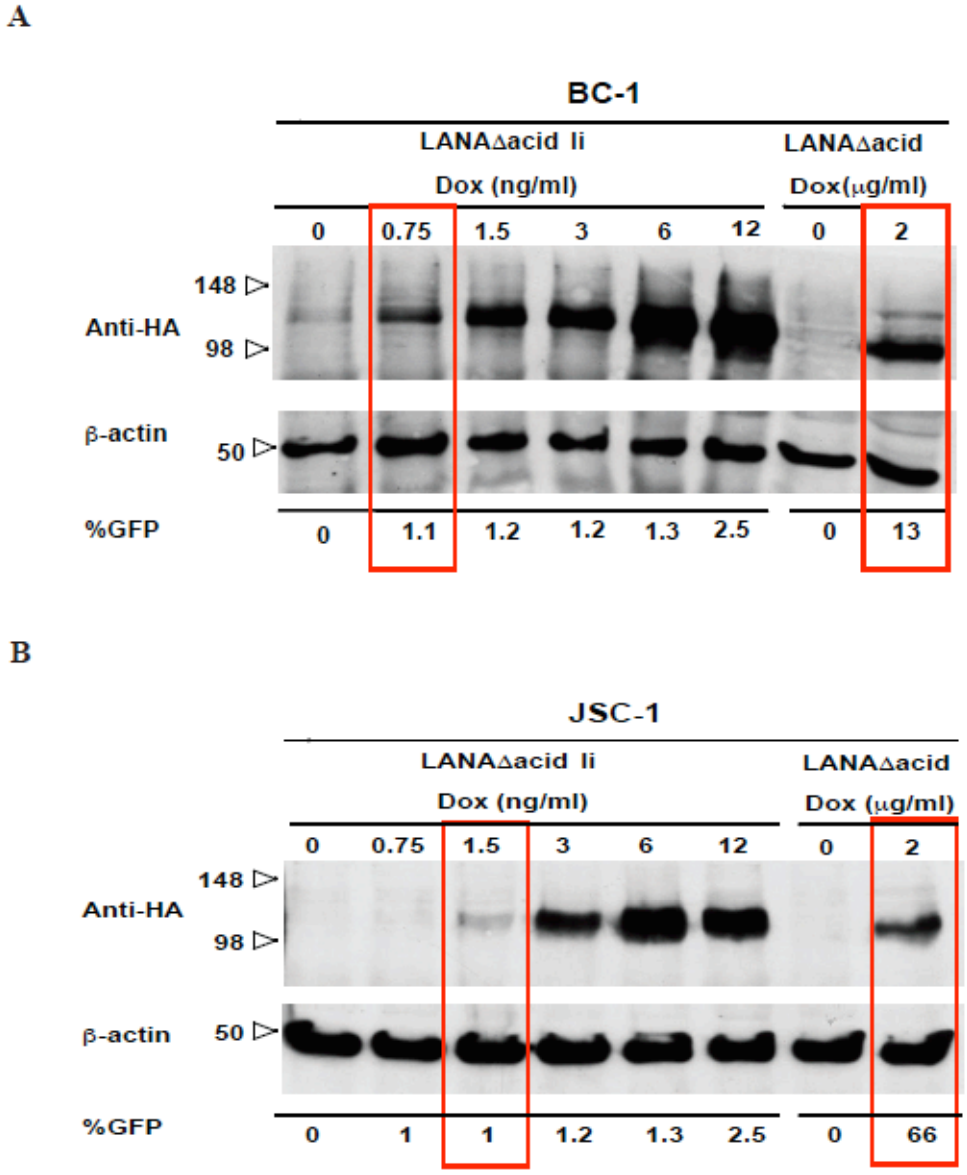


Figure 5.12 Recombinant LANA construct protein expression in transfected PELs BC-1 and JSC-1, following induction with different concentrations of dox. Immunoblot showing LANA construct protein expression following dox induction for 72hrs of LANAΔacid li and LANAΔacid transfected PELs (A) BC-1 and (B) JSC-1. LANAΔAcid transfected PEL cells were induced with dox at 2μg/ml. LANAΔacid li transfected PEL cells induced with titrated concentrations of dox from 12-0.75ng/ml. The percentage of cells expressing GFP at each dox concentration is shown under the appropriate gel lane. Recombinant LANA protein levels were detected using monoclonal HA antibody. Actin levels served as a loading control detected using monoclonal β-actin antibody. The red box highlights the selected concentrations of dox to be used.

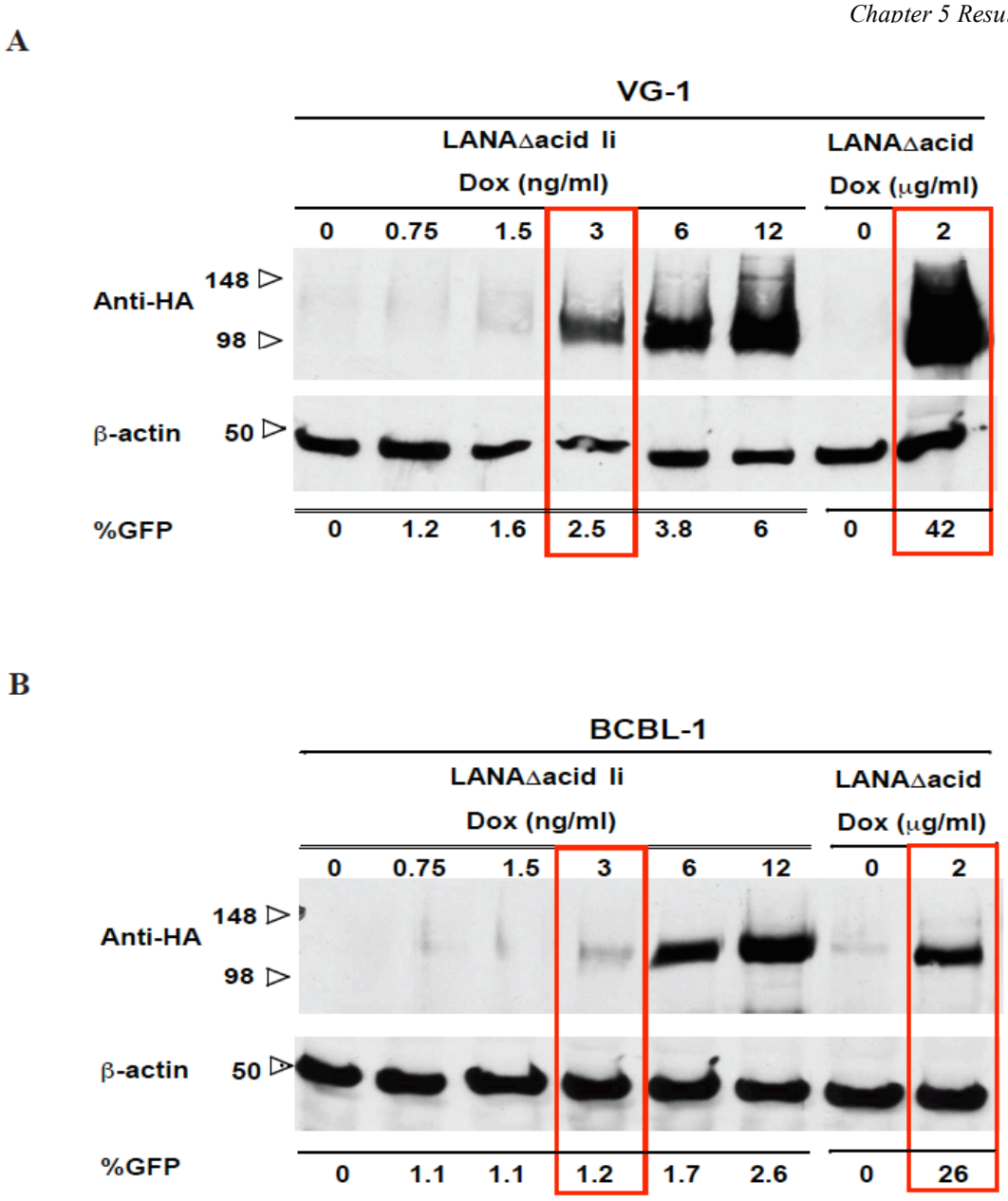


Figure 5.13 Recombinant LANA construct protein expression in the transfected PELs VG-1 and BCBL-1, following induction with different concentrations of dox. Immunoblot showing recombinant LANA construct protein expression following dox induction for 72hrs of LANAΔacid li and LANAΔacid transfected PELs (A) VG-1 and (B) BCBL-1. LANAΔacid transfected PEL cells were induced with dox at 2μg/ml. LANAΔacid li transfected PEL cells were induced with titrated concentrations of dox from 12-0.75ng/ml. The percentage of cells expressing GFP at each dox concentration is shown under the appropriate gel lane. Recombinant LANA protein levels were detected using monoclonal HA antibody. Actin levels served as a loading control detected using monoclonal β-actin antibody. The red box highlights the selected concentrations of dox to be used.

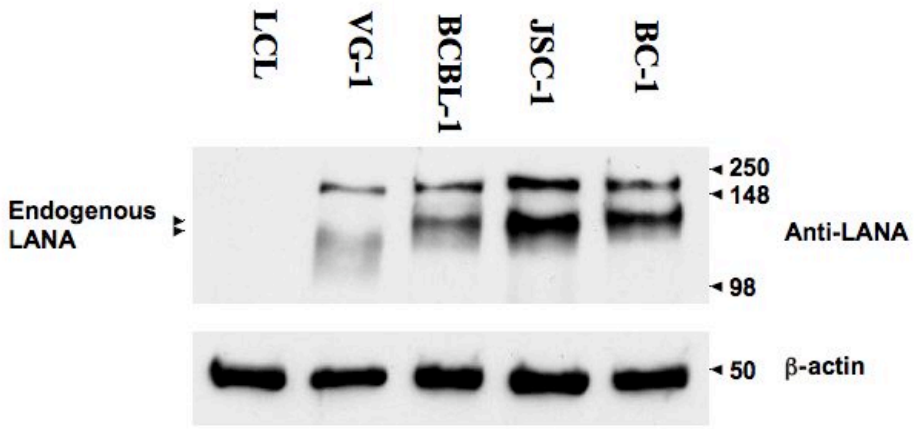


Figure 5.14 Endogenous LANA protein expression in PELs. Immunoblot showing endogenous LANA protein levels from four PELs BC-1, JSC-1, BCBL-1 and VG-1. The blot was probed with the rat monoclonal LANA (acidic repeat)-specific antibody. Actin levels served as a loading control detected using monoclonal β-actin antibody

of LANA protein, despite the 7% GFP compared to the 42.5% GFP of the LANAΔacid transfected BC-1 cells, highlighting that the LANAΔacid li transfected cells produce higher levels of protein for reasons that is unclear. In the case of JSC-1, expression of both LANAΔacid and LANAΔacid li constructs was detected.

We next tested whether the LANA-specific CD4+ T cell clones could recognise the endogenous LANA expressed within these PELs or the control overexpressed recombinant LANA constructs that were previously recognised in LCLs. Recognition assays were conducted assaying the HLA-DQ7 restricted KS48c10 GSP-specific clone against the HLA matched PELs BC-1 and JSC-1. The clone was incubated with either the uninduced or DMSO sensitised PELs to measure recognition of endogenous LANA, or the control dox induced transfected PELs, or GSP peptide-sensitised PELs. Results of IFN-γ secretion by the clones are shown in Figures 5.15 and 5.16(B). The GSP peptide-sensitised BC-1 and JSC-1 cells were recognised by the GSP-specific CD4+ T cells with minimal recognition of these cells when pulsed with DMSO. The LANAΔacid li construct expressing BC-1 and JSC-1 cells were recognised by the GSP-specific CD4+ T cell clone, showing that LANA protein directly routed into endo-lysosomal compartment in PELs can be processed and presented for CD4+ T cell recognition. However most importantly, there was little if any recognition by the CD4+ T cell clone of endogenous LANA expressed in these PELs (uninduced cells) or any additional recognition of cells expressing the LANAΔacid construct. These results suggest that BC-1 and JSC-1 cannot process and present endogenous LANA or ectopically expressed LANAΔacid to LANA-specific CD4+ T cells. These experiments were repeated on three different occasions with KS48 LANA GSP-

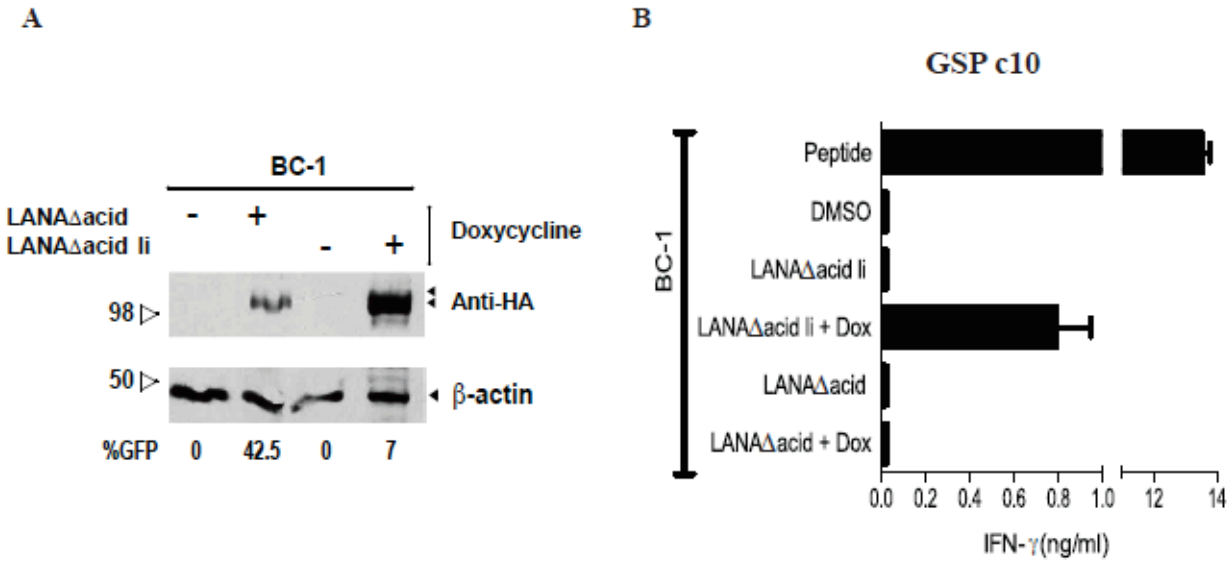


Figure 5.15 Recombinant LANA construct protein expression and T cell recognition of the transfected PEL BC-1. (A) Immunoblot showing recombinant LANA protein expression levels of the LANAΔacid and LANAΔacid li transfected PEL BC-1, following 72hrs of dox induction at 2μg/ml and 0.75ng/ml, respectively. The percentage of cells expressing GFP is shown under the appropriate gel lane. Recombinant LANA protein levels were detected using monoclonal HA antibody and actin levels served as a loading control detected using monoclonal β-actin antibody. (B) The dox induced and uninduced BC-1 cells were incubated overnight with HLA DQ-7 restricted LANA peptide GSP-specific CD4+ T cell clone 10 and recognition quantified by IFN-γ ELISA. As a positive control the BC-1 cells were peptide pulsed with the LANA peptide GSP at 5μg/ml or as a negative control with peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

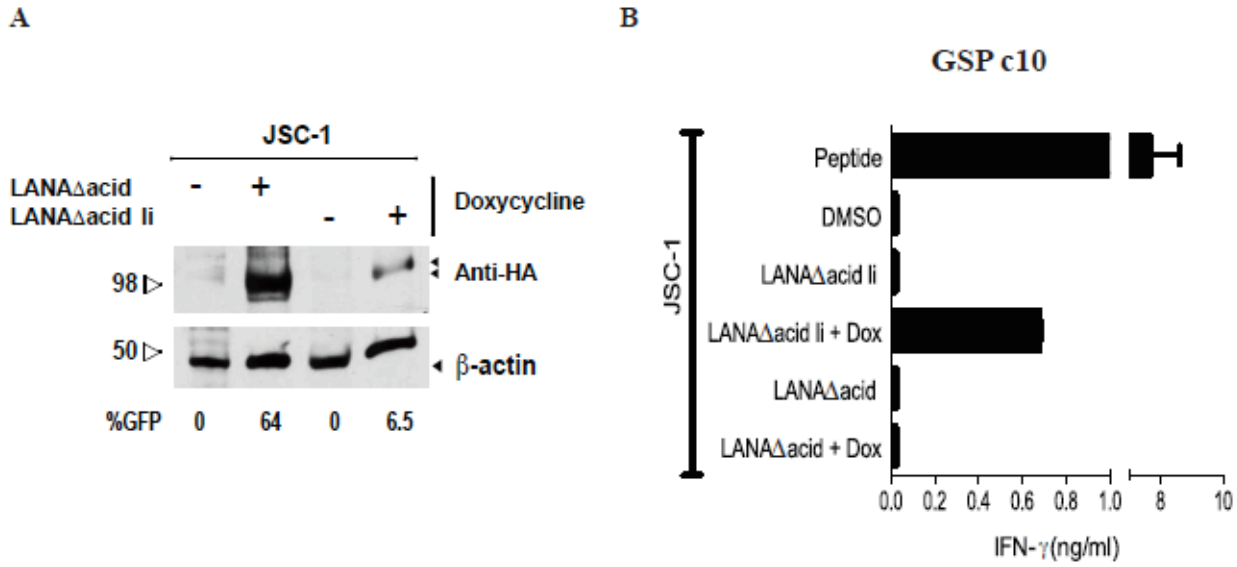


Figure 5.16 Recombinant LANA construct protein expression and T cell recognition of the transfected PEL JSC-1. (A) Immunoblot showing recombinant LANA protein expression levels of the LANAΔacid and LANAΔacid li transfected PEL JSC-1, following 72hrs of dox induction at 2μg/ml and 1.5ng/ml, respectively. The percentage of cells expressing GFP is shown under the appropriate gel lane. Recombinant LANA protein levels were detected using monoclonal HA antibody and actin levels served as a loading control detected using monoclonal β-actin antibody. (B) The dox induced and uninduced JSC-1 cells were incubated overnight with the HLA DQ7-restricted LANA peptide GSP-specific CD4+ T cell clone 10 and recognition quantified by IFN-γ ELISA. As a positive control the JSC-1 cells were pulsed with the LANA peptide GSP at 5μg/ml or as a negative control with the peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

specific clone 10, and consistently showed no recognition of the endogenous LANA expressed by unmanipulated PELs or overexpressed LANAΔacid.

LANA-specific CD4+ recognition experiments were performed on an additional two PELs, VG-1 and BCBL-1. As described above, in addition to recognition of the endogenous LANA expressed by the PELs, LANAΔacid construct expression was induced in these PELs by incubation with the selected titrated dox concentration for 72hrs and cells expressing LANAΔacid further enriched by selecting for the vector expressed reporter protein NGFR. Figure 5.17(A) shows LANA protein expression levels in VG-1 by immunoblotting with a monoclonal mouse antibody specific to the N'-terminus of LANA. This allows a comparison between the levels of endogenous LANA and the ectopically expressed LANAΔacid constructs, showing higher levels of the ectopically expressed LANAΔacid proteins compared to the endogenous protein. Recognition assays measuring IFN-γ production from two independent HLA-matched LANA-specific clones incubated with the dox induced and uninduced VG-1 transfected cells, or as a positive control, cognate epitope-peptide sensitised VG-1 cells are shown in Figure 5.17. Here the KSB1 derived T cell clones c204, restricted through DQ6 specific for peptide WGD (B) and clone c33 restricted through DR13 specific for peptide LRS (C) were assayed for recognition of the different VG-1 targets. For each of the LANA-specific clones, the positive control peptide pulsed VG-1 cells were recognised by the LANA-specific CD4+ T cells, but there was minimal recognition of VG-1 cells pulsed with DMSO. Recognition of the VG-1 transfected cells gave a similar pattern of results as to those seen using the KS48c10 clone against BC-1 and JSC-1. Here the LANAΔacid li construct expressing VG-1 cells were recognised by the LANA-specific CD4+ T cell clones, however the

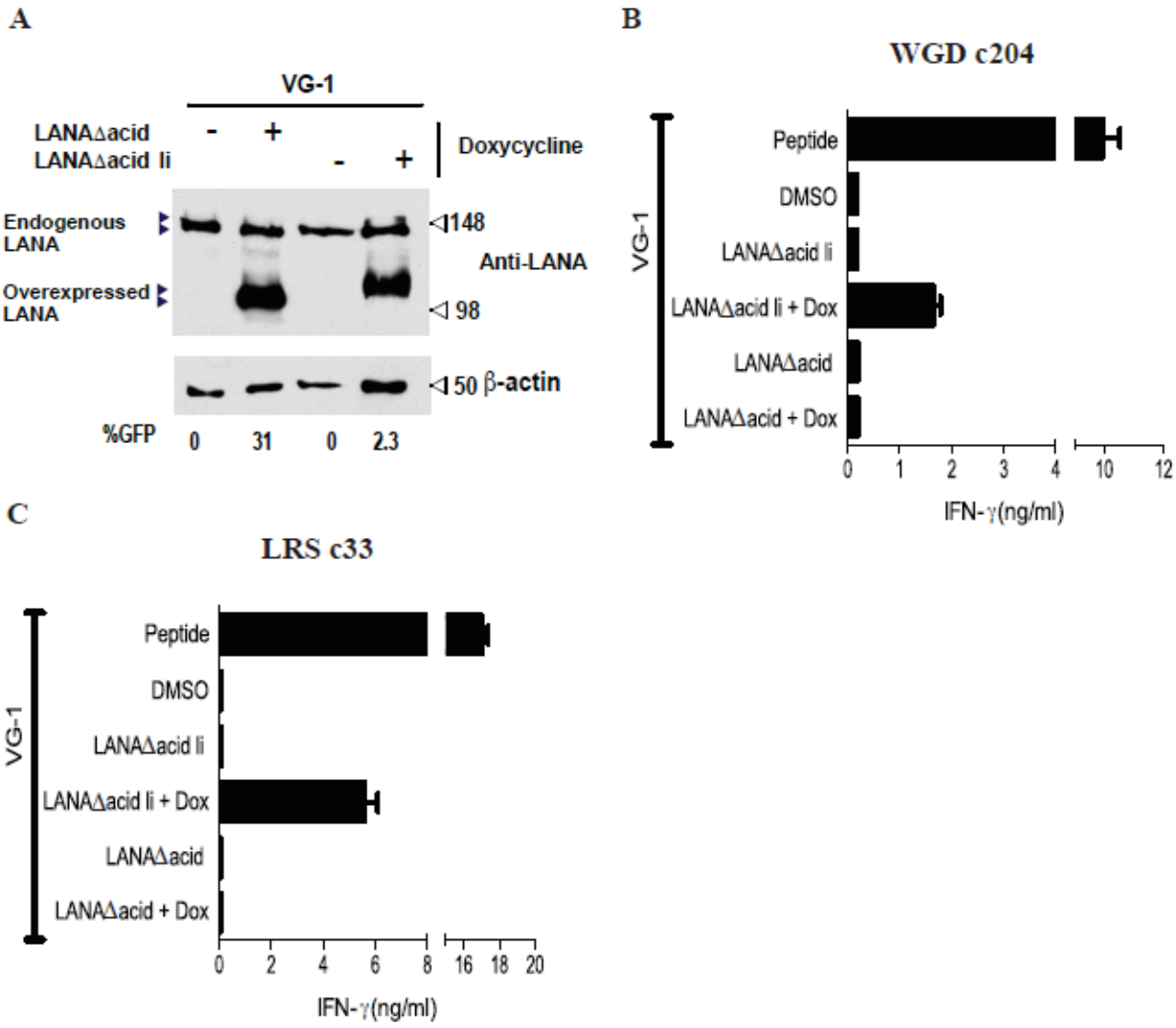


Figure 5.17 Recombinant LANA construct protein expression and T cell recognition of the transfected PEL VG-1. (A) Immunoblot showing recombinant LANA protein expression levels of the LANAΔacid and LANAΔacid li transfected PEL VG-1, following 72hrs of dox induction at 2μg/ml and 3ng/ml, respectively. The percentage of cells expressing GFP is shown under the appropriate gel lane. Recombinant LANA protein levels were detected using mouse monoclonal N'-terminus LANA antibody and actin levels served as a loading control detected using monoclonal β-actin antibody. The dox induced and uninduced transfected VG-1 cells were incubated overnight with two LANA specific CD4+ T cell clones with different peptide specificities and recognition quantified by IFN-γ ELISA. Results shown from (B) the HLA DR13-restricted clone 204 specific for peptide WGD and (C) the HLA DR13-restricted clone 33 specific for peptide LRS. As a positive control the VG-1 cells were pulsed with the cognate epitope-peptide at 5μg/ml or as a negative control with the peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

uninduced PELs, i.e. only those expressing the endogenous form of LANA showed minimal recognition by the CD4+ T cells with no increased recognition of cells over those expressing the LANAΔacid protein. This suggests that VG-1 is also unable to process and present endogenous LANA or ectopically expressed LANAΔacid to LANA-specific CD4+ T cells. This recognition assay was repeated three times with all seven VG-1 HLA-matched restricted CD4+ T cell clones, in no experiments was there recognition of the endogenous LANA expressed in VG-1 or the LANAΔacid expressing VG-1 by any of the T cell clones.

Figure 5.18 shows the result of similar experiments carried out on the PEL BCBL-1. As with the other PELs, the BCBL-1 cells transfected with the LANAΔacid and LANAΔacid li expressing constructs were induced with dox and expression confirmed by immunoblotting with the monoclonal HA antibody as illustrated in figure 5.18(A). Lysates from the LANAΔacid transfected cells showed higher levels of the recombinant LANA protein, when compared to the LANAΔacid li transfected cells, likely reflecting the higher percentage of cells transfected with LANAΔacid. The BCBL-1 cells were incubated with the HLA-DQ6 restricted LANA EYR peptide-specific CD4+ T cell clone 63 to test for recognition of LANA antigens. The positive control EYR peptide pulsed BCBL-1 cells were recognised by the EYR-specific CD4+ T cells and showed an increased response over the DMSO sensitised BCBL-1 cells. However the BCBL-1 cells either pulsed with peptide solvent DMSO, dox induced or uninduced LANAΔacid and LANAΔacid li transfected BCBL-1 cells all induced IFN-γ release by the EYR-specific CD4+ T cells. However, no increase in IFN-γ secretion above background DMSO levels was detected from LANAΔacid and

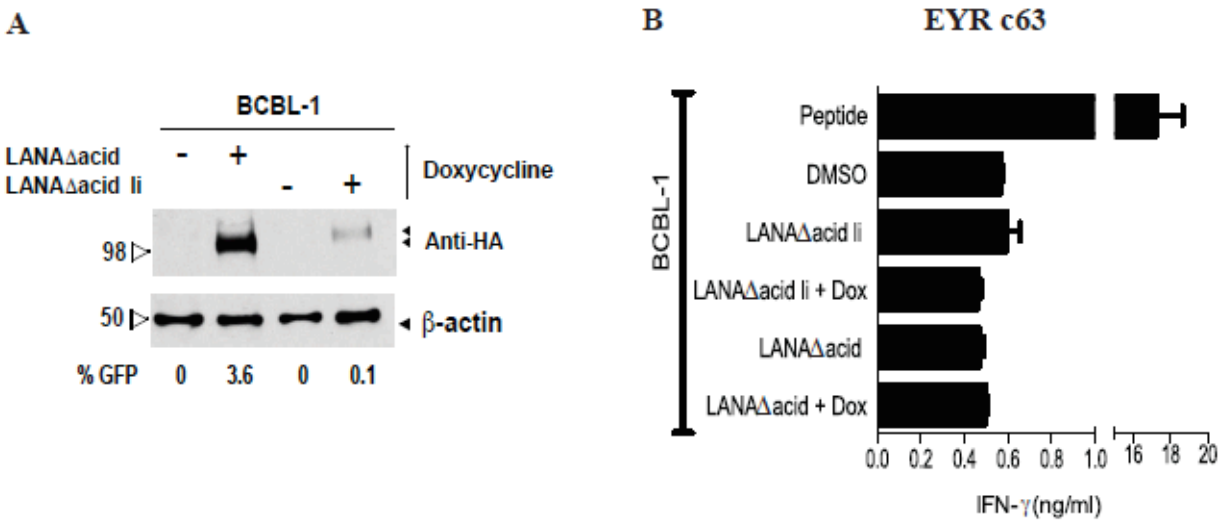


Figure 5.18 Recombinant LANA construct protein expression and T cell recognition of the transfected PEL BCBL-1. (A) Immunoblot showing recombinant LANA protein expression levels of the LANAΔacid and LANAΔacid Ii transfected PEL BCBL-1, following 72hrs of dox induction at 2μg/ml and 3ng/ml, respectively. The percentage of cells expressing GFP is shown under the appropriate gel lane. Recombinant LANA protein levels were detected using monoclonal HA antibody and actin levels served as a loading control detected using monoclonal β-actin antibody. (B) The dox induced and uninduced BCBL-1 cells were incubated overnight with the HLA DQ6-restricted LANA peptide EYR-specific CD4+ T cell clone 63 and recognition quantified by IFN-γ ELISA. As a positive control the BCBL-1 cells were pulsed with the LANA peptide EYR at 5μg/ml or as a negative control with the peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

LANAΔacid li expressing BCBL-1 cells, suggesting no recognition of the overexpressed recombinant LANA protein. To ensure the EYR-specific CD4+ T cell clone was not responding non-specifically to BCBL-1 or that it is a phenomenon specific to this clone, all of the LANA-specific CD4+ T cell clones that are restricted through HLA-DQ6 were tested against BCBL-1 and, in parallel, the HLA-DQ6 matched PEL VG-1. In these assays the BCBL-1 and VG-1 cells were either unmanipulated, sensitised with either the clone's cognate epitope-peptide as a positive control, or with peptide solvent DMSO as a negative control. These target PEL cells were incubated with the LANA-specific CD4+ T cell clones and recognition quantified by IFN-γ ELISA. Figure 5.19 shows the results of this assay using the four HLA-DQ6 restricted LANA-specific clones derived from KSB1: (A) clone 63 specific for peptide EYR, (B) clone 71 specific for peptide PAF, (C) clone 204 specific for peptide WGD and (D) c110 specific for peptides LAP/LRS. Each of the clones secreted IFN-γ in response to challenge with the peptide-sensitised BCBL-1 and VG-1 cells substantially above the levels induced when incubated with the DMSO sensitised or unmanipulated PEL. However, the unmanipulated and DMSO sensitised BCBL-1 cells induced IFN-γ secretion by each of the four T cell clones while in parallel assays, VG-1 induced little response. This finding supports the BCBL-1 result shown previously in figure 5.18, suggesting the HLA-DQ6 restricted T cell clones are likely recognising LANA epitopes expressed on these BCBL-1 cells.

5.7 CD4+ T cell recognition of PELs exogenously fed LANA protein

To further characterise the antigen processing function of the PELs, these cells were fed enriched LANA protein preparations and used as targets for the LANA-specific

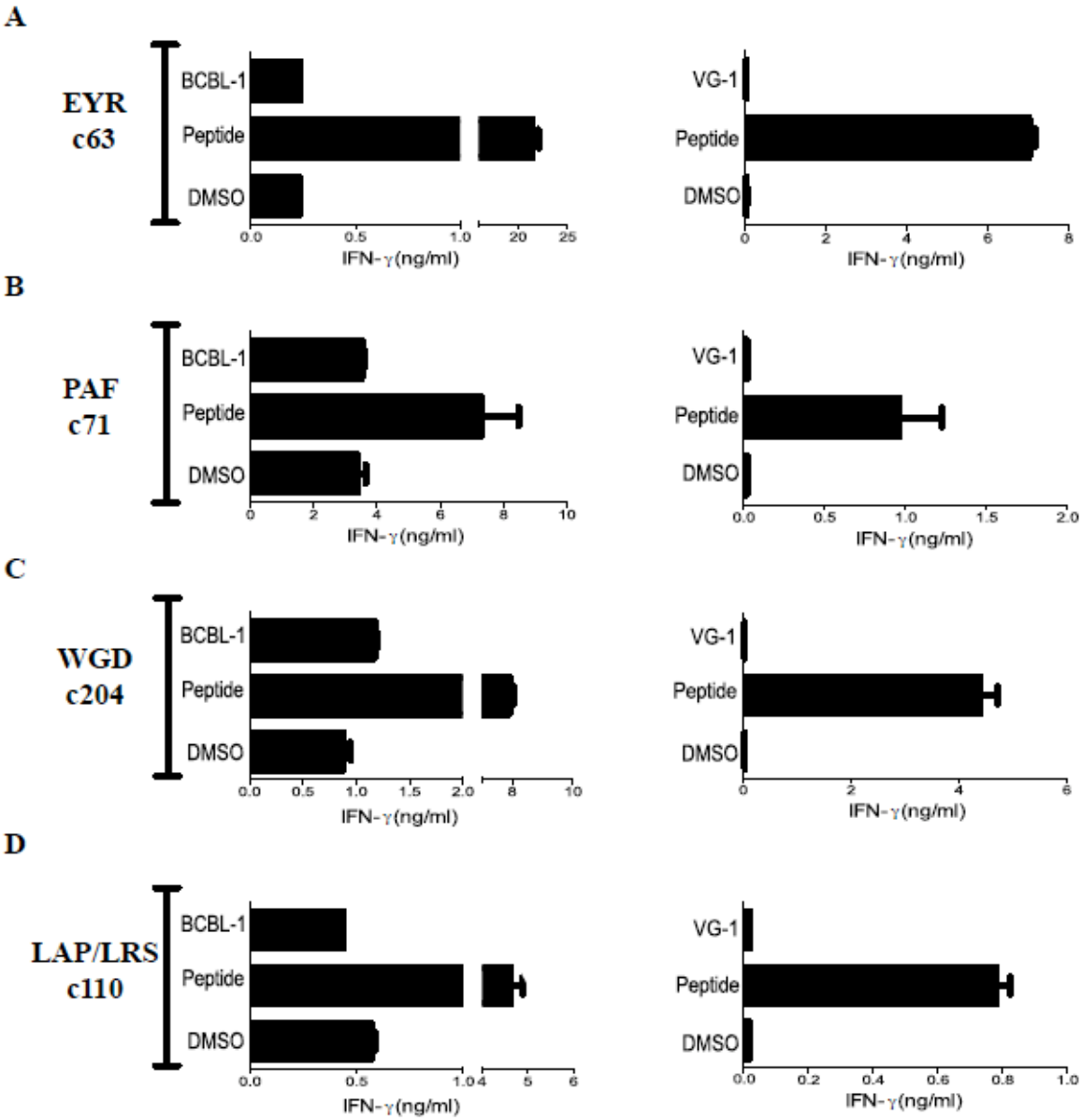


Figure 5.19 CD4+ T cell recognition of PEL lines BCBL-1 and VG-1. The PELs BCBL-1 and VG-1 were incubated with HLA-matched DQ6 restricted LANA-specific CD4+ T cell clones, specific for LANA peptides (A) EYR clone 63, (B) PAF clone 71 (C) WGD clone 204 and (D) LAP/LRS clone 110. As a positive control the BCBL-1 and VG-1 cells were sensitised with their cognate epitope-peptide and as a negative control with the peptide solvent DMSO. T cell recognition was quantified by IFN-γ ELISA and the results are expressed as the mean IFN-γ release from triplicate wells.

CD4+ T cells in recognition assays. These experiments were conducted similar to those using LCLs as targets as described in section 4.3, with the PELs fed either a LANA protein preparation from BCBL-1 cells or a control preparation from DG75 cells. The PELs were incubated with the protein for 18 hours before being used in recognition assays with a range of HLA-matched LANA-specific CD4+ T cell clones and recognition quantified by IFN-γ ELISA. Figure 5.20 shows the results of this protein feeding experiment for the PELs (A) BC-1 and (B) JSC-1 incubated with the HLA-DQ7 restricted LANA-specific GSP clone 10 from donor KS48. The protein fed PEL (C) VG-1 was incubated with the HLA-DR13 restricted LANA-specific LRS clone 33 and (D) BCBL-1 with the HLA-DQ6 restricted LANA-specific EYR clone 63. For each of the LANA-specific CD4+ clones, the positive control peptide sensitised PELs were recognised by the LANA-specific CD4+ T cells, but the clones did not recognise PELs pulsed with peptide solvent DMSO apart from the PEL BCBL-1 in which there was some background recognition. In contrast to the experiments conducted with LCLs fed these protein preparations (Figure 5.7), no recognition of the BC-1, JSC-1 and VG-1 PELs fed exogenous LANA protein at both 80μg/ml and 40μg/ml was observed, nor was recognition of the PELs fed the control DG75 protein by all the LANA-specific CD4+ T cell clones. These results suggest that LANA antigen cannot be processed and presented for LANA-specific CD4+ T cell recognition using the classic MHC class II processing pathway by BC-1, JSC-1 and VG-1. For the BCBL-1 cells there was no increase in recognition of the cells fed LANA protein and this result was consistent across all the HLA-DQ6 restricted LANA-specific clones (data not shown). These experiments were repeated twice for each of the PELs with their HLA-matched LANA-specific clones, all yielding a similar result.

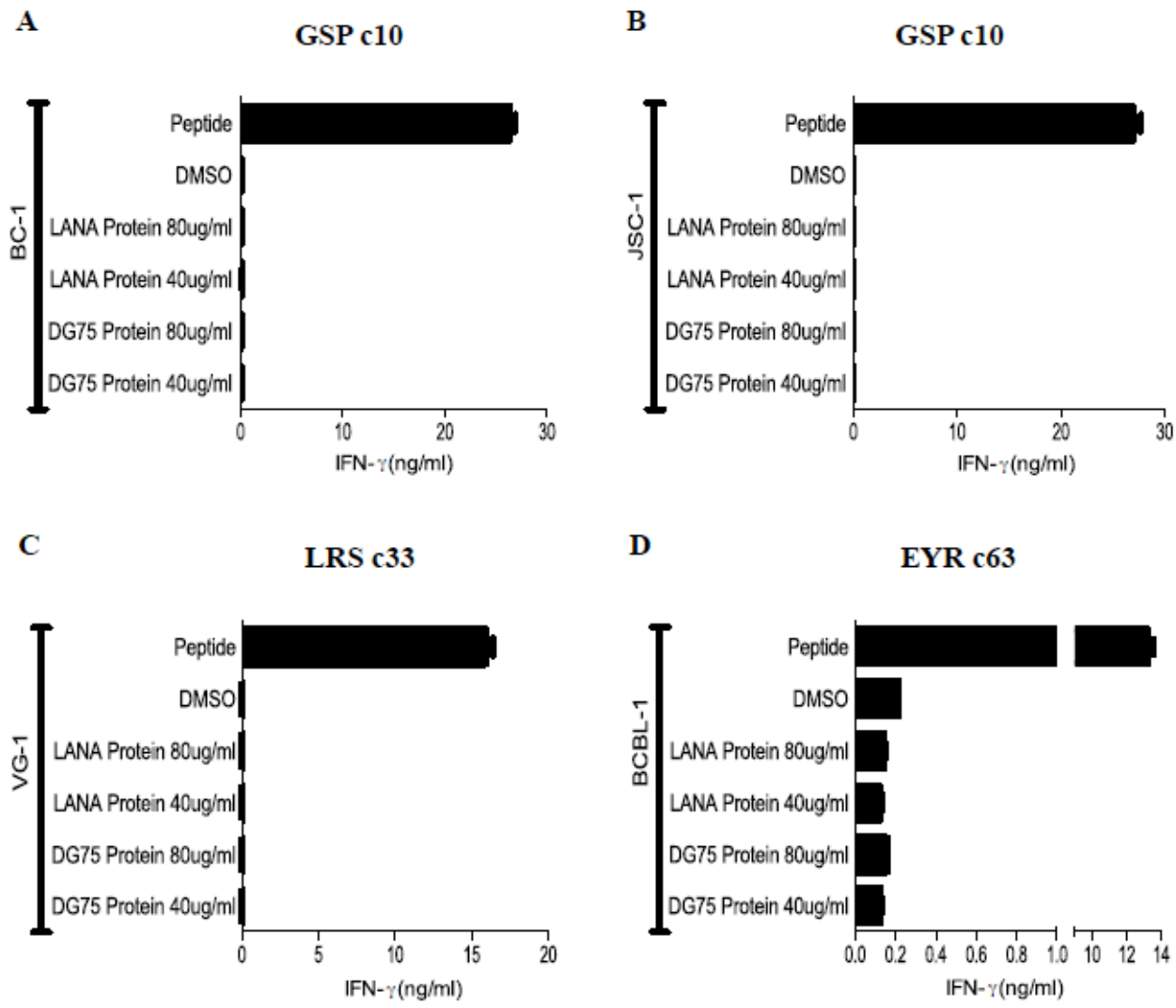


Figure 5.20 CD4+ T cell recognition of PEL cells exogenously fed LANA protein. LANA protein derived from nuclear extracts of the PEL BCBL-1 or a control preparation derived from the KSHV negative cell line DG75 were incubated with the PELs for 18 hours at 80μg/ml and 40μg/ml. These protein loaded cells were then used as targets in recognition assays by overnight incubation with HLA-matched LANA-specific CD4+ T cell clones and T cell recognition was quantified by IFN-γ ELISA. PELs (A) BC-1 and (B) JSC-1 were incubated with the DQ7-restricted KS48 derived clone 10, specific for peptide GSP. The PEL (C) VG-1 was incubated with the DR13-restricted KSB1 derived clone 33, specific for peptide LRS and PEL (D) BCBL-1 with the DQ6-restricted KSB1 clone 63, specific for peptide EYR. As a positive control each of the PEL targets were sensitised with the T cell clone's cognate epitope-peptide at 5μg/ml and as a negative control with peptide solvent DMSO. Results are expressed as the mean IFN-γ release from triplicate wells.

5.8 Levels of CLIP on the MHC class II molecules of PELs suggest appropriate peptide exchange takes place in at least some PELs

The T cell recognition assays carried out in this section have shown that unlike LCLs most PELs are incapable of processing and presenting ectopically expressed, exogenously fed or endogenously expressed LANA for CD4+ recognition. The exception to this was the PEL BCBL-1, which consistently induced IFN-γ production by the HLA-matched LANA-specific clones. This raises the question how does BCBL-1 differ in its MHC class II processing machinery from the other three PELs tested?

The MHC class II processing pathway involves a number of steps as described previously in the introduction. A crucial part of this pathway for CD4+ T cell presentation is the loading of the MHC class II molecule with the CD4+ T cell's cognate epitope-peptide. In order for this to occur, the MHC class II-like molecule HLA-DM catalyses the removal of the class II associated invariant chain peptide (CLIP) to allow binding of the target epitope-peptide. Previous studies have shown that mutant B-cell lines with a defect in antigen presentation often fail to bind peptides derived from the internalised proteins and often arrive at the cell surface with the CLIP peptide still bound as a result of a defect in HLA-DM function (Weenink et al., 1997; Riberdy et al., 1992; Sette et al., 1992). To test whether this phenomenon was occurring in BC-1, JSC-1 and VG-1, these PELs were stained for surface MHC class II and CLIP in parallel and analysed by flow cytometry. For comparison BCBL-1 and donor LCLs, KS48 and KSB1 were also analysed. Figure 5.21 shows the results of this staining, the levels of surface MHC class II and CLIP on the different PELs. Staining with this antibody was validated using the mutant cell line T2 DR5 which

lacks HLA-DM as a positive control, which showed high levels of surface CLIP. The cell line T2 DR5 engineered to express HLA-DM was used as a negative control and showed no CLIP staining on the surface of the cells (data not shown). The donor LCLs, KS48 and KSB1, showed approximately 25-30% surface CLIP expression compared to their surface MHC class II. The PELs varied greatly in CLIP expression, with JSC-1 and BCBL-1 showing almost no CLIP surface expression, while BC-1 showed high levels of CLIP expression compared to its surface MHC class II. VG-1 showed similar levels of CLIP expression compared to its surface MHC class II, however it is difficult to interpret this result as the VG-1 surface MHC class II levels were so low. These results suggest that for the PEL BC-1, HLA-DM may be a limiting factor in the MHC Class II antigen processing and presentation pathway.

5.9 Restoration of HLA class II expression on PELs and recognition by LANA-specific CD4+ T cells

The MHC class II surface staining on the PELs shown in figure 5.8 and figure 5.21 above have consistently shown that higher levels of surface MHC class II is expressed by BCBL-1 compared to the other PELs, being at levels similar to those seen on the LCLs. Furthermore, the previous experiments have shown that only LCLs and the PEL BCBL-1 are capable of presenting LANA antigen for CD4+ T cell recognition. Recent evidence from studying the function of the KSHV-encoded vIRF3 gene, which is expressed in PELs provides a potential explanation as to why PELs express low surface MHC class II. The KSHV protein vIRF3, expressed during latency in PELs, functions to disrupt type I IFN signaling and is required for the continuous proliferation of PEL cells (Wies et al., 2009; Wies et al., 2008). However this protein has now been shown to inhibit the function of both the IFN-γ-sensitive CIITA

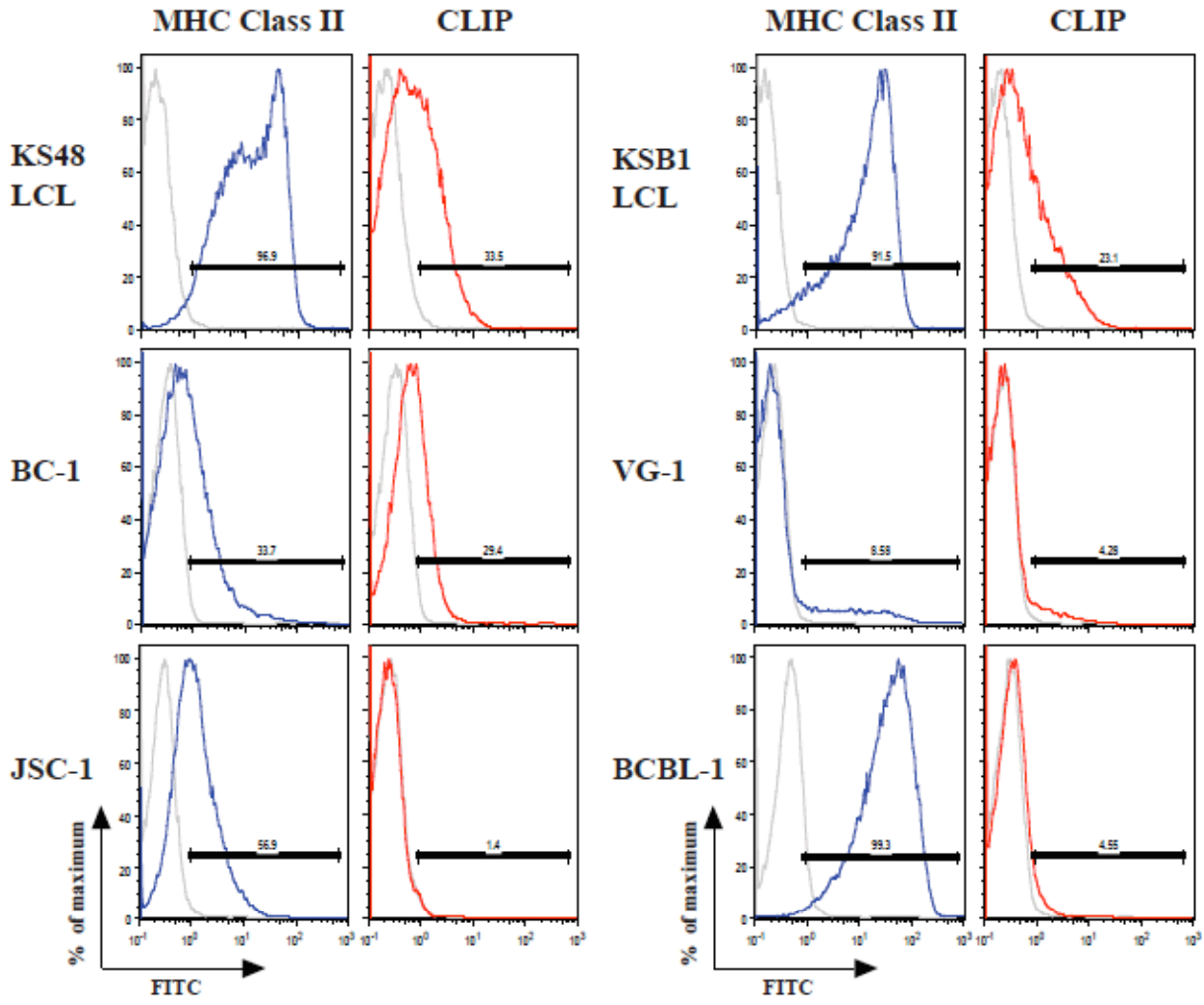


Figure 5.21 Staining for MHC class II and CLIP on the surface of donor LCLs and PELs. LCLs KS48 and KSB1 and the PEL lines BC-1, JSC-1, VG-1 and BCBL-1 were stained with mouse monoclonal unconjugated antibodies specific for MHC class II (blue line) and CLIP (red line). Each of the cell lines were also stained with an unconjugated IgG2a isotype control in parallel. Bound primary antibody was detected by staining with a secondary anti-mouse FITC conjugated antibody and fluorescence intensity measured by flow cytometry.

promoter pIV and the lymphoid cell-specific CIITA promoter pIII (Schmidt et al., 2011). These both drive expression of the class II transactivator (CIITA) gene, whose product binds to the MHC class II enhanceosome where it recruits factors that drive the expression of MHC class II genes amongst others. The expression of vIRF3 in the different PEL lines used in this study was analysed by western blot analysis using a mouse monoclonal vIRF3 antibody and actin was used as a loading control. Figure 5.22 shows that as expected all the PEL lines express vIRF3, interestingly BCBL-1 which was consistently recognised by the LANA-specific CD4+ T cells showed the lowest level of expression of this protein.

It has been shown that ectopic expression of vIRF3 decreased surface class II expression in B cells and siRNA knockdown of vIRF3 in PELs increased surface class II expression (Schmidt et al., 2011). In an attempt to reverse the effects of vIRF-3 expression on the PELs BC-1, JSC-1 and VG-1, a panel of siRNAs and protocols that have been previously described in the above study were used to knock down expression in the PELs. However, these attempts to knockdown the vIRF3 protein were unsuccessful and no change in surface class II expression, vIRF3 protein expression levels or recognition by the LANA-specific CD4+ T cells was detected (data not shown). Alternatively, as vIRF3 is thought to interfere with the CIITA promoter, CIITA was expressed from a different promoter, these experiments were carried out by Dr Andrew Hislop, as I was unable to complete this part of the study due to time restrictions. Here the PELs were transduced with a retrovirus expressing either CIITA or a control construct (HLA-B*81) from the retroviral long terminal repeats, immediately downstream from this gene was an IRES and the truncated NGFR. The transduced cells were selected by magnetically sorting for NGFR using

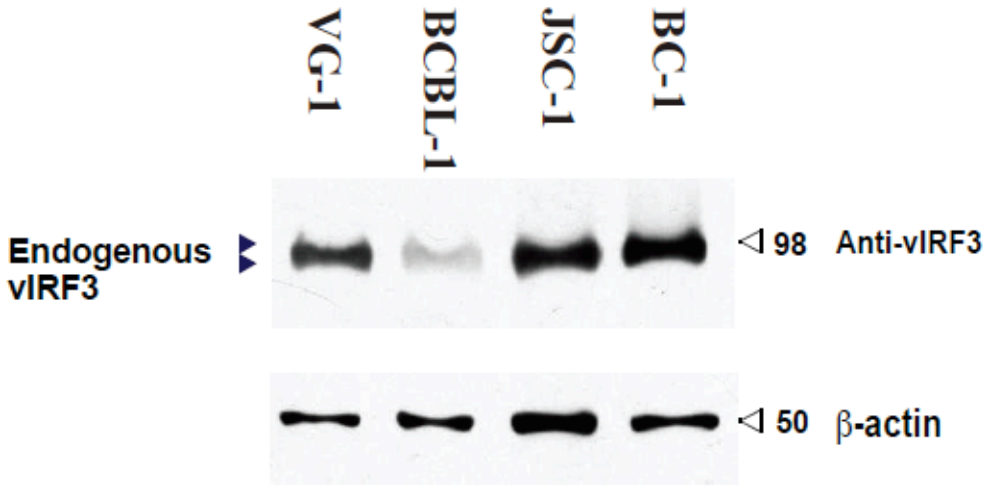


Figure 5.22 vIRF3 protein expression in PEL cell lines. Immunoblot showing endogenous vIRF3 protein levels from the four PEL cell lines BC-1, JSC-1, BCBL-1 and VG-1. The blot was probed with the mouse monoclonal vIRF3 specific antibody. Actin levels served as a loading control detected using monoclonal β-actin antibody.

MACS NGFR-specific beads. Lysates of the CIITA transduced cells compared to control transduced cells analysed by western blot showed expression of CIITA and proteins that it transactivates, namely CD74 (MHC class II invariant chain) and HLA-DR, demonstrating function of the CIITA transgene (data not shown). Expression of surface levels of class II were also measured by flow cytometry on the transduced PELs and compared to levels on BCBL-1, an LCL and class II negative fibroblasts. Surface HLA class II levels were measured on NGFR-expressing cells by co-staining with anti-NGFR antibody and an anti-class II MHC antibody. The control transduced cells showed little difference in surface class II expression compared to non-transduced cells (data not shown), however figure 5.23 shows that the CIITA transduced PELs VG-1, BC-1 and JSC-1 expressed increased levels of class II, comparable to those expressed by the LCL or BCBL-1.

As the CIITA transduced PELs now expressed increased levels of surface class II, the ability of the different LANA-specific CD4+ T cell clones to recognise these cells was reexamined. The non-transduced PELs, control-transduced PELs and autologous LCLs were compared to the CIITA transduced PELs when incubated with HLA-matched LANA-specific CD4+ T cell clones and recognition quantified by IFN-γ ELISA. As controls, aliquots of these cells were sensitised with the cognate peptide-epitope. Figure 5.24 shows representative results of these assays, when (A) BC-1 and (B) JSC-1 cells were assayed against the HLA-DQ7 restricted KS48 clone 10 specific for LANA peptide GSP and (C) VG-1 cells against the HLA-DQ6 restricted KSB1 clone 110 specific for LANA peptides LAP and LRS. For all three PELs, little or no IFN-γ was released in response to the non-transduced PELs, consistent with the

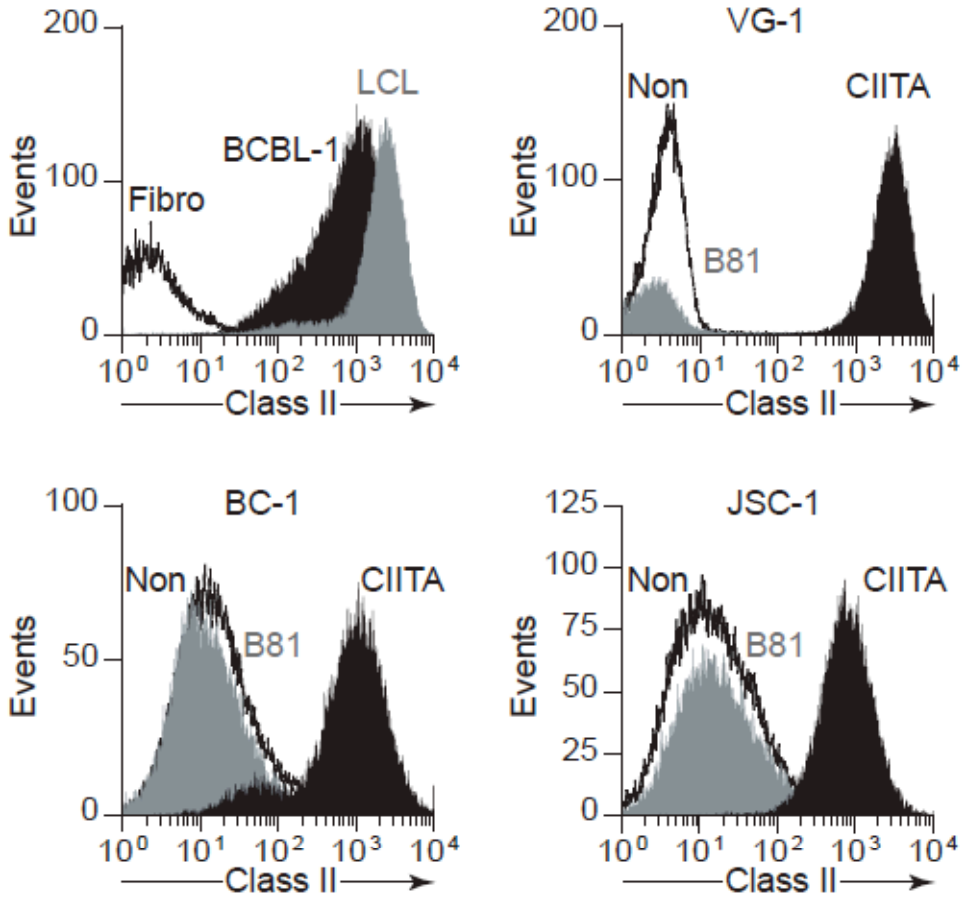


Figure 5.23 Expression of CIITA and MHC class II after CIITA transduction in PELs. The PELs were transduced with CIITA and analysed by flow cytometry for surface MHC class II levels on BCBL-1, an LCL or a fibroblast (upper left panel), VG-1 transductants (upper right panel), BC-1 transductants (lower left panel) and JSC-1 transductants (lower right panel). CIITA transduced PELs are shown in black, non-transduced PELs in grey and isotype control staining is open.

previous observations. The clones also showed a similar pattern of recognition to the control HLA-B*81 transduced cells. Similarly HLA mis-matched LANA-specific clones were assayed against each of the PELs in parallel assays secreting minimal amounts of IFN-γ. Each of the CIITA transduced PELs however were able to induce IFN-γ secretion from their HLA-matched clone, indicating the clones could now recognise these PELs. The PEL VG-1 was further tested against a range of HLA-DR, DQ and DP restricted and HLA-matched LANA-specific clones which consistently showed recognition of the CIITA transduced VG-1 cells (data not shown). These results show that the overexpression of CIITA in the PELs allows the recognition of endogenously expressed LANA antigen by the CD4+ T cells, overcoming the potential immune evasion function elicited by vIRF-3.

Discussion

This study used LANA-specific CD4+ T cell clones to probe recognition of LANA-expressing B cells. EBV-transformed B cells (LCLs) which have an intact class II antigen processing pathway were capable of efficiently processing and presenting LANA antigens for CD4+ T cell recognition. However the majority of the KSHV B cell malignant PELs, which natively express LANA, were not recognised by the LANA-specific CD4+ T cells; the exception being the BCBL-1 PEL. Only when LANA was ectopically expressed in PELs as a fusion protein with the MHC class II invariant chain was T cell recognition of JSC-1, BC-1 and VG-1 induced. Interestingly, BCBL-1 cells relative to the other PELs expressed lower amounts of vIRF3 and expressed higher levels of MHC class II on their surface. The inhibition of CIITA transcription by vIRF3 provides an explanation as to why there is poor T cell recognition of the PELs JSC-1, BC-1 and VG-1. Consequently, bypassing the

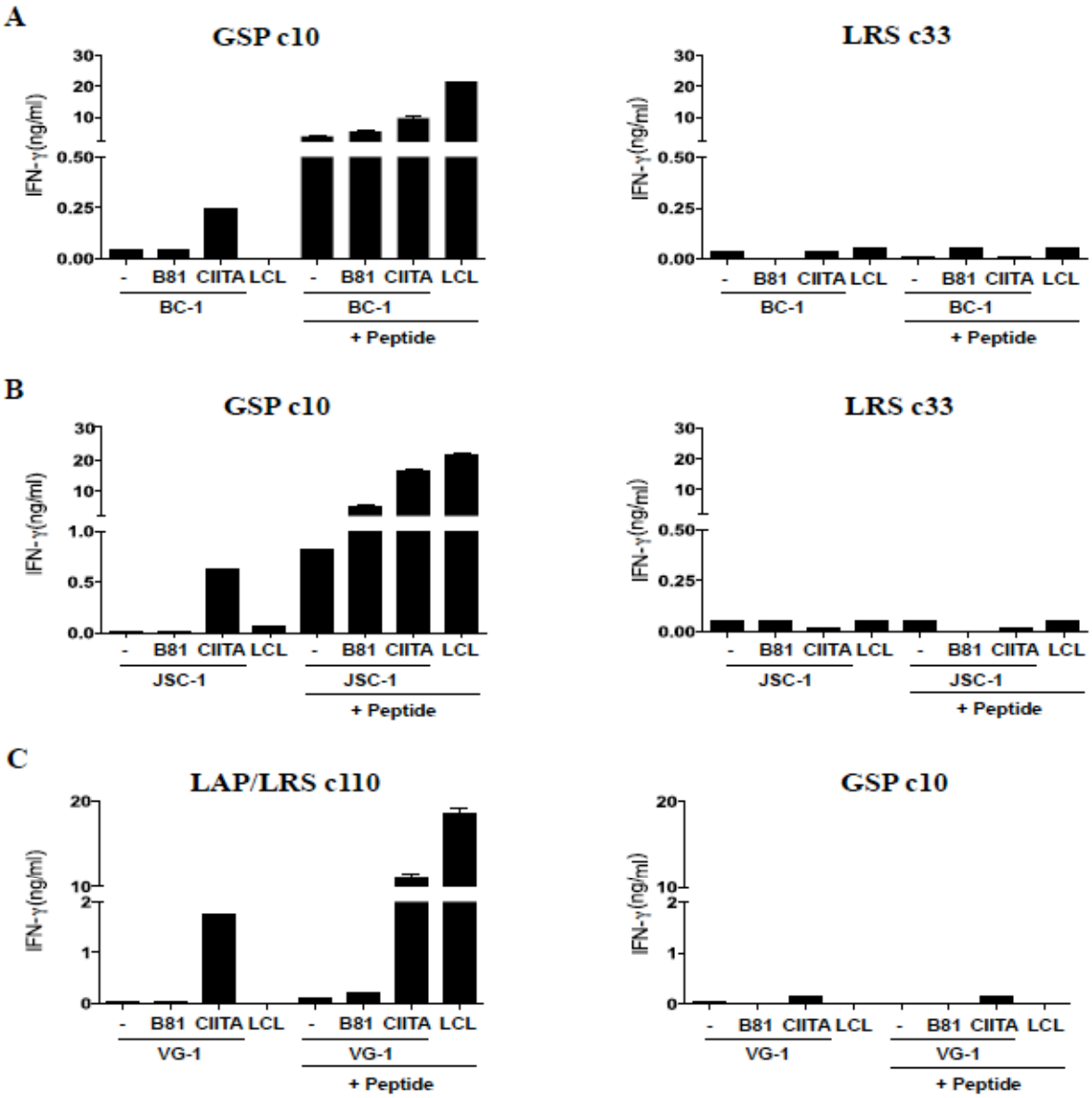


Figure 5.24 CD4+ T cell recognition of PEL lines transduced with CIITA expression constructs. Recognition of non-transduced PELs, PELs transduced with a control construct, PELs transduced with CIITA or autologous LCL by HLA-matched and HLA-mismatched LANA-specific CD4+ T cell clones was measured in parallel assays and recognition quantified by IFN-γ ELISA. As controls, aliquots of these cells were sensitised with the cognate peptide-epitope at a concentration of 0.5 μg/ml. PELs (A) BC-1 and (B) JSC-1 cells were assayed against the HLA-matched DQ7-restricted KS48 derived clone 10 specific for the LANA peptide GSP and the HLA mismatched KSB1 derived clone 33 specific for LANA peptide LRS. (C) VG-1 cells were assayed against the KSB1 derived HLA DQ6-restricted clone 110 specific for LANA peptides LAP and LRS and the HLA mismatched the KS48 derived clone 10 specific for the LANA peptide GSP. Results are expressed as the mean IFN-γ release from triplicate wells.

inhibitory effects of vIRF-3 on CIITA transcription by ectopically expressing CIITA in these PELs increases MHC Class II levels on their surface and allows CD4+ T cell recognition by the LANA-specific T cell clones. This is the first study to investigate the recognition of LANA expressing cells or malignancies using CD4+ LANA-specific T cells. In contrast, a number of studies have investigated T cell recognition of the EBV genome maintenance protein EBNA1 using EBNA1-specific CD4+ T cells, however each with variable results. The first reported EBNA1-specific CTL CD4+ clone studied did not lyse target LCLs (Khanna et al., 1995). This result was further supported by another group that reported EBNA1 evades direct immune recognition by CD4+ T cells (Mautner et al., 2004). However, other studies have shown that cytotoxic CD4+ T cell clones reactive to EBNA1 inhibited B-cell proliferation in vitro (Nikiforow et al., 2003). Further investigations into this variability in EBNA1 CD4+ T cell recognition was carried out by the Rickinson group which appear to support the theory that the majority of EBNA1-specific CD4+ T cell clone do not efficiently recognise LCLs. This group further suggested that efficiency of recognition may be an EBNA1 CD4+ epitope-specific phenomenon (Long et al., 2005; Tsang et al., 2006; Leung et al., 2010).

Similar to what is seen toward PELs, the MHC Class I restricted CTL response has been shown to be ineffective against cells from the EBV associated B cell malignancy Burkitts lymphoma (Rooney et al., 1985), highlighting the potential role of the CD4+ response in the control of these malignancies. For Burkitt's lymphoma this was confirmed in an *in vitro* study in which EBNA1-specific T helper 1 (Th1) CD4+ CTLs were capable of recognising Burkitt Lymphoma lines (Paludan et al., 2002). Some PELs are co-infected with EBV and similar to Burkitts lymphoma have an EBV

latency I phenotype, in which they only express EBNA1. In preliminary studies we have examined the ability of EBNA1-specific CD4+ T cell clones to recognise the EBV co-infected PEL BC-1. Similar to what we found with the LANA-specific clones, no recognition of endogenous EBNA1 was observed. Only when an invariant chain tagged form of EBNA1 was expressed in the PELs was recognition observed (data not shown). These results highlight that unlike Burkitt's lymphoma, PELs potentially have an immune evasion mechanism that interferes with the CD4+ T cell recognition of expressed antigens.

An interesting phenomenon observed in these studies was the higher level of expression of the LANAΔacid li protein compared with LANAΔacid when expression was induced from the pRTS-CD2 vectors. Given these constructs have been cloned into the same vector at the same sites and the LANA sequences are the same, the observed effect seems attributable to the invariant chain sequence fused to the amino terminus of the LANAΔacid li construct. When others have used a similar strategies and constructs for the expression of EBNA1, the invariant chain tagged construct was detected at lower levels compared to the non-tagged version. This was attributed to the endosomal routing of the fusion protein resulting in its rapid degradation (Leung et al., 2010). Why this is not seen in the comparable experiments conducted here with the equivalent LANA construct, expressed from the same vector is not clear. An emerging concept in the regulation of protein expression to minimise epitope production is the idea that translation initiation may be a mechanism by which viruses can restrict protein expression and consequently epitope-peptide generation (Apcher, et al. 2010; Apcher et al., 2009). One may speculate that in this instance, sequences in the 5' region of LANA may have such activity and are not conducive to efficient

transcription. However when a separate sequence taken from a human gene which is likely optimised for efficient expression is fused to the 5' terminus of the gene, such an effect is overcome. This could be tested by assessing *in vitro* translation of *in vitro* synthesised mRNA for both constructs. Alternatively, non-coding changes could be inserted into the 5' sequence of the LANAΔacid construct and changes in levels of expressed protein monitored.

As well as PELs expressing the latency profile of infected cells in KS lesions, an additional two latency proteins are expressed, the viral IL-6 and the immunomodulatory and anti-apoptotic protein vIRF3. A previous study highlighted the role of vIRF3 in inhibiting the transcription of CIITA. This transactivator functions by binding to four transacting factors that act as an enhanceosome bound to regulatory modules within the promoter regions of genes associated with the class II pathway such as HLA-DP, HLA-DQ, HLA-DR, the invariant chain and the non-classical MHC class II molecules HLA-DM and HLA-DO (Steimle et al., 1994; Harton et al., 2000: Reith et al., 2005). When bound to the enhanceosome, CIITA promotes transcription by recruiting and activating elements of the transcription machinery. Inhibiting CIITA expression then represents an effective strategy to restrict expression of many components of the HLA class II processing pathway. The importance of CIITA function in immune control is highlighted by the observations that herpesviruses, EBV and CMV also interfere with CIITA expression (Apcher et al., 2009; Miller et al., 1998).

The only PEL that was consistently recognised by the LANA-specific CD4+ T cell clone was BCBL-1. Interestingly, BCBL-1 expressed lower levels of vIRF3 and

higher levels of MHC Class II on its surface in comparison to the other three PELs studied BC-1, JSC-1 and VG-1. A previous study had demonstrated that siRNA knockdown of vIRF3 decreased the inhibition of CIITA transcription and increased surface MHC Class II expression on PELs (Schmidt, et al. 2011). Using similar protocols and siRNA vIRF3 sequences described in that study, our attempts were unsuccessful and we were unable to replicate these results. The study did not show efficient knockdown of vIRF3, suggesting sub optimal sequences of vIRF3 siRNAs were used or perhaps poor transfection efficiency. Given vIRF3 is required for proliferation of cells one may speculate these cells may be outgrown by non-transfected cells within the culture. A more controlled approach may be to transduce the PELs with retroviral based vectors expressing inhibitory shRNA sequences under the control of an inducible promoter which also expresses reporter proteins.

These findings are consistent with the idea that vIRF3 inhibition of CIITA promoter usage was at least one factor in preventing efficient class II processing and presentation of LANA epitopes. However the observation that some PELs such as VG-1 which express similar levels of vIRF3 as compared to BC-1 or JSC-1 but have much lower levels of surface class II compared to these cells may suggest other factors are involved in subverting class II processing in PELs. In this context vFLIP has been proposed to be an inhibitor of autophagy (Lee et al., 2009), a process known to be important in the generation of CD4 epitopes (Paludan et al., 2005). Nevertheless, bypassing the CIITA promoter blockage by vIRF3 through the ectopic expression of CIITA restored expression of class II and other CIITA targets in the PELs sufficient to overcome any other potential inhibitory mechanisms.

Chapter 6

Final Discussion

The adaptive T cell immune response plays a key role in the control of viral infections, especially as effectors of long-term immune surveillance against viruses in the infected host. The molecular targets of T cells are peptide-epitopes derived from viral antigens that are processed and presented by the MHC complexes. Therefore, determining the identity of these viral peptide antigens is highly important, as they can be used to track the cognate T cell responses in disease, allowing a better understanding of their role in the control of virus pathogenesis. Ultimately these epitopes may find use as the basis of immunotherapeutic strategies to control virus infection and disease progression.

The previous observations of control of KSHV-associated disease such as KS in HIV patients and immunosuppressed transplant patients when their cellular response is restored by therapeutic intervention, argues strongly for a role of these effectors in control of at least some KSHV-associated diseases (Duman et al., 2002; Bihl et al., 2007). In light of this, several groups have attempted to define the viral antigens that are responsible for initiating and maintaining CTL immune responses in KSHV infected hosts. However to date, only a limited number of viral peptide-epitopes have been identified. These have mainly been derived from studying HIV co-infected individuals, identifying epitopes from lytic cycle proteins which are mostly HLA-A*0201 restricted (Bourboulia et al., 2004; Guihot et al., 2006; Wang et al., 2002;

Wang et al., 2001; Ribechini et al., 2006). Furthermore, the responses elicited by these epitopes are generally weak, especially when compared to other herpesviruses such as EBV and CMV, and it is unclear whether the weakness of these responses is the normal state or were consequences of the patients pathological state (Bihl et al., 2007). Very few studies have examined the T cell response to KSHV in healthy donors who are controlling their infection, in the absence of an immune disregulating HIV setting.

To address this relatively unexplored area of KSHV T cell immunity, this study investigated the T cell response to the four well-defined KSHV latent proteins LANA, vFLIP, vCyclin and Kaposin in healthy Gambian KSHV infected donors using overlapping peptide libraries spanning their sequences to stimulate T cell responses. This gave an overall estimation of the T cell response to these antigens across all HLA types. The ELISpot screens showed that the *ex vivo* responses, as measured in the periphery to these overlapping peptide pools, were very weak and substantially lower than responses made to EBV by this donor population, implying the number of T cells controlling KSHV latent infection appear lower than those controlling EBV latent infections (Njie et al., 2009). These KSHV-specific responses were also lower than those reported for similar studies which have used peptide libraries to examine the T cell response in HIV co-infected donors to lytic and latent antigens. However, these studies were consistent with LANA being more frequently targeted than Kaposin (Woodberry et al., 2005: Bihl et al., 2007). Furthermore, the present analysis of *ex vivo* T cell responses also suggests that the other two latent proteins, vCyclin and vFLIP, induce and maintain weak responses in healthy donors. Thus unlike T cell responses to the EBNA3 family in EBV infected donors (Steven et al., 1996), KSHV-

specific T cell responses do not appear to be focused on particular latent antigens, but appear to elicit weak responses to all latent antigens. Consequently, identifying novel and immunodominant epitopes using these methods has proven very challenging for KSHV immunologists.

In order to discover the targets of these weak responses, this study used an alternative approach to isolate latent T cell specificities from the healthy KSHV infected cohort. KSHV-specific T cell clones were generated by *ex vivo* expansion, stimulating donors PBMCs with overlapping peptides spanning the sequences of LANA, vFLIP and vCyclin. Most of these studies were focussed on LANA as this appeared to be the dominant antigen in the *ex vivo* ELISpot screens. As such the majority of PBMCs from the KSHV seropositive donors were stimulated with the LANA peptide library to derive LANA-specific T cell clones. The T cell cloning primarily yielded CD4+ LANA-specific T cell epitopes, generating clones specific to one CD8+ and thirteen CD4+ targets from five donors, restricted by a wide range of HLA types. In terms of numbers of clones isolated, CD4 clones were more frequently isolated than CD8s. T cell epitopes from vFLIP and vCyclin were also identified in responses from one local donor. PBMCs from the Gambian cohort were limited and so not used to identify epitopes from these proteins. Although the vFLIP and vCyclin responses were sourced from one donor's response, four CD4+ targets to vFLIP, two to vCyclin and one CD8+ target were identified to each protein. Interestingly in this case the number of CD8 clones isolated to these epitopes was clearly greater than the more diverse CD4 clones.

The differential isolation of CD4 and CD8 T cell clones from the donors may give some idea as to the abundance of these responses in vivo. We cannot exclude the possibility that the *ex vivo* expansion and culture of these cells may have biased the expansion of specificities and lymphocyte subsets, indeed the use of 15 mer peptides to expand responses has been reported to show some bias to stimulating CD8 responses (Draenert et al., 2003). However the higher frequency of isolated CD4+ T cell clones specific for LANA supports the idea that these may be the numerically dominant form of cellular immunity to LANA, while the preferential isolation of CD8+ T cells specific to vFLIP and vCyclin may be the dominant form of cellular immunity for these targets. This data is based on responses from one carefully studied donor, clearly other healthy donors must be assessed to confirm or disprove these contentions. Having now identified CD4 and CD8 epitopes in these proteins across a range of HLA types it would be of great interest to repeat the ELISpot analysis on healthy KSHV-infected donors and determine if CD4 responses dominate the LANA-specific response and whether CD8 responses dominate the vFLIP and vCyclin responses.

To date very few CD4+ and CD8+ targets have been identified within the latent proteins. Consequently studies on the recognition of latent KSHV expressing cells have been very limited. This study investigated the recognition of both ectopically and natively expressed latent antigens in various cell backgrounds by the CD4+ and CD8+ KSHV-specific T cells identified in this study. Revealing that these genes show features which indicate that they have evolved strategies to minimise CD8+ T cell targeting.

Initially recognition of ectopically expressed vFLIP constructs using CD8+ T cells for both model and native vFLIP epitopes was examined. The wildtype (Wt) vFLIP sequence was poorly expressed and poorly recognised when expressed in MJS and LCL cell backgrounds. However, recognition was restored upon the expression of a human codon-optimised sequence of vFLIP, suggesting that vFLIP uses sub-optimal codon sequences to reduce protein expression, which consequently limits the supply of viral peptides available for T cell recognition. At present, it is difficult to determine whether the lack of detectable Wt vFLIP protein expression and recognition are due to the expression system used in this study or whether it is indeed the result of the poor codon usage in the Wt sequence resulting in inefficient protein translation as a potential immune evasion mechanism. Indeed, previous work has detected Wt vFLIP protein expression *in vitro* using a lentiviral expression system in DCs (Rowe et al., 2009), however native expression in KSHV-infected B cells and endothelial cells appears low (Low et al., 2001; Alkharsah et al., 2011). In order to determine the protein translation and expression levels of vFLIP in a more physiologically relevant setting, a different expression system would need to be employed. The Hu and Wt vFLIP sequences would need to be transcribed from a tricistronic or bicistronic transcript mimicking vFLIP expression in vivo. In addition to this measuring vFLIP expression levels in primary B cells or endothelial cells latently infected with KSHV *in vitro* would provide a good indication of how well vFLIP is expressed from the virus genome in a KSHV relevant cell background.

This study also provided further evidence that the acidic repeat sequence encoded within LANA interferes with its own processing and presentation, reducing its recognition by LANA-specific CD8+ T cells in an LCL background. It has been

previously shown that the acidic repeat sequence inhibits degradation and its own translation efficiency (Kwun et al., 2007); indeed previous studies using a murine T cell epitope inserted in LANA has suggested that LANA expressing cells are not recognised by CD8+ T cells (Zaldumbide et al., 2007). This is the first study to test this recognition in a native setting using a LANA-specific CD8+ T cell clone. The results of this study are similar to CD8+ T cell recognition studies on EBV genome maintenance protein EBNA1, which have shown that the GAr domain within its sequence is responsible for reduced CD8+ T cell recognition by downregulating the rate of protein translation and proteasomal degradation (Tellam, 2007; Levitskaya et al., 1995). As the primary source of endogenous antigen for EBNA1 presentation has been shown to be derived from DRiPs, the translation efficiency of EBNA1 directly correlated with the efficiency at which DRiPs were produced and presented for CD8+ T cell recognition (Tellam, 2004 et al.; Tellam, 2007 et al.). We hypothesise that LANA antigens for presentation to CD8+ T cells are derived from a similar route, as previous work has shown the acidic repeat sequence interferes with LANA translation and proteasomal degradation (Kwun et al., 2007; Zaldumbide et al., 2007), supporting the inefficient recognition of FL-LANA expressing cells by LANA-specific CD8+ T cells used in this study. This would need to be confirmed in this system by measuring rates of translation and degradation to determine whether this correlates with the CD8+ T cell recognition of the respective constructs. Furthermore, this finding would need to be confirmed using different LANA-specific CD8+ T cell targets to exclude the possibility that this is an epitope-specific phenomenon.

The CD8+ T cell recognition of LANA was also assessed in *in vitro* KSHV-infected HMEC-1 cells. However despite successful infection of the cells and detectable levels

of LANA protein expression, there was no CD8+ T cell recognition of LANA. The HMEC-1 cells were confirmed to be capable of processing and presenting antigen, yet when surface MHC class I levels were tested on infected cells, they were found to be reduced compared to uninfected cells. This result was somewhat unexpected as the KSHV proteins primarily thought to be responsible for downregulating MHC class I surface expression, K3 and K5, are classified as lytic cycle antigens. However, studies have shown that K5 protein can be detected in endothelial cells up to 5 days later following KSHV infection and the levels of expression correlate with MOI used (Krishnan et al., 2004; Adang et al., 2007). Ideally, we would have checked for K5 expression levels in the endothelial cells in parallel to MHC class I staining, as well as titrating the MOI of the virus used for infection in these assays. We hypothesise that the combined roles of the acidic repeat sequence within LANA with the reduced levels of MHC class I expression was sufficient to overcome any level of CD8+ T cell recognition that may have been present in these cells.

This work so far has shown that in these models, the latent antigens vFLIP and LANA are inefficiently recognised by CD8+ T cells. This is most likely as a result of the virus employing strategies to restrict protein translation, consequently limiting the amount of DRiPs available (Yewdell et al., 1996), which are thought to be the major source of peptide-epitopes which feed into the class I processing pathway (Tellam et al., 2007; Mackay et al., 2009). In contrast, CD4+ T cells are thought to derive their peptide-epitopes from the mature protein pool (Mackay et al., 2009) and so may not be affected by these evasion strategies. As such the ability of LANA-specific CD4+ T cells to recognise LANA-expressing LCLs and PELs was investigated. The CD4+ T cells recognised the LANA-expressing LCLs but could not recognise the majority of

the KSHV-infected B cell malignant PEL lines which natively express LANA, as they appear to have defects in their ability to process and present antigen. Part of this defect may relate to the function of vIRF3 which has been shown to inhibit the promoter activities of both the IFN-γ and CIITA genes resulting in reduced MHC class II surface expression on these PELs (Schmidt et al., 2011). However, BCBL-1 cells relative to the other PELs expressed lower amounts of vIRF3 and expressed higher levels of MHC class II on their surface and was the only PEL line to be recognised by the LANA-specific CD4+ T cells. Although the LANA-specific CD4+ T cells did not recognise most PELs, it is not clear whether they would recognise B cells which had been infected with KSHV as part of the normal biology of KSHV infection. Thus although the LANA-specific CD4+ T cells may be ineffective against most PELs, they may have a role for control when the virus establishes latency in B cells. In this context, the recent ability to infect B cells in vitro with KSHV would allow for this to be tested *in vitro* at least (Myoung et al., 2011; Hassman et al., 2011). It would be of interest to know whether vIRF3 is also expressed at this stage of infection to determine whether it potentially protects newly infected cells from CD4+ T cell recognition.

Taking the results of the recognition assays using CD8+ T cell clones specific for LANA and vFLIP together, it appears for these two latent proteins at least, the virus is going to great lengths to minimise CD8+ T cell targeting of these proteins. This raises the idea that CD4+ T cells may have a greater role in the control of KSHV infection and at least some KSHV-associated malignancies. Such an argument may help to explain why HIV co-infected donors are so sensitive to the development of KSHV-associated malignancies. Thus, levels of CD4+ T cells will progressively decline in

untreated HIV patients and these patients appear to develop KSHV-associated disease before diseases associated with other herpesviruses (Rosenberg et al., 1997; Moore et al., 1996). Now that we have better defined epitopes, it would be of interest to measure the KSHV-specific CD4+ T cell responses to these proteins in untreated HIV patients with KSHV-associated disease when they initiate HAART and as they resolve disease. Previous studies have shown that peripheral levels of global KSHV-specific T cells and KSHV-specific CD8+ T cells slowly increase with time after initiation of anti-retroviral treatment (Bihl et al., 2007; Bourboulia et al., 2004), however the KSHV-specific CD4 response has been largely untested in this population. A careful observational study of KSHV-specific CD4 responses with monitoring viral loads and disease resolution would help give some idea as to whether KSHV-specific CD4+ T cells have a role in control of KSHV-associated disease after HAART initiation.

The importance of the CD4+ T cell response in controlling virus infection has been highlighted in recent years as CD4+ T cells have been shown to act as CTLs in their own right. It is now coming to light that a number of immune evasion mechanisms for CD4+ T cells are at play in herpesvirus infections. In KSHV infected PELs it has been shown that vIRF3 restricts CIITA gene expression by blocking expression from the lymphoid origin-specific promoter pIII and IFN-γ responsive promoter pIV of this gene. Consistent with this, we found stimulating PELs with recombinant IFN-γ did not increase expression of surface MHC class II, despite inducing surface MHC class II on cell types such as fibroblasts in parallel assays. However, the inhibitory effects of vIRF3 on CIITA transcription were bypassed by ectopically expressing CIITA in these PELs, increasing MHC class II levels on their surface and allowing CD4+ T cell

recognition by the LANA-specific T cell clones. However we were unable to determine whether vIRF3 is the sole determinant of the poor recognition of PELs by the LANA-specific T cells, or whether other mechanisms are at play in preventing CIITA transcription. In order to address this, vIRF3 expression will need to be knocked down using siRNAs or shRNAs with more optimal sequences and where the efficiency of transfection can be measured in parallel to CIITA and surface class II levels. However, if there is no significant restoration of function following vIRF3 knockdown, this would suggest that other inhibitory mechanisms are responsible and other components of the MHC class II processing pathway would need to be examined. It may also be of interest to determine whether, similar to some EBNA1 CD4+ epitopes (Leung et al., 2010), LANA epitopes are generated through the autophagy pathway. A recent study has highlighted a role for vFLIP in the inhibition of the autophagy pathway in the PEL BCBL-1 (Lee et al., 2009). However as BCBL-1 was the only PEL capable of presenting LANA antigen for CD4+ T cell recognition in this study, it would suggest that it is unlikely that the specific LANA epitopes presented by BCBL-1 were generated through the autophagy pathway.

The importance of evading CD4+ T cell recognition during herpesvirus replication is highlighted by studies on other herpesviruses which also downregulate surface MHC class II on infected cells and subsequently evade CD4+ T cell recognition. In EBV, the transcriptional regulator BZLF1 inhibits MHC class II expression at the transcriptional level by binding and repressing the CIITA promoter PIII, using a similar mechanism employed by vIRF3 (Li et al., 2009). While human CMV (HCMV) infection of human endothelial cells and fibroblasts also results in a defect in IFN-γ signal transduction disrupting MHC class II expression (Miller et al., 1998).

HCMV also encodes two proteins US2 and US3, which lead to the proteasomal degradation of MHC class II molecules (Tomazin et al., 1999) and inhibit the assembly of the peptide:MHC class II complex (Hegde et al., 2002), respectively.

In contrast to studies on other herpesviruses, very few studies have investigated the CD4+ T cell response in KSHV infected cells. Recent work has highlighted a role for non-MHC restricted CD4+ T cells in the non-cytolytic control of KSHV lytic replication in tonsillar B cells, preventing lytic reactivation and promoting latency (Myoung et al., 2011). However, to our knowledge the data presented in this thesis is the first to investigate CD4+ T cell recognition of any KSHV malignancy. Our results have shown that, based on *in vitro* studies, direct recognition of most PEL cell lines may not lead to successful control of these infected cells by CD4+ T cells. However inducing CIITA expression in the PELs represents a potential therapeutic intervention to restore recognition by CD4+ T cells.

The second KSHV infected cell type which may be subject to direct control by KSHV-specific CD4+ T cells are the infected B cells of MCD lesions. In contrast to HIV-associated KS patients, MCD patients show relatively conserved immune function, with higher levels of peripheral CD4+ T cells, yet may develop disease even when on HAART; indeed the incidence of MCD is increasing in HAART treated patients (Powles et al., 2009). Previous studies have suggested that KSHV-specific CD8+ T cell responses in MCD patients are of an equivalent frequency and functionality compared to asymptomatic KSHV carriers co-infected with HIV (Guihot et al., 2008). Although good levels of peripheral CD4+ T cells may be seen in MCD patients, little is known about the frequency of KSHV-specific CD4+ T cells. As B

cells express HLA class II, these KSHV-infected B cells conceivably may be targeted by specific CD4+ T cells. However, vIRF3 can be expressed in infected cells in MCD. Importantly, not all infected cells in MCD lesions express vIRF3, suggesting at least some may be targets for the CD4 response (Rivas et al., 2001). Also of interest is the observation that when follicular dendritic cells are found to express LANA in these lesions, this is associated with increased T cell infiltration and lower viral loads, implying a role for T cell control (El-Daly et al., 2010). As KSHV-specific CD8 responses appear equivalent to HIV KSHV co-infected donors who control their disease, it would be of interest to examine the KSHV-specific CD4 response with the epitopes identified in this study, during the relapsing and remitting phases of this disease to determine if any correlates with KSHV-specific CD4+ T cell control exist.

In this study, for the first time well-characterised CD8+ and CD4+ KSHV-specific T cells were used to test the recognition of ectopically and endogenously expressed latent antigens in various cell backgrounds including LCLs, KSHV-infected endothelial cells and KSHV malignant B cells in the form of PEL cell lines. This work has highlighted the potential roles of the latent proteins in disrupting recognition by the adaptive immune T cell response in a native setting. The strategies used by the virus to minimise recognition by CD8+ T cells explored in this study will be difficult to overcome in a therapeutic setting, as these evasion mechanisms primarily focus on restricting protein expression. Similarly, the virus has mechanisms in place to prevent efficient CD4+ T cell recognition. However these latter evasion strategies, as we have demonstrated, are more susceptible to manipulation and represent potential therapeutics which can be used to favour immune control and restore the virus-host balance.

References

Ablashi, D.V., L.G. Chatlynne, J.E. Whitman, Jr., and E. Cesarman. 2002. Spectrum of Kaposi's sarcoma-associated herpesvirus, or human herpesvirus 8, diseases. *Clin Microbiol Rev* 15:439-464.

Ackerman, A.L., and P. Cresswell. 2004. Cellular mechanisms governing cross-presentation of exogenous antigens. *Nat Immunol* 5:678-684.

Adang, L.A., C. Tomescu, W.K. Law, and D.H. Kedes. 2007. Intracellular Kaposi's sarcoma-associated herpesvirus load determines early loss of immune synapse components. *J Virol* 81:5079-5090.

Ades, E.W., F.J. Candal, R.A. Swerlick, V.G. George, S. Summers, D.C. Bosse, and T.J. Lawley. 1992. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol* 99:683-690.

Aichinger, G., L. Karlsson, M.R. Jackson, M. Vestberg, J.H. Vaughan, L. Teyton, R.I. Lechler, and P.A. Peterson. 1997. Major histocompatibility complex class II-dependent unfolding, transport, and degradation of endogenous proteins. *J Biol Chem* 272:29127-29136.

Albanesi, C., A. Cavani, and G. Girolomoni. 1998. Interferon-gamma-stimulated human keratinocytes express the genes necessary for the production of peptide-loaded MHC class II molecules. *J Invest Dermatol* 110:138-142.

Albert, M.L., B. Sauter, and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 392:86-89.

Alkharsah, K.R., V.V. Singh, R. Bosco, S. Santag, A. Grundhoff, A. Konrad, M. Sturzl, D. Wirth, O. Dittrich-Breiholz, M. Kracht, and T.F. Schulz. 2011. Deletion of Kaposi's Sarcoma-Associated Herpesvirus FLICE Inhibitory Protein, vFLIP, from the Viral Genome Compromises the Activation of STAT1-Responsive Cellular Genes and Spindle Cell Formation in Endothelial Cells. *J Virol* 85:10375-10388.

Ambroziak, J.A., D.J. Blackbourn, B.G. Herndier, R.G. Glogau, J.H. Gullett, A.R. McDonald, E.T. Lennette, and J.A. Levy. 1995. Herpes-like sequences in HIV-infected and uninfected Kaposi's sarcoma patients. *Science* 268:582-583.

Amyes, E., C. Hatton, D. Montamat-Sicotte, N. Gudgeon, A.B. Rickinson, A.J. McMichael, and M.F. Callan. 2003. Characterization of the CD4+ T cell response to Epstein-Barr virus during primary and persistent infection. *J Exp Med* 198:903-911.

Anderson, M.S., and J. Miller. 1992. Invariant chain can function as a chaperone protein for class II major histocompatibility complex molecules. *Proc Natl Acad Sci U S A* 89:2282-2286.

Andreoni, M., L. Sarmati, E. Nicastri, G. El Sawaf, M. El Zalabani, I. Uccella, R. Bugarini, S.G. Parisi, and G. Rezza. 2002. Primary human herpesvirus 8 infection in immunocompetent children. *JAMA* 287:1295-1300.

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:e1001151.

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* 83:1289-1298.

Barry, M., and R.C. Bleackley. 2002. Cytotoxic T lymphocytes: all roads lead to death. *Nat Rev Immunol* 2:401-409.

Bechtel, J.T., Y. Liang, J. Hvidding, and D. Ganem. 2003. Host range of Kaposi's sarcoma-associated herpesvirus in cultured cells. *J Virol* 77:6474-6481.

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* 83:2783-2788.

Bestetti, G., G. Renon, P. Mauclere, A. Ruffie, F.X. Mbopi Keou, D. Eme, C. Parravicini, M. Corbellino, G. de The, and A. Gessain. 1998. High seroprevalence of human herpesvirus-8 in pregnant women and prostitutes from Cameroon. *AIDS* 12:541-543.

Bettelli, E., Y. Carrier, W. Gao, T. Korn, T.B. Strom, M. Oukka, H.L. Weiner, and V.K. Kuchroo. 2006. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-238.

Bevan, M.J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* 143:1283-1288.

Bihl, F., C. Berger, J.V. Chisholm, 3rd, L.M. Henry, B. Bertisch, A. Trojan, D. Nadal, R.F. Speck, M. Flepp, C. Brander, and N.J. Mueller. 2009. Cellular immune responses and disease control in acute AIDS-associated Kaposi's sarcoma. *AIDS* 23:1918-1922.

Bihl, F., A. Mosam, L.N. Henry, J.V. Chisholm, 3rd, S. Dollard, P. Gumbi, E. Cassol, T. Page, N. Mueller, P. Kiepiela, J.N. Martin, H.M. Coovadia, D.T. Scadden, and C. Brander. 2007. Kaposi's sarcoma-associated herpesvirus-specific immune reconstitution and antiviral effect of combined HAART/chemotherapy in HIV clade C-infected individuals with Kaposi's sarcoma. *AIDS* 21:1245-1252.

Bihl, F., M. Narayan, J.V. Chisholm, III, L.M. Henry, T.J. Suscovich, E.E. Brown, T.M. Welzel, D.E. Kaufmann, T.M. Zaman, S. Dollard, J.N. Martin, F. Wang, D.T. Scadden, K.M. Kaye, and C. Brander. 2007. Lytic and Latent Antigens of the Human Gammaherpesviruses Kaposi's Sarcoma-Associated Herpesvirus and Epstein-Barr Virus Induce T-Cell Responses with Similar Functional Properties and Memory Phenotypes. *J. Virol.* 81:4904-4908.

Blackbourn, D.J., E. Lennette, B. Klencke, A. Moses, B. Chandran, M. Weinstein, R.G. Glogau, M.H. Witte, D.L. Way, T. Kutzkey, B. Herndier, and J.A. Levy. 2000. The restricted cellular host range of human herpesvirus 8. *AIDS* 14:1123-1133.

Blake, N., T. Haigh, G. Shaka'a, D. Croom-Carter, and A. Rickinson. 2000. The importance of exogenous antigen in priming the human CD8+ T cell response: lessons from the EBV nuclear antigen EBNA1. *J Immunol* 165:7078-7087.

Blott, E.J., and G.M. Griffiths. 2002. Secretory lysosomes. *Nat Rev Mol Cell Biol* 3:122-131.

Bonnerot, C., M.S. Marks, P. Cosson, E.J. Robertson, E.K. Bikoff, R.N. Germain, and J.S. Bonifacino. 1994. Association with BiP and aggregation of class II MHC molecules synthesized in the absence of invariant chain. *EMBO J* 13:934-944.

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:e137.

Boshoff, C., and R. Weiss. 2002. AIDS-related malignancies. *Nat Rev Cancer* 2:373-382.

Bour-Jordan, H., and J.A. Blueston. 2002. CD28 function: a balance of costimulatory and regulatory signals. *J Clin Immunol* 22:1-7.

Bourboulia, D., D. Aldam, D. Lagos, E. Allen, I. Williams, D. Cornforth, A. Copas, and C. Boshoff. 2004. Short- and long-term effects of highly active antiretroviral therapy on Kaposi sarcoma-associated herpesvirus immune responses and viraemia. *AIDS* 18:485-493.

Bouvard, V., R. Baan, K. Straif, Y. Grosse, B. Secretan, F. El Ghissassi, L. Benbrahim-Tallaa, N. Guha, C. Freeman, L. Galichet, and V. Cogliano. 2009. A review of human carcinogens--Part B: biological agents. *Lancet Oncol* 10:321-322.

Bouvier, M. 2003. Accessory proteins and the assembly of human class I MHC molecules: a molecular and structural perspective. *Mol Immunol* 39:697-706.

Bouvier, M., and D.C. Wiley. 1994. Importance of peptide amino and carboxyl termini to the stability of MHC class I molecules. *Science* 265:398-402.

Bower, M. 2010. How I treat HIV-associated multicentric Castleman disease. *Blood* 116:4415-4421.

Brander, C., P. O'Connor, T. Suscovich, N.G. Jones, Y. Lee, D. Kedes, D. Ganem, J. Martin, D. Osmond, S. Southwood, A. Sette, B.D. Walker, and D.T. Scadden. 2001. Definition of an Optimal Cytotoxic T Lymphocyte Epitope in the Latently Expressed Kaposi's Sarcoma‚ÄìAssociated Herpesvirus Kaposin Protein. *The Journal of Infectious Diseases* 184:119-126.

Brander, C., T. Suscovich, Y. Lee, P.T. Nguyen, P. O'Connor, J. Seebach, N.G. Jones, M. van Gorder, B.D. Walker, and D.T. Scadden. 2000. Impaired CTL Recognition of Cells Latently Infected with Kaposi's Sarcoma-Associated Herpes Virus. *J Immunol* 165:2077-2083.

Brazil, M.I., S. Weiss, and B. Stockinger. 1997. Excessive degradation of intracellular protein in macrophages prevents presentation in the context of major histocompatibility complex class II molecules. *Eur J Immunol* 27:1506-1514.

Brown, J.H., T. Jardetzky, M.A. Saper, B. Samraoui, P.J. Bjorkman, and D.C. Wiley. 1988. A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. *Nature* 332:845-850.

Bryan, B.A., O.F. Dyson, J.A. McCubrey, and S.M. Akula. 2005. Biology of Kaposi's sarcoma-associated herpesvirus. *Front Biosci* 10:2882-2891.

Cadwell, K., and L. Coscoy. 2008. The specificities of Kaposi's sarcoma-associated herpesvirus-encoded E3 ubiquitin ligases are determined by the positions of lysine or cysteine residues within the intracytoplasmic domains of their targets. *J Virol* 82:4184-4189.

Callan, M.F., C. Fazou, H. Yang, T. Rostron, K. Poon, C. Hatton, and A.J. McMichael. 2000. CD8(+) T-cell selection, function, and death in the primary immune response in vivo. *J Clin Invest* 106:1251-1261.

Cannon, J.S., D. Ciufo, A.L. Hawkins, C.A. Griffin, M.J. Borowitz, G.S. Hayward, and R.F. Ambinder. 2000. A new primary effusion lymphoma-derived cell line yields a highly infectious Kaposi's sarcoma herpesvirus-containing supernatant. *J Virol* 74:10187-10193.

Casper, C., A. Wald, J. Pauk, S.R. Tabet, L. Corey, and C.L. Celum. 2002. Correlates of prevalent and incident Kaposi's sarcoma-associated herpesvirus infection in men who have sex with men. *J Infect Dis* 185:990-993.

Cesarman, E., P.S. Moore, P.H. Rao, G. Inghirami, D.M. Knowles, and Y. Chang. 1995. In vitro establishment and characterization of two acquired immunodeficiency syndrome-related lymphoma cell lines (BC-1 and BC-2) containing Kaposi's sarcoma-associated herpesvirus-like (KSHV) DNA sequences. *Blood* 86:2708-2714.

Chandran, B. 2010. Early events in Kaposi's sarcoma-associated herpesvirus infection of target cells. *J Virol* 84:2188-2199.

Chandriani, S., and D. Ganem. 2010. Array-based transcript profiling and limiting-dilution reverse transcription-PCR analysis identify additional latent genes in Kaposi's sarcoma-associated herpesvirus. *J Virol* 84:5565-5573.

Chang, Y., E. Cesarman, M.S. Pessin, F. Lee, J. Culpepper, D.M. Knowles, and P.S. Moore. 1994. Identification of herpesvirus-like DNA sequences in AIDS-associated Kaposi's sarcoma. *Science* 266:1865-1869.

Chatterjee, M., J. Osborne, G. Bestetti, Y. Chang, and P.S. Moore. 2002. Viral IL-6-induced cell proliferation and immune evasion of interferon activity. *Science* 298:1432-1435.

Chaudhary, P.M., M.T. Eby, A. Jasmin, A. Kumar, L. Liu, and L. Hood. 2000. Activation of the NF-kappaB pathway by caspase 8 and its homologs. *Oncogene* 19:4451-4460.

Chicz, R.M., R.G. Urban, J.C. Gorga, D.A. Vignali, W.S. Lane, and J.L. Strominger. 1993. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J Exp Med* 178:27-47.

Choi, J.K., B.S. Lee, S.N. Shim, M. Li, and J.U. Jung. 2000. Identification of the novel K15 gene at the rightmost end of the Kaposi's sarcoma-associated herpesvirus genome. *J Virol* 74:436-446.

Christensen, J.P., R.D. Cardin, K.C. Branum, and P.C. Doherty. 1999. CD4(+) T cell-mediated control of a gamma-herpesvirus in B cell-deficient mice is mediated by IFN-gamma. *Proc Natl Acad Sci U S A* 96:5135-5140.

Coscoy, L., D.J. Sanchez, and D. Ganem. 2001. A novel class of herpesvirus-encoded membrane-bound E3 ubiquitin ligases regulates endocytosis of proteins involved in immune recognition. *J Cell Biol* 155:1265-1273.

Del Prete, G. 1992. Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. *Allergy* 47:450-455.

Dissanayake, S.K., N. Tuera, and S. Ostrand-Rosenberg. 2005. Presentation of endogenously synthesized MHC class II-restricted epitopes by MHC class II cancer vaccines is independent of transporter associated with Ag processing and the proteasome. *J Immunol* 174:1811-1819.

Dittmer, D., M. Lagunoff, R. Renne, K. Staskus, A. Haase, and D. Ganem. 1998. A cluster of latently expressed genes in Kaposi's sarcoma-associated herpesvirus. *J Virol* 72:8309-8315.

Doumas, B.T. 1975. Standards for total serum protein assays--a collaborative study. *Clin Chem* 21:1159-1166.

Dourmishev, L.A., A.L. Dourmishev, D. Palmeri, R.A. Schwartz, and D.M. Lukac. 2003. Molecular genetics of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) epidemiology and pathogenesis. *Microbiol Mol Biol Rev* 67:175-212, table of contents.

Draenert, R., M. Altfeld, C. Brander, N. Basgoz, C. Corcoran, A.G. Wurcel, D.R. Stone, S.A. Kalams, A. Trocha, M.M. Addo, P.J. Goulder, and B.D. Walker. 2003. Comparison of overlapping peptide sets for detection of antiviral CD8 and CD4 T cell responses. *J Immunol Methods* 275:19-29.

Dugan, G.E., and E.W. Hewitt. 2009. Dependence of the localization and function of the human cytomegalovirus protein US6 on the transporter associated with antigen processing. *J Gen Virol* 90:2234-2238.

Duman, S., H. Toz, G. Asci, S. Alper, M. Ozkahya, I. Unal, A. Celik, E. Ok, and A. Basci. 2002. Successful treatment of post-transplant Kaposi's sarcoma by reduction of immunosuppression. *Nephrol Dial Transplant* 17:892-896.

Duncan, L.M., S. Piper, R.B. Dodd, M.K. Saville, C.M. Sanderson, J.P. Luzio, and P.J. Lehner. 2006. Lysine-63-linked ubiquitination is required for endolysosomal degradation of class I molecules. *EMBO J* 25:1635-1645.

Ebert, L.M., P. Schaerli, and B. Moser. 2005. Chemokine-mediated control of T cell traffic in lymphoid and peripheral tissues. *Mol Immunol* 42:799-809.

El-Daly, H., M. Bower, and K.N. Naresh. 2010. Follicular dendritic cells in multicentric Castleman disease present human herpes virus type 8 (HHV8)-latent nuclear antigen 1 (LANA1) in a proportion of cases and is associated with an enhanced T-cell response. *Eur J Haematol* 84:133-136.

Elliott, T. 1991. How do peptides associate with MHC class I molecules? *Immunol Today* 12:386-388.

Engelhard, V.H. 1994. Structure of peptides associated with class I and class II MHC molecules. *Annu Rev Immunol* 12:181-207.

Fabiola Micheletti, P.M.C.F.P.R.M.B.M.A.M.G.S.T.B.E.R.G. 2002. Identification of cytotoxic T lymphocyte epitopes of human herpesvirus 8. *Immunology* 106:395-403.

Faint, J.M., N.E. Annels, S.J. Curnow, P. Shields, D. Pilling, A.D. Hislop, L. Wu, A.N. Akbar, C.D. Buckley, P.A. Moss, D.H. Adams, A.B. Rickinson, and M. Salmon. 2001. Memory T cells constitute a subset of the human CD8+CD45RA+ pool with distinct phenotypic and migratory characteristics. *J Immunol* 167:212-220.

Fakhari, F.D., J.H. Jeong, Y. Kanan, and D.P. Dittmer. 2006. The latency-associated nuclear antigen of Kaposi sarcoma-associated herpesvirus induces B cell hyperplasia and lymphoma. *J Clin Invest* 116:735-742.

Falk, K., O. Rotzschke, S. Stevanovic, G. Jung, and H.G. Rammensee. 1991. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* 351:290-296.

Fernandez, J.A., F. Zavala, and M. Tsuji. 1999. Phenotypic and functional characterization of CD8(+) T cell clones specific for a mouse cytomegalovirus epitope. *Virology* 255:40-49.

Ferrara, N. 2004. Vascular endothelial growth factor as a target for anticancer therapy. *Oncologist* 9 Suppl 1:2-10.

Fogg, M.H., L.J. Wirth, M. Posner, and F. Wang. 2009. Decreased EBNA-1-specific CD8+ T cells in patients with Epstein-Barr virus-associated nasopharyngeal carcinoma. *Proc Natl Acad Sci U S A* 106:3318-3323.

Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4:330-336.

Frances, C., C. Mouquet, A.G. Marcelin, S. Barete, R. Agher, D. Charron, H. Benalia, N. Dupin, J.C. Piette, M.O. Bitker, and V. Calvez. 2000. Outcome of kidney transplant recipients with previous human herpesvirus-8 infection. *Transplantation* 69:1776-1779.

Friborg, J., Jr., W. Kong, M.O. Hottiger, and G.J. Nabel. 1999. p53 inhibition by the LANA protein of KSHV protects against cell death. *Nature* 402:889-894.

Fujimuro, M., F.Y. Wu, C. ApRhys, H. Kajumbula, D.B. Young, G.S. Hayward, and S.D. Hayward. 2003. A novel viral mechanism for dysregulation of beta-catenin in Kaposi's sarcoma-associated herpesvirus latency. *Nat Med* 9:300-306.

Fuld, S., C. Cunningham, K. Klucher, A.J. Davison, and D.J. Blackbourn. 2006. Inhibition of interferon signaling by the Kaposi's sarcoma-associated herpesvirus full-length viral interferon regulatory factor 2 protein. *J Virol* 80:3092-3097.

Ganem, D. 2007. Kaposi’s sarcoma-associated herpesvirus. In Fields virology, 5th ed. S.E. Straus, editor Lippincott Williams & Wilkins, Philadelphia, PA. p. 2875–2888.

Gao, S.J., L. Kingsley, M. Li, W. Zheng, C. Parravicini, J. Ziegler, R. Newton, C.R. Rinaldo, A. Saah, J. Phair, R. Detels, Y. Chang, and P.S. Moore. 1996. KSHV antibodies among Americans, Italians and Ugandans with and without Kaposi's sarcoma. *Nat Med* 2:925-928.

Geijtenbeek, T.B., A. Engering, and Y. Van Kooyk. 2002. DC-SIGN, a C-type lectin on dendritic cells that unveils many aspects of dendritic cell biology. *J Leukoc Biol* 71:921-931.

Geraminejad, P., O. Memar, I. Aronson, P.L. Rady, U. Hengge, and S.K. Tyring. 2002. Kaposi's sarcoma and other manifestations of human herpesvirus 8. *Journal of the American Academy of Dermatology* 47:641-655.

Glaunsinger, B., and D. Ganem. 2004. Highly selective escape from KSHV-mediated host mRNA shutoff and its implications for viral pathogenesis. *J Exp Med* 200:391-398.

Glaunsinger, B., and D. Ganem. 2004. Lytic KSHV infection inhibits host gene expression by accelerating global mRNA turnover. *Mol Cell* 13:713-723.

Goudsmit, J., N. Renwick, N.H. Dukers, R.A. Coutinho, S. Heisterkamp, M. Bakker, T.F. Schulz, M. Cornelissen, and G.J. Weverling. 2000. Human herpesvirus 8 infections in the Amsterdam Cohort Studies (1984-1997): analysis of seroconversions to ORF65 and ORF73. *Proc Natl Acad Sci U S A* 97:4838-4843.

Gourley, T.S., E.J. Wherry, D. Masopust, and R. Ahmed. 2004. Generation and maintenance of immunological memory. *Semin Immunol* 16:323-333.

Green, D.R., N. Droin, and M. Pinkoski. 2003. Activation-induced cell death in T cells. *Immunol Rev* 193:70-81.

Gromme, M., F.G. Uytdehaag, H. Janssen, J. Calafat, R.S. van Binnendijk, M.J. Kenter, A. Tulp, D. Verwoerd, and J. Neefjes. 1999. Recycling MHC class I molecules and endosomal peptide loading. *Proc Natl Acad Sci U S A* 96:10326-10331.

Grossmann, C., S. Podgrabinska, M. Skobe, and D. Ganem. 2006. Activation of NF-kappaB by the latent vFLIP gene of Kaposi's sarcoma-associated herpesvirus is required for the spindle shape of virus-infected endothelial cells and contributes to their proinflammatory phenotype. *J Virol* 80:7179-7185.

Grundhoff, A., and D. Ganem. 2001. Mechanisms governing expression of the v-FLIP gene of Kaposi's sarcoma-associated herpesvirus. *J Virol* 75:1857-1863.

Guasparri, I., S.A. Keller, and E. Cesarman. 2004. KSHV vFLIP is essential for the survival of infected lymphoma cells. *J Exp Med* 199:993-1003.

Guggemoos, S., D. Hangel, S. Hamm, A. Heit, S. Bauer, and H. Adler. 2008. TLR9 contributes to antiviral immunity during gammaherpesvirus infection. *J Immunol* 180:438-443.

Guihot, A., L.J. Couderc, F. Agbalika, L. Galicier, P. Bossi, E. Rivaud, A. Scherrer, D. Zucman, C. Katlama, and E. Oksenhendler. 2005. Pulmonary manifestations of multicentric Castleman's disease in HIV infection: a clinical, biological and radiological study. *Eur Respir J* 26:118-125.

Guihot, A., N. Dupin, A.G. Marcelin, I. Gorin, A.S. Bedin, P. Bossi, L. Galicier, E. Oksenhendler, B. Autran, and G. Carcelain. 2006. Low T cell responses to human herpesvirus 8 in patients with AIDS-related and classic Kaposi sarcoma. *J Infect Dis* 194:1078-1088.

Guihot, A., E. Oksenhendler, L. Galicier, A.G. Marcelin, L. Papagno, A.S. Bedin, F. Agbalika, N. Dupin, J. Cadranel, B. Autran, and G. Carcelain. 2008. Multicentric Castleman disease is associated with polyfunctional effector memory HHV-8-specific CD8+ T cells. *Blood* 111:1387-1395.

Haigh, T.A., X. Lin, H. Jia, E.P. Hui, A.T. Chan, A.B. Rickinson, and G.S. Taylor. 2008. EBV latent membrane proteins (LMPs) 1 and 2 as immunotherapeutic targets: LMP-specific CD4+ cytotoxic T cell recognition of EBV-transformed B cell lines. *J Immunol* 180:1643-1654.

Hammer, G.E., F. Gonzalez, E. James, H. Nolla, and N. Shastri. 2007. In the absence of aminopeptidase ERAAP, MHC class I molecules present many unstable and highly immunogenic peptides. *Nat Immunol* 8:101-108.

Harton, J.A., and J.P. Ting. 2000. Class II transactivator: mastering the art of major histocompatibility complex expression. *Mol Cell Biol* 20:6185-6194.

Hassman, L.M., T.J. Ellison, and D.H. Kedes. 2011. KSHV infects a subset of human tonsillar B cells, driving proliferation and plasmablast differentiation. *J Clin Invest* 121:752-768.

Hegde, N.R., R.A. Tomazin, T.W. Wisner, C. Dunn, J.M. Boname, D.M. Lewinsohn, and D.C. Johnson. 2002. Inhibition of HLA-DR assembly, transport, and loading by human cytomegalovirus glycoprotein US3: a novel mechanism for evading major histocompatibility complex class II antigen presentation. *J Virol* 76:10929-10941.

Hiltbold, E.M., and P.A. Roche. 2002. Trafficking of MHC class II molecules in the late secretory pathway. *Curr Opin Immunol* 14:30-35.

Hirata, Y., A.H. Broquet, L. Menchen, and M.F. Kagnoff. 2007. Activation of innate immune defense mechanisms by signaling through RIG-I/IPS-1 in intestinal epithelial cells. *J Immunol* 179:5425-5432.

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* 195:893-905.

Hogquist, K.A., S.C. Jameson, W.R. Heath, J.L. Howard, M.J. Bevan, and F.R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell* 76:17-27.

Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi. 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* 434:772-777.

Hsieh, C.S., P. deRoos, K. Honey, C. Beers, and A.Y. Rudensky. 2002. A role for cathepsin L and cathepsin S in peptide generation for MHC class II presentation. *J Immunol* 168:2618-2625.

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* 74:5300-5309.

Jager, A., and V.K. Kuchroo. 2010. Effector and regulatory T-cell subsets in autoimmunity and tissue inflammation. *Scand J Immunol* 72:173-184.

Jensen, P.E. 1998. Antigen processing: HLA-DO--a hitchhiking inhibitor of HLA-DM. *Curr Biol* 8:R128-131.

Joo, C.H., Y.C. Shin, M. Gack, L. Wu, D. Levy, and J.U. Jung. 2007. Inhibition of interferon regulatory factor 7 (IRF7)-mediated interferon signal transduction by the Kaposi's sarcoma-associated herpesvirus viral IRF homolog vIRF3. *J Virol* 81:8282-8292.

Kappler, J.W., N. Roehm, and P. Marrack. 1987. T cell tolerance by clonal elimination in the thymus. *Cell* 49:273-280.

Kaushik, S., A.C. Massey, N. Mizushima, and A.M. Cuervo. 2008. Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. *Mol Biol Cell* 19:2179-2192.

Kellam, P., C. Boshoff, D. Whitby, S. Matthews, R.A. Weiss, and S.J. Talbot. 1997. Identification of a major latent nuclear antigen, LNA-1, in the human herpesvirus 8 genome. *J Hum Virol* 1:19-29.

Kelly, G.L., H.M. Long, J. Stylianou, W.A. Thomas, A. Leese, A.I. Bell, G.W. Bornkamm, J. Mautner, A.B. Rickinson, and M. Rowe. 2009. An Epstein-Barr virus anti-apoptotic protein constitutively expressed in transformed cells and implicated in burkitt lymphomagenesis: the Wp/BHRF1 link. *PLoS Pathog* 5:e1000341.

Khanna, R., S.R. Burrows, M.G. Kurilla, C.A. Jacob, I.S. Misko, T.B. Sculley, E. Kieff, and D.J. Moss. 1992. Localization of Epstein-Barr virus cytotoxic T cell epitopes using recombinant vaccinia: implications for vaccine development. *J Exp Med* 176:169-176.

Khanna, R., S.R. Burrows, D.J. Moss, and S.L. Silins. 1996. Peptide transporter (TAP-1 and TAP-2)-independent endogenous processing of Epstein-Barr virus (EBV) latent membrane protein 2A: implications for cytotoxic T-lymphocyte control of EBV-associated malignancies. *J Virol* 70:5357-5362.

Khanna, R., S.R. Burrows, P.M. Steigerwald-Mullen, S.A. Thomson, M.G. Kurilla, and D.J. Moss. 1995. Isolation of cytotoxic T lymphocytes from healthy seropositive individuals specific for peptide epitopes from Epstein-Barr virus nuclear antigen 1: implications for viral persistence and tumor surveillance. *Virology* 214:633-637.

Khanna, R., S.R. Burrows, S.A. Thomson, D.J. Moss, P. Cresswell, L.M. Poulsen, and L. Cooper. 1997. Class I processing-defective Burkitt's lymphoma cells are recognized efficiently by CD4+ EBV-specific CTLs. *J Immunol* 158:3619-3625.

Kimball, L.E., C. Casper, D.M. Koelle, R. Morrow, L. Corey, and J. Vieira. 2004. Reduced levels of neutralizing antibodies to Kaposi sarcoma-associated herpesvirus in persons with a history of Kaposi sarcoma. *J Infect Dis* 189:2016-2022.

Korn, T., E. Bettelli, W. Gao, A. Awasthi, A. Jager, T.B. Strom, M. Oukka, and V.K. Kuchroo. 2007. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448:484-487.

Krishnan, H.H., P.P. Naranatt, M.S. Smith, L. Zeng, C. Bloomer, and B. Chandran. 2004. Concurrent expression of latent and a limited number of lytic genes with immune modulation and antiapoptotic function by Kaposi's sarcoma-associated herpesvirus early during infection of primary endothelial and fibroblast cells and subsequent decline of lytic gene expression. *J Virol* 78:3601-3620.

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* 412:357-365.

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* 81:8225-8235.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Lagos, D., M.W. Trotter, R.J. Vart, H.W. Wang, N.C. Matthews, A. Hansen, O. Flore, F. Gotch, and C. Boshoff. 2007. Kaposi sarcoma herpesvirus-encoded vFLIP and vIRF1 regulate antigen presentation in lymphatic endothelial cells. *Blood* 109:1550-1558.

Lagos, D., R.J. Vart, F. Gratrix, S.J. Westrop, V. Emuss, P.P. Wong, R. Robey, N. Imami, M. Bower, F. Gotch, and C. Boshoff. 2008. Toll-like receptor 4 mediates innate immunity to Kaposi sarcoma herpesvirus. *Cell Host Microbe* 4:470-483.

Lagunoff, M., J. Bechtel, E. Venetsanakos, A.M. Roy, N. Abbey, B. Herndier, M. McMahon, and D. Ganem. 2002. De novo infection and serial transmission of Kaposi's sarcoma-associated herpesvirus in cultured endothelial cells. *J Virol* 76:2440-2448.

Lallemand, F., N. Desire, W. Rozenbaum, J.C. Nicolas, and V. Marechal. 2000. Quantitative analysis of human herpesvirus 8 viral load using a real-time PCR assay. *J Clin Microbiol* 38:1404-1408.

Lalvani, A., T. Dong, G. Ogg, A.A. Patham, H. Newell, A.V. Hill, A.J. McMichael, and S. Rowland-Jones. 1997. Optimization of a peptide-based protocol employing IL-7 for in vitro restimulation of human cytotoxic T lymphocyte precursors. *J Immunol Methods* 210:65-77.

Lambert, M., M. Gannage, A. Karras, M. Abel, C. Legendre, D. Kerob, F. Agbalika, P.-M. Girard, C. Lebbe, and S. Caillat-Zucman. 2006. Differences in the frequency and function of HHV8-specific CD8 T cells between asymptomatic HHV8 infection and Kaposi sarcoma. *Blood* 108:3871-3880.

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* 194:1053-1068.

Lebbe, C., P. de Cremoux, G. Millot, M.P. Podgorniak, O. Verola, R. Berger, P. Morel, and F. Calvo. 1997. Characterization of in vitro culture of HIV-negative Kaposi's sarcoma-derived cells. In vitro responses to alfa interferon. *Arch Dermatol Res* 289:421-428.

Lechler, R., G. Aichinger, and L. Lightstone. 1996. The endogenous pathway of MHC class II antigen presentation. *Immunol Rev* 151:51-79.

Ledergerber, B., A. Telenti, and M. Egger. 1999. Risk of HIV related Kaposi's sarcoma and non-Hodgkin's lymphoma with potent antiretroviral therapy: prospective cohort study. Swiss HIV Cohort Study. *BMJ* 319:23-24.

Lee, J.S., Q. Li, J.Y. Lee, S.H. Lee, J.H. Jeong, H.R. Lee, H. Chang, F.C. Zhou, S.J. Gao, C. Liang, and J.U. Jung. 2009. FLIP-mediated autophagy regulation in cell death control. *Nat Cell Biol* 11:1355-1362.

Leen, A., P. Meij, I. Redchenko, J. Middeldorp, E. Bloemena, A. Rickinson, and N. Blake. 2001. Differential immunogenicity of Epstein-Barr virus latent-cycle proteins for human CD4(+) T-helper 1 responses. *J Virol* 75:8649-8659.

Lefort, S., A. Soucy-Faulkner, N. Grandvaux, and L. Flamand. 2007. Binding of Kaposi's sarcoma-associated herpesvirus K-bZIP to interferon-responsive factor 3 elements modulates antiviral gene expression. *J Virol* 81:10950-10960.

Lennon-Dumenil, A.M., A.H. Bakker, P. Wolf-Bryant, H.L. Ploegh, and C. Lagaudriere-Gesbert. 2002. A closer look at proteolysis and MHC-class-II-restricted antigen presentation. *Curr Opin Immunol* 14:15-21.

Lepone, L., G. Rappocciolo, E. Knowlton, M. Jais, P. Piazza, F.J. Jenkins, and C.R. Rinaldo. 2010. Monofunctional and polyfunctional CD8+ T cell responses to human herpesvirus 8 lytic and latency proteins. *Clin Vaccine Immunol* 17:1507-1516.

Leung, C.S., T.A. Haigh, L.K. Mackay, A.B. Rickinson, and G.S. Taylor. 2010. Nuclear location of an endogenously expressed antigen, EBNA1, restricts access to macroautophagy and the range of CD4 epitope display. *Proc Natl Acad Sci U S A* 107:2165-2170.

Levitskaya, J., M. Coram, V. Levitsky, S. Imreh, P.M. Steigerwald-Mullen, G. Klein, M.G. Kurilla, and M.G. Masucci. 1995. Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* 375:685-688.

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* 182:1799-1809.

Li, Q., R. Means, S. Lang, and J.U. Jung. 2007. Downregulation of gamma interferon receptor 1 by Kaposi's sarcoma-associated herpesvirus K3 and K5. *J Virol* 81:2117-2127.

Li, Y., L.X. Wang, G. Yang, F. Hao, W.J. Urba, and H.M. Hu. 2008. Efficient cross-presentation depends on autophagy in tumor cells. *Cancer Res* 68:6889-6895.

Lich, J.D., J.F. Elliott, and J.S. Blum. 2000. Cytoplasmic processing is a prerequisite for presentation of an endogenous antigen by major histocompatibility complex class II proteins. *J Exp Med* 191:1513-1524.

Lieberman, J. 2003. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* 3:361-370.

Liljedahl, M., O. Winqvist, C.D. Surh, P. Wong, K. Ngo, L. Teyton, P.A. Peterson, A. Brunmark, A.Y. Rudensky, W.P. Fung-Leung, and L. Karlsson. 1998. Altered antigen presentation in mice lacking H2-O. *Immunity* 8:233-243.

Lim, C.S., B.T. Seet, R.J. Ingham, G. Gish, L. Matskova, G. Winberg, I. Ernberg, and T. Pawson. 2007. The K15 protein of Kaposi's sarcoma-associated herpesvirus recruits the endocytic regulator intersectin 2 through a selective SH3 domain interaction. *Biochemistry* 46:9874-9885.

Liu, L., M.T. Eby, N. Rathore, S.K. Sinha, A. Kumar, and P.M. Chaudhary. 2002. The human herpes virus 8-encoded viral FLICE inhibitory protein physically associates with and persistently activates the Ikappa B kinase complex. *J Biol Chem* 277:13745-13751.

Liu, W.J., F. Gao, K.N. Zhao, W. Zhao, G.J. Fernando, R. Thomas, and I.H. Frazer. 2002. Codon modified human papillomavirus type 16 E7 DNA vaccine enhances cytotoxic T-lymphocyte induction and anti-tumour activity. *Virology* 301:43-52.

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* 79:4896-4907.

Low, W., M. Harries, H. Ye, M.Q. Du, C. Boshoff, and M. Collins. 2001. Internal ribosome entry site regulates translation of Kaposi's sarcoma-associated herpesvirus FLICE inhibitory protein. *J Virol* 75:2938-2945.

Lukac, D.M., J.R. Kirshner, and D. Ganem. 1999. Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells. *J Virol* 73:9348-9361.

Lunemann, J.D., and C. Munz. 2009. Autophagy in CD4+ T-cell immunity and tolerance. *Cell Death Differ* 16:79-86.

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:e1000699.

Madden, D.R. 1995. The three-dimensional structure of peptide-MHC complexes. *Annu Rev Immunol* 13:587-622.

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* 165:5729-5737.

Mallery, D.L., W.A. McEwan, S.R. Bidgood, G.J. Towers, C.M. Johnson, and L.C. James. 2010. Antibodies mediate intracellular immunity through tripartite motif-containing 21 (TRIM21). *Proc Natl Acad Sci U S A* 107:19985-19990.

Malnati, M.S., M. Marti, T. LaVaute, D. Jaraquemada, W. Biddison, R. DeMars, and E.O. Long. 1992. Processing pathways for presentation of cytosolic antigen to MHC class II-restricted T cells. *Nature* 357:702-704.

Marks, M.S., J.S. Blum, and P. Cresswell. 1990. Invariant chain trimers are sequestered in the rough endoplasmic reticulum in the absence of association with HLA class II antigens. *J Cell Biol* 111:839-855.

Masood, R., E. Cesarman, D.L. Smith, P.S. Gill, and O. Flore. 2002. Human herpesvirus-8-transformed endothelial cells have functionally activated vascular endothelial growth factor/vascular endothelial growth factor receptor. *Am J Pathol* 160:23-29.

Matta, H., and P.M. Chaudhary. 2004. Activation of alternative NF-kappa B pathway by human herpes virus 8-encoded Fas-associated death domain-like IL-1 beta-converting enzyme inhibitory protein (vFLIP). *Proc Natl Acad Sci U S A* 101:9399-9404.

Matthews, N.C., M.R. Goodier, R.C. Robey, M. Bower, and F.M. Gotch. 2011. Killing of Kaposi's sarcoma-associated herpesvirus-infected fibroblasts during latent infection by activated natural killer cells. *Eur J Immunol* 41:1958-1968.

Mautner, J., D. Pich, F. Nimmerjahn, S. Milosevic, D. Adhikary, H. Christoph, K. Witter, G.W. Bornkamm, W. Hammerschmidt, and U. Behrends. 2004. Epstein-Barr virus nuclear antigen 1 evades direct immune recognition by CD4+ T helper cells. *Eur J Immunol* 34:2500-2509.

McCormick, C., and D. Ganem. 2005. The kaposin B protein of KSHV activates the p38/MK2 pathway and stabilizes cytokine mRNAs. *Science* 307:739-741.

Mesri, E.A., E. Cesarman, and C. Boshoff. 2010. Kaposi's sarcoma and its associated herpesvirus. *Nat Rev Cancer* 10:707-719.

Miller, D.M., B.M. Rahill, J.M. Boss, M.D. Lairmore, J.E. Durbin, J.W. Waldman, and D.D. Sedmak. 1998. Human cytomegalovirus inhibits major histocompatibility complex class II expression by disruption of the Jak/Stat pathway. *J Exp Med* 187:675-683.

Moore, R.D., and R.E. Chaisson. 1996. Natural history of opportunistic disease in an HIV-infected urban clinical cohort. *Ann Intern Med* 124:633-642.

Mosmann, T.R. 1991. Regulation of immune responses by T cells with different cytokine secretion phenotypes: role of a new cytokine, cytokine synthesis inhibitory factor (IL10). *Int Arch Allergy Appl Immunol* 94:110-115.

Mosmann, T.R., and S. Sad. 1996. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 17:138-146.

Munoz-Fernandez, M.A., M.A. Fernandez, and M. Fresno. 1992. Synergism between tumor necrosis factor-alpha and interferon-gamma on macrophage activation for the killing of intracellular Trypanosoma cruzi through a nitric oxide-dependent mechanism. *Eur J Immunol* 22:301-307.

Muralidhar, S., A.M. Pumfery, M. Hassani, M.R. Sadaie, M. Kishishita, J.N. Brady, J. Doniger, P. Medveczky, and L.J. Rosenthal. 1998. Identification of kaposin (open reading frame K12) as a human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) transforming gene. *J Virol* 72:4980-4988.

Murray, R.J., M.G. Kurilla, J.M. Brooks, W.A. Thomas, M. Rowe, E. Kieff, and A.B. Rickinson. 1992. Identification of target antigens for the human cytotoxic T cell response to Epstein-Barr virus (EBV): implications for the immune control of EBV-positive malignancies. *J Exp Med* 176:157-168.

Myoung, J., and D. Ganem. 2011. Active lytic infection of human primary tonsillar B cells by KSHV and its noncytolytic control by activated CD4+ T cells. *J Clin Invest* 121:1130-1140.

Myoung, J., and D. Ganem. 2011. Infection of Lymphoblastoid Cell Lines by Kaposi's Sarcoma-Associated Herpesvirus: Critical Role of Cell-Associated Virus. *J Virol* 85:9767-9777.

Myoung, J., and D. Ganem. 2011. Infection of primary human tonsillar lymphoid cells by KSHV reveals frequent but abortive infection of T cells. *Virology* 413:1-11.

Neefjes, J.J., F. Momburg, and G.J. Hammerling. 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science* 261:769-771.

Ngumbela, K.C., K.P. Ryan, R. Sivamurthy, M.A. Brockman, R.T. Gandhi, N. Bhardwaj, and D.G. Kavanagh. 2008. Quantitative effect of suboptimal codon usage on translational efficiency of mRNA encoding HIV-1 gag in intact T cells. *PLoS One* 3:e2356.

Nikiforow, S., K. Bottomly, G. Miller, and C. Munz. 2003. Cytolytic CD4(+)-T-cell clones reactive to EBNA1 inhibit Epstein-Barr virus-induced B-cell proliferation. *J Virol* 77:12088-12104.

Nimmerjahn, F., S. Milosevic, U. Behrends, E.M. Jaffee, D.M. Pardoll, G.W. Bornkamm, and J. Mautner. 2003. Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. *Eur J Immunol* 33:1250-1259.

Njie, R., A.I. Bell, H. Jia, D. Croom-Carter, S. Chaganti, A.D. Hislop, H. Whittle, and A.B. Rickinson. 2009. The effects of acute malaria on Epstein-Barr virus (EBV) load and EBV-specific T cell immunity in Gambian children. *J Infect Dis* 199:31-38.

Offermann, M.K. 2007. Kaposi sarcoma herpesvirus-encoded interferon regulator factors. *Curr Top Microbiol Immunol* 312:185-209.

Ojala, P.M., M. Tiainen, P. Salven, T. Veikkola, E. Castanos-Velez, R. Sarid, P. Biberfeld, and T.P. Makela. 1999. Kaposi's sarcoma-associated herpesvirus-encoded v-cyclin triggers apoptosis in cells with high levels of cyclin-dependent kinase 6. *Cancer Res* 59:4984-4989.

Ojala, P.M., K. Yamamoto, E. Castanos-Velez, P. Biberfeld, S.J. Korsmeyer, and T.P. Makela. 2000. The apoptotic v-cyclin-CDK6 complex phosphorylates and inactivates Bcl-2. *Nat Cell Biol* 2:819-825.

Omiya, R., C. Buteau, H. Kobayashi, C.V. Paya, and E. Celis. 2002. Inhibition of EBV-induced lymphoproliferation by CD4(+) T cells specific for an MHC class II promiscuous epitope. *J Immunol* 169:2172-2179.

Osborne, J., P.S. Moore, and Y. Chang. 1999. KSHV-encoded viral IL-6 activates multiple human IL-6 signaling pathways. *Hum Immunol* 60:921-927.

Osmond, D.H., S. Buchbinder, A. Cheng, A. Graves, E. Vittinghoff, C.K. Cossen, B. Forghani, and J.N. Martin. 2002. Prevalence of Kaposi Sarcoma-Associated Herpesvirus Infection in Homosexual Men at Beginning of and During the HIV Epidemic. *JAMA* 287:221-225.

Paludan, C., K. Bickham, S. Nikiforow, M.L. Tsang, K. Goodman, W.A. Hanekom, J.F. Fonteneau, S. Stevanovic, and C. Munz. 2002. Epstein-Barr nuclear antigen 1-specific CD4(+) Th1 cells kill Burkitt's lymphoma cells. *J Immunol* 169:1593-1603.

Paludan, C., D. Schmid, M. Landthaler, M. Vockerodt, D. Kube, T. Tuschl, and C. Munz. 2005. Endogenous MHC class II processing of a viral nuclear antigen after autophagy. *Science* 307:593-596.

Parham, P., S.G. Marsh, L.D. Butler. 2000. THE HLA FactsBook. ACADEMIC PRESS, London, UK. 413 pp.

Park, H., Z. Li, X.O. Yang, S.H. Chang, R. Nurieva, Y.H. Wang, Y. Wang, L. Hood, Z. Zhu, Q. Tian, and C. Dong. 2005. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133-1141.

Parker, D.C. 1993. T cell-dependent B cell activation. *Annu Rev Immunol* 11:331-360.

Parravicini, C., M. Corbellino, M. Paulli, U. Magrini, M. Lazzarino, P.S. Moore, and Y. Chang. 1997. Expression of a virus-derived cytokine, KSHV vIL-6, in HIV-seronegative Castleman's disease. *Am J Pathol* 151:1517-1522.

Pearce, M., S. Matsumura, and A.C. Wilson. 2005. Transcripts encoding K12, v-FLIP, v-cyclin, and the microRNA cluster of Kaposi's sarcoma-associated herpesvirus originate from a common promoter. *J Virol* 79:14457-14464.

Peck, A., and E.D. Mellins. 2010. Plasticity of T-cell phenotype and function: the T helper type 17 example. *Immunology* 129:147-153.

Perkins, E.M., D. Anacker, A. Davis, V. Sankar, R.F. Ambinder, and P. Desai. 2008. Small capsid protein pORF65 is essential for assembly of Kaposi's sarcoma-associated herpesvirus capsids. *J Virol* 82:7201-7211.

Piolot, T., M. Tramier, M. Coppey, J.C. Nicolas, and V. Marechal. 2001. Close but distinct regions of human herpesvirus 8 latency-associated nuclear antigen 1 are responsible for nuclear targeting and binding to human mitotic chromosomes. *J Virol* 75:3948-3959.

Pluger, E.B., M. Boes, C. Alfonso, C.J. Schroter, H. Kalbacher, H.L. Ploegh, and C. Driessen. 2002. Specific role for cathepsin S in the generation of antigenic peptides in vivo. *Eur J Immunol* 32:467-476.

Polson, A.G., L. Huang, D.M. Lukac, J.D. Blethrow, D.O. Morgan, A.L. Burlingame, and D. Ganem. 2001. Kaposi's sarcoma-associated herpesvirus K-bZIP protein is phosphorylated by cyclin-dependent kinases. *J Virol* 75:3175-3184.

Powles, T., J. Stebbing, A. Bazeos, E. Hatzimichael, S. Mandalia, M. Nelson, B. Gazzard, and M. Bower. 2009. The role of immune suppression and HHV-8 in the increasing incidence of HIV-associated multicentric Castleman's disease. *Ann Oncol* 20:775-779.

Pu, Z., S.B. Lovitch, E.K. Bikoff, and E.R. Unanue. 2004. T cells distinguish MHC-peptide complexes formed in separate vesicles and edited by H2-DM. *Immunity* 20:467-476.

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* 201:349-360.

Pulendran, B., K. Palucka, and J. Banchereau. 2001. Sensing pathogens and tuning immune responses. *Science* 293:253-256.

Qian, S.B., E. Reits, J. Neefjes, J.M. Deslich, J.R. Bennink, and J.W. Yewdell. 2006. Tight linkage between translation and MHC class I peptide ligand generation implies specialized antigen processing for defective ribosomal products. *J Immunol* 177:227-233.

Qu, L., F. Jenkins, and D.J. Triulzi. 2010. Human herpesvirus 8 genomes and seroprevalence in United States blood donors. *Transfusion* 50:1050-1056.

Qunibi, W., M. Akhtar, K. Sheth, H.E. Ginn, O. Al-Furayh, E.B. DeVol, and S. Taher. 1988. Kaposi's sarcoma: the most common tumor after renal transplantation in Saudi Arabia. *Am J Med* 84:225-232.

Raab, M.S., J.C. Albrecht, A. Birkmann, S. Yaguboglu, D. Lang, B. Fleckenstein, and F. Neipel. 1998. The immunogenic glycoprotein gp35-37 of human herpesvirus 8 is encoded by open reading frame K8.1. *J Virol* 72:6725-6731.

Radcliffe, C.M., G. Diedrich, D.J. Harvey, R.A. Dwek, P. Cresswell, and P.M. Rudd. 2002. Identification of specific glycoforms of major histocompatibility complex class I heavy chains suggests that class I peptide loading is an adaptation of the quality control pathway involving calreticulin and ERp57. *J Biol Chem* 277:46415-46423.

Radkov, S.A., P. Kellam, and C. Boshoff. 2000. The latent nuclear antigen of Kaposi sarcoma-associated herpesvirus targets the retinoblastoma-E2F pathway and with the oncogene Hras transforms primary rat cells. *Nat Med* 6:1121-1127.

Rammensee, H., J. Bachmann, N.P. Emmerich, O.A. Bachor, and S. Stevanovic. 1999. SYFPEITHI: database for MHC ligands and peptide motifs. *Immunogenetics* 50:213-219.

Randall, R.E., and S. Goodbourn. 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. *J Gen Virol* 89:1-47.

Rappocciolo, G., H.R. Hensler, M. Jais, T.A. Reinhart, A. Pegu, F.J. Jenkins, and C.R. Rinaldo. 2008. Human herpesvirus 8 infects and replicates in primary cultures of activated B lymphocytes through DC-SIGN. *J Virol* 82:4793-4806.

Reid, P.A., and C. Watts. 1990. Cycling of cell-surface MHC glycoproteins through primaquine-sensitive intracellular compartments. *Nature* 346:655-657.

Reis e Sousa, C. 2004. Activation of dendritic cells: translating innate into adaptive immunity. *Curr Opin Immunol* 16:21-25.

Reith, W., S. LeibundGut-Landmann, and J.M. Waldburger. 2005. Regulation of MHC class II gene expression by the class II transactivator. *Nat Rev Immunol* 5:793-806.

Renne, R., C. Barry, D. Dittmer, N. Compitello, P.O. Brown, and D. Ganem. 2001. Modulation of cellular and viral gene expression by the latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus. *J Virol* 75:458-468.

Renne, R., D. Blackbourn, D. Whitby, J. Levy, and D. Ganem. 1998. Limited transmission of Kaposi's sarcoma-associated herpesvirus in cultured cells. *J Virol* 72:5182-5188.

Renne, R., W. Zhong, B. Herndier, M. McGrath, N. Abbey, D. Kedes, and D. Ganem. 1996. Lytic growth of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in culture. *Nat Med* 2:342-346.

Ressing, M.E., D. Horst, B.D. Griffin, J. Tellam, J. Zuo, R. Khanna, M. Rowe, and E.J. Wiertz. 2008. Epstein-Barr virus evasion of CD8(+) and CD4(+) T cell immunity via concerted actions of multiple gene products. *Semin Cancer Biol* 18:397-408.

Ressing, M.E., and E.J. Wiertz. 2008. Manipulation of the immune response by Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus: consequences for tumor development. *Semin Cancer Biol* 18:379-380.

Rezaee, S.A., C. Cunningham, A.J. Davison, and D.J. Blackbourn. 2006. Kaposi's sarcoma-associated herpesvirus immune modulation: an overview. *J Gen Virol* 87:1781-1804.

Rezaee, S.A., J.A. Gracie, I.B. McInnes, and D.J. Blackbourn. 2005. Inhibition of neutrophil function by the Kaposi's sarcoma-associated herpesvirus vOX2 protein. *AIDS* 19:1907-1910.

Ribechini, E., C. Fortini, M. Marastoni, S. Traniello, S. Spisani, P. Monini, and R. Gavioli. 2006. Identification of CD8+ T Cell Epitopes within Lytic Antigens of Human Herpes Virus 8. *J Immunol* 176:923-930.

Riberdy, J.M., J.R. Newcomb, M.J. Surman, J.A. Barbosa, and P. Cresswell. 1992. HLA-DR molecules from an antigen-processing mutant cell line are associated with invariant chain peptides. *Nature* 360:474-477.

Rivas, C., A.E. Thlick, C. Parravicini, P.S. Moore, and Y. Chang. 2001. Kaposi's sarcoma-associated herpesvirus LANA2 is a B-cell-specific latent viral protein that inhibits p53. *J Virol* 75:429-438.

Robertson, K.A., E.J. Usherwood, and A.A. Nash. 2001. Regression of a murine gammaherpesvirus 68-positive b-cell lymphoma mediated by CD4 T lymphocytes. *J Virol* 75:3480-3482.

Robey, R.C., D. Lagos, F. Gratrix, S. Henderson, N.C. Matthews, R.J. Vart, M. Bower, C. Boshoff, and F.M. Gotch. 2009. The CD8 and CD4 T-cell response against Kaposi's sarcoma-associated herpesvirus is skewed towards early and late lytic antigens. *PLoS One* 4:e5890.

Robinson, J.H., and A.A. Delvig. 2002. Diversity in MHC class II antigen presentation. *Immunology* 105:252-262.

Roncarolo, M.G., and M.K. Levings. 2000. The role of different subsets of T regulatory cells in controlling autoimmunity. *Curr Opin Immunol* 12:676-683.

Rooney, C.M., M. Rowe, L.E. Wallace, and A.B. Rickinson. 1985. Epstein-Barr virus-positive Burkitt's lymphoma cells not recognized by virus-specific T-cell surveillance. *Nature* 317:629-631.

Roost, H.P., M.F. Bachmann, A. Haag, U. Kalinke, V. Pliska, H. Hengartner, and R.M. Zinkernagel. 1995. Early high-affinity neutralizing anti-viral IgG responses without further overall improvements of affinity. *Proc Natl Acad Sci U S A* 92:1257-1261.

Rosenberg, E.S., J.M. Billingsley, A.M. Caliendo, S.L. Boswell, P.E. Sax, S.A. Kalams, and B.D. Walker. 1997. Vigorous HIV-1-specific CD4+ T cell responses associated with control of viremia. *Science* 278:1447-1450.

Rowe, H.M., L. Lopes, N. Brown, S. Efklidou, T. Smallie, S. Karrar, P.M. Kaye, and M.K. Collins. 2009. Expression of vFLIP in a lentiviral vaccine vector activates NF-(kappa)B, matures dendritic cells, and increases CD8+ T-cell responses. *J Virol* 83:1555-1562.

Rudensky, A., P. Preston-Hurlburt, S.C. Hong, A. Barlow, and C.A. Janeway, Jr. 1991. Sequence analysis of peptides bound to MHC class II molecules. *Nature* 353:622-627.

Russo, J.J., R.A. Bohenzky, M.C. Chien, J. Chen, M. Yan, D. Maddalena, J.P. Parry, D. Peruzzi, I.S. Edelman, Y. Chang, and P.S. Moore. 1996. Nucleotide sequence of the Kaposi sarcoma-associated herpesvirus (HHV8). *Proc Natl Acad Sci U S A* 93:14862-14867.

Sadler, R., L. Wu, B. Forghani, R. Renne, W. Zhong, B. Herndier, and D. Ganem. 1999. A complex translational program generates multiple novel proteins from the latently expressed kaposin (K12) locus of Kaposi's sarcoma-associated herpesvirus. *J Virol* 73:5722-5730.

Sakaguchi, S. 2000. Regulatory T cells: key controllers of immunologic self-tolerance. *Cell* 101:455-458.

Sallusto, F., D. Lenig, R. Forster, M. Lipp, and A. Lanzavecchia. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.

Sanderson, S., K. Frauwirth, and N. Shastri. 1995. Expression of endogenous peptide-major histocompatibility complex class II complexes derived from invariant chain-antigen fusion proteins. *Proc Natl Acad Sci U S A* 92:7217-7221.

Sandri-Goldin, R.M. 2001. Nuclear export of herpes virus RNA. *Curr Top Microbiol Immunol* 259:2-23.

Schifferli, J.A., and R.P. Taylor. 1989. Physiological and pathological aspects of circulating immune complexes. *Kidney Int* 35:993-1003.

Schmid, D., M. Pypaert, and C. Munz. 2007. Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes. *Immunity* 26:79-92.

Schmidt, K., E. Wies, and F. Neipel. 2011. Kaposi's Sarcoma-Associated Herpesvirus Viral Interferon Regulatory Factor 3 Inhibits Gamma Interferon and Major Histocompatibility Complex Class II Expression. *J Virol* 85:4530-4537.

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* 404:770-774.

Schulz, T.F. 2000. Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8): epidemiology and pathogenesis. *J Antimicrob Chemother* 45 Suppl T3:15-27.

Schulz, T.F. 2001. KSHV/HHV8-associated lymphoproliferations in the AIDS setting. *Eur J Cancer* 37:1217-1226.

Serada, S., M. Fujimoto, M. Mihara, N. Koike, Y. Ohsugi, S. Nomura, H. Yoshida, T. Nishikawa, F. Terabe, T. Ohkawara, T. Takahashi, B. Ripley, A. Kimura, T. Kishimoto, and T. Naka. 2008. IL-6 blockade inhibits the induction of myelin antigen-specific Th17 cells and Th1 cells in experimental autoimmune encephalomyelitis. *Proc Natl Acad Sci U S A* 105:9041-9046.

Sette, A., S. Ceman, R.T. Kubo, K. Sakaguchi, E. Appella, D.F. Hunt, T.A. Davis, H. Michel, J. Shabanowitz, R. Rudersdorf, and et al. 1992. Invariant chain peptides in most HLA-DR molecules of an antigen-processing mutant. *Science* 258:1801-1804.

Sha, W.C., C.A. Nelson, R.D. Newberry, D.M. Kranz, J.H. Russell, and D.Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature* 336:73-76.

Shastri, N., and F. Gonzalez. 1993. Endogenous generation and presentation of the ovalbumin peptide/Kb complex to T cells. *J Immunol* 150:2724-2736.

Sherman, M.A., D.A. Weber, and P.E. Jensen. 1995. DM enhances peptide binding to class II MHC by release of invariant chain-derived peptide. *Immunity* 3:197-205.

Shore, A.M., P.C. White, R.C. Hui, A. Essafi, E.W. Lam, M. Rowe, and P. Brennan. 2006. Epstein-Barr virus represses the FoxO1 transcription factor through latent membrane protein 1 and latent membrane protein 2A. *J Virol* 80:11191-11199.

Sigal, L.J., S. Crotty, R. Andino, and K.L. Rock. 1999. Cytotoxic T-cell immunity to virus-infected non-haematopoietic cells requires presentation of exogenous antigen. *Nature* 398:77-80.

Sigal, L.J., and K.L. Rock. 2000. Bone marrow-derived antigen-presenting cells are required for the generation of cytotoxic T lymphocyte responses to viruses and use transporter associated with antigen presentation (TAP)-dependent and -independent pathways of antigen presentation. *J Exp Med* 192:1143-1150.

Simpson, G.R., T.F. Schulz, D. Whitby, P.M. Cook, C. Boshoff, L. Rainbow, M.R. Howard, S.J. Gao, R.A. Bohenzky, P. Simmonds, C. Lee, A. de Ruiter, A. Hatzakis, R.S. Tedder, I.V. Weller, R.A. Weiss, and P.S. Moore. 1996. Prevalence of Kaposi's sarcoma associated herpesvirus infection measured by antibodies to recombinant capsid protein and latent immunofluorescence antigen. *Lancet* 348:1133-1138.

Soulier, J., L. Grollet, E. Oksenhendler, P. Cacoub, D. Cazals-Hatem, P. Babinet, M.F. d'Agay, J.P. Clauvel, M. Raphael, L. Degos, and et al. 1995. Kaposi's sarcoma-associated herpesvirus-like DNA sequences in multicentric Castleman's disease. *Blood* 86:1276-1280.

Spiller, O.B., L. Mark, C.E. Blue, D.G. Proctor, J.A. Aitken, A.M. Blom, and D.J. Blackbourn. 2006. Dissecting the regions of virion-associated Kaposi's sarcoma-associated herpesvirus complement control protein required for complement regulation and cell binding. *J Virol* 80:4068-4078.

Stebbing, J., C. Adams, A. Sanitt, S. Mletzko, M. Nelson, B. Gazzard, T. Newsom-Davis, and M. Bower. 2011. Plasma HHV8 DNA predicts relapse in individuals with HIV-associated multicentric Castleman disease. *Blood* 118:271-275.

Steimle, V., C.A. Siegrist, A. Mottet, B. Lisowska-Grospierre, and B. Mach. 1994. Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA. *Science* 265:106-109.

Steven, N.M., A.M. Leese, N.E. Annels, S.P. Lee, and A.B. Rickinson. 1996. Epitope focusing in the primary cytotoxic T cell response to Epstein-Barr virus and its relationship to T cell memory. *J Exp Med* 184:1801-1813.

Sullivan, S.G., H.H. Hirsch, S. Franceschi, I. Steffen, E.B. Amari, N.J. Mueller, I. Magkouras, R.J. Biggar, M. Rickenbach, and G.M. Clifford. 2010. Kaposi sarcoma herpes virus antibody response and viremia following highly active antiretroviral therapy in the Swiss HIV Cohort study. *AIDS* 24:2245-2252.

Sun, Q., H. Matta, G. Lu, and P.M. Chaudhary. 2006. Induction of IL-8 expression by human herpesvirus 8 encoded vFLIP K13 via NF-kappaB activation. *Oncogene* 25:2717-2726.

Suscovich, T.J., M. Paulose-Murphy, J.D. Harlow, Y. Chen, S.Y. Thomas, T.J. Mellott, B.D. Walker, D.T. Scadden, S. Zeichner, and C. Brander. 2004. Defective immune function of primary effusion lymphoma cells is associated with distinct KSHV gene expression profiles. *Leuk Lymphoma* 45:1223-1238.

Sutton, C.E., S.J. Lalor, C.M. Sweeney, C.F. Brereton, E.C. Lavelle, and K.H. Mills. 2009. Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17 responses and autoimmunity. *Immunity* 31:331-341.

Swanton, C., D.J. Mann, B. Fleckenstein, F. Neipel, G. Peters, and N. Jones. 1997. Herpes viral cyclin/Cdk6 complexes evade inhibition by CDK inhibitor proteins. *Nature* 390:184-187.

Tellam, J., G. Connolly, K.J. Green, J.J. Miles, D.J. Moss, S.R. Burrows, and R. Khanna. 2004. Endogenous presentation of CD8+ T cell epitopes from Epstein-Barr virus-encoded nuclear antigen 1. *J Exp Med* 199:1421-1431.

Tellam, J., M.H. Fogg, M. Rist, G. Connolly, D. Tscharke, N. Webb, L. Heslop, F. Wang, and R. Khanna. 2007. Influence of translation efficiency of homologous viral proteins on the endogenous presentation of CD8+ T cell epitopes. *J Exp Med* 204:525-532.

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* 105:9319-9324.

Tempera, I., and P.M. Lieberman. 2010. Chromatin organization of gammaherpesvirus latent genomes. *Biochim Biophys Acta* 1799:236-245.

Tewari, M.K., G. Sinnathamby, D. Rajagopal, and L.C. Eisenlohr. 2005. A cytosolic pathway for MHC class II-restricted antigen processing that is proteasome and TAP dependent. *Nat Immunol* 6:287-294.

Thien, C.B., F.D. Blystad, Y. Zhan, A.M. Lew, V. Voigt, C.E. Andoniou, and W.Y. Langdon. 2005. Loss of c-Cbl RING finger function results in high-intensity TCR signaling and thymic deletion. *EMBO J* 24:3807-3819.

Toes, R.E., A.K. Nussbaum, S. Degermann, M. Schirle, N.P. Emmerich, M. Kraft, C. Laplace, A. Zwinderman, T.P. Dick, J. Muller, B. Schonfisch, C. Schmid, H.J. Fehling, S. Stevanovic, H.G. Rammensee, and H. Schild. 2001. Discrete cleavage motifs of constitutive and immunoproteasomes revealed by quantitative analysis of cleavage products. *J Exp Med* 194:1-12.

Tomazin, R., J. Boname, N.R. Hegde, D.M. Lewinsohn, Y. Altschuler, T.R. Jones, P. Cresswell, J.A. Nelson, S.R. Riddell, and D.C. Johnson. 1999. Cytomegalovirus US2 destroys two components of the MHC class II pathway, preventing recognition by CD4+ T cells. *Nat Med* 5:1039-1043.

Tomkowicz, B., S.P. Singh, M. Cartas, and A. Srinivasan. 2002. Human herpesvirus-8 encoded Kaposin: subcellular localization using immunofluorescence and biochemical approaches. *DNA Cell Biol* 21:151-162.

Tsang, C.W., X. Lin, N.H. Gudgeon, G.S. Taylor, H. Jia, E.P. Hui, A.T. Chan, C.K. Lin, and A.B. Rickinson. 2006. CD4+ T-cell responses to Epstein-Barr virus nuclear antigen EBNA1 in Chinese populations are highly focused on novel C-terminal domain-derived epitopes. *J Virol* 80:8263-8266.

Uebel, S., and R. Tampe. 1999. Specificity of the proteasome and the TAP transporter. *Curr Opin Immunol* 11:203-208.

Uhl, M., O. Kepp, H. Jusforgues-Saklani, J.M. Vicencio, G. Kroemer, and M.L. Albert. 2009. Autophagy within the antigen donor cell facilitates efficient antigen cross-priming of virus-specific CD8+ T cells. *Cell Death Differ* 16:991-1005.

Uldrick, T.S., and D. Whitby. 2011. Update on KSHV epidemiology, Kaposi Sarcoma pathogenesis, and treatment of Kaposi Sarcoma. *Cancer Lett* 305:150-162.

Valle, A., C.E. Zuber, T. Defrance, O. Djossou, M. De Rie, and J. Banchereau. 1989. Activation of human B lymphocytes through CD40 and interleukin 4. *Eur J Immunol* 19:1463-1467.

Veldhoen, M., R.J. Hocking, C.J. Atkins, R.M. Locksley, and B. Stockinger. 2006. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-189.

Verschuren, E.W., J. Klefstrom, G.I. Evan, and N. Jones. 2002. The oncogenic potential of Kaposi's sarcoma-associated herpesvirus cyclin is exposed by p53 loss in vitro and in vivo. *Cancer Cell* 2:229-241.

Vieira, J., and P.M. O'Hearn. 2004. Use of the red fluorescent protein as a marker of Kaposi's sarcoma-associated herpesvirus lytic gene expression. *Virology* 325:225-240.

Voo, K.S., T. Fu, H.Y. Wang, J. Tellam, H.E. Heslop, M.K. Brenner, C.M. Rooney, and R.F. Wang. 2004. Evidence for the presentation of major histocompatibility complex class I-restricted Epstein-Barr virus nuclear antigen 1 peptides to CD8+ T lymphocytes. *J Exp Med* 199:459-470.

Wang, Q.J., X.-L. Huang, G. Rappocciolo, F.J. Jenkins, W.H. Hildebrand, Z. Fan, E.K. Thomas, and C.R. Rinaldo, Jr. 2002. Identification of an HLA A*0201-restricted CD8+ T-cell epitope for the glycoprotein B homolog of human herpesvirus 8. *Blood* 99:3360-3366.

Wang, Q.J., F.J. Jenkins, L.P. Jacobson, L.A. Kingsley, R.D. Day, Z.-W. Zhang, Y.-X. Meng, P.E. Pellet, K.G. Kousoulas, A. Baghian, and C.R. Rinaldo, Jr. 2001. Primary human herpesvirus 8 infection generates a broadly specific CD8+ T-cell response to viral lytic cycle proteins. *Blood* 97:2366-2373.

Watts, C. 2001. Antigen processing in the endocytic compartment. *Curr Opin Immunol* 13:26-31.

Wearsch, P.A., and P. Cresswell. 2008. The quality control of MHC class I peptide loading. *Curr Opin Cell Biol* 20:624-631.

Weenink, S., H. Averdunk, T. Boston, V. Boswarva, J.C. Guery, L. Adorini, E. Mellins, J. McCluskey, and A.M. Gautam. 1997. Impaired antigen presentation by murine I-Ad class II MHC molecules expressed in normal and HLA-DM-defective human B cell lines. *Int Immunol* 9:889-896.

Wei, M.L., and P. Cresswell. 1992. HLA-A2 molecules in an antigen-processing mutant cell contain signal sequence-derived peptides. *Nature* 356:443-446.
West, J., and B. Damania. 2008. Upregulation of the TLR3 pathway by Kaposi's sarcoma-associated herpesvirus during primary infection. *J Virol* 82:5440-5449.

Wherry, E.J., V. Teichgraber, T.C. Becker, D. Masopust, S.M. Kaech, R. Antia, U.H. von Andrian, and R. Ahmed. 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. *Nat Immunol* 4:225-234.

Wies, E., A.S. Hahn, K. Schmidt, C. Viebahn, N. Rohland, A. Lux, T. Schellhorn, A. Holzer, J.U. Jung, and F. Neipel. 2009. The Kaposi's Sarcoma-associated Herpesvirus-encoded vIRF-3 Inhibits Cellular IRF-5. *J Biol Chem* 284:8525-8538.

Wies, E., Y. Mori, A. Hahn, E. Kremmer, M. Sturzl, B. Fleckenstein, and F. Neipel. 2008. The viral interferon-regulatory factor-3 is required for the survival of KSHV-infected primary effusion lymphoma cells. *Blood* 111:320-327.

Wilkinson, J., A. Cope, J. Gill, D. Bourboulia, P. Hayes, N. Imami, T. Kubo, A. Marcelin, V. Calvez, R. Weiss, B. Gazzard, C. Boshoff, and F. Gotch. 2002. Identification of Kaposi's Sarcoma-Associated Herpesvirus (KSHV)-Specific Cytotoxic T-Lymphocyte Epitopes and Evaluation of Reconstitution of KSHV-Specific Responses in Human Immunodeficiency Virus Type 1-Infected Patients Receiving Highly Active Antiretroviral Therapy. *J. Virol.* 76:2634-2640.

Williams, A., C.A. Peh, and T. Elliott. 2002. The cell biology of MHC class I antigen presentation. *Tissue Antigens* 59:3-17.

Wilson, S.J., E.H. Tsao, B.L. Webb, H. Ye, L. Dalton-Griffin, C. Tsantoulas, C.V. Gale, M.Q. Du, A. Whitehouse, and P. Kellam. 2007. X box binding protein XBP-1s transactivates the Kaposi's sarcoma-associated herpesvirus (KSHV) ORF50 promoter, linking plasma cell differentiation to KSHV reactivation from latency. *J Virol* 81:13578-13586.

Woodberry, T., T.J. Suscovich, L.M. Henry, J.N. Martin, S. Dollard, P.G. O'Connor, J.K. Davis, D. Osmond, T.-H. Lee, D.H. Kedes, A. Khatri, J. Lee, B.D. Walker, D.T. Scadden, and C. Brander. 2005. Impact of Kaposi Sarcoma‚ÄìAssociated Herpesvirus (KSHV) Burden and HIV Coinfection on the Detection of T Cell Responses to KSHV ORF73 and ORF65 Proteins. *The Journal of Infectious Diseases* 192:622-629.

Xu, Y., D.P. AuCoin, A.R. Huete, S.A. Cei, L.J. Hanson, and G.S. Pari. 2005. A Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 ORF50 deletion mutant is defective for reactivation of latent virus and DNA replication. *J Virol* 79:3479-3487.

Yewdell, J.W. 2007. Plumbing the sources of endogenous MHC class I peptide ligands. *Curr Opin Immunol* 19:79-86.

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* 157:1823-1826.

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

Yu, Y., S.E. Wang, and G.S. Hayward. 2005. The KSHV immediate-early transcription factor RTA encodes ubiquitin E3 ligase activity that targets IRF7 for proteosome-mediated degradation. *Immunity* 22:59-70.

Zaldumbide, A., M. Ossevoort, E.J. Wiertz, and R.C. Hoeben. 2007. In cis inhibition of antigen processing by the latency-associated nuclear antigen I of Kaposi sarcoma herpes virus. *Mol Immunol* 44:1352-1360.

Zhao, K.N., W.J. Liu, and I.H. Frazer. 2003. Codon usage bias and A+T content variation in human papillomavirus genomes. *Virus Res* 98:95-104.

Zhang, Y., T. Davis, X. Wang, J. Deng, J. Baillargeon, T. Yeh, H. Jenson, and S. Gao. 2000. Distinct distribution of rare us kshv genotypes in south texas. Implications for kshv epidemiology and evolution. *Ann Epidemiol* 10:470.

Zhou, D., P. Li, Y. Lin, J.M. Lott, A.D. Hislop, D.H. Canaday, R.R. Brutkiewicz, and J.S. Blum. 2005. Lamp-2a facilitates MHC class II presentation of cytoplasmic antigens. *Immunity* 22:571-581.

Zhu, F.X., S.M. King, E.J. Smith, D.E. Levy, and Y. Yuan. 2002. A Kaposi's sarcoma-associated herpesviral protein inhibits virus-mediated induction of type I interferon by blocking IRF-7 phosphorylation and nuclear accumulation. *Proc Natl Acad Sci U S A* 99:5573-5578.

Zinkernagel, R.M., M.B. Dunlop, R.V. Blanden, P.C. Doherty, and D.C. Shreffler. 1976. H-2 compatibility requirement for virus-specific T-cell-mediated cytolysis. Evaluation of the role of H-2I region and non-H-2 genes in regulating immune response. *J Exp Med* 144:519-532.

Zong, J., D.M. Ciufo, R. Viscidi, L. Alagiozoglou, S. Tyring, P. Rady, J. Orenstein, W. Boto, H. Kalumbuja, N. Romano, M. Melbye, G.H. Kang, C. Boshoff, and G.S. Hayward. 2002. Genotypic analysis at multiple loci across Kaposi's sarcoma herpesvirus (KSHV) DNA molecules: clustering patterns, novel variants and chimerism. *J Clin Virol* 23:119-148.

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:e1000255.

Zuo, J., L.L. Quinn, J. Tamblyn, W.A. Thomas, R. Feederle, H.J. Delecluse, A.D. Hislop, and M. Rowe. 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* 85:1604-1614.

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* 82:2385-2393.