Characterising the Adaptive T-cell Immune Response Against Kaposi's Sarcoma-associated Herpesvirus

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DECLARATION

I, Rebecca Caroline Robey, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

Kaposi's sarcoma-associated herpesvirus (KSHV) is causally related to Kaposi's sarcoma (KS), the most common malignancy in individuals with untreated HIV/AIDS. Several lines of evidence indicate that KS oncogenesis is associated with loss of T cell-mediated control of KSHV-infected cells. However, the adaptive CD8 and CD4 T-cell responses against KSHV have not been fully characterised. Neither the antigenic repertoire nor the immunodominant targets of CD8 and CD4 KSHV-specific T cells are fully understood, and the phenotypes and functions of these cells remain largely unexplored.

To investigate the targets of the CD8 and CD4 T-cell responses against KSHV, a novel approach for a large-scale screen of KSHV antigens was proposed that used lentiviral-transduced monocyte-derived dendritic cells (moDCs) expressing a panel of KSHV open reading frames (ORFs). Transduced moDCs naturally process the KSHV gene products and present the resulting antigenic peptides in the context of MHC class I and II. Transduced moDCs were cultured with autologous T cells and the CD8 and CD4 proliferative responses to each KSHV ORF (or pool of ORFs) were assessed.

CD8 and CD4 KSHV-specific responses were investigated in 14 KSHV-seropositive individuals. Unexpectedly, both the CD8 and CD4 T-cell responses against KSHV were found to be skewed towards ORFs expressed in the early and late phases of the viral lytic cycle. The most frequently recognised CD8 target was a pool of late lytic KSHV ORFs, [ORF28/ORF36/ORF37].

Identification of novel KSHV CD8 epitopes from within the late lytic ORF pool was attempted. Peptide-MHC binding and denaturation assays identified peptides that had the highest affinity for HLA-A*0201. Recognition of these potential epitopes was tested in clinical samples by IFNγ ELISpot, and compared with recognition of nine previously published HLA-A*0201-restricted KSHV epitopes. Finally, the use of pentamers as tools to investigate the memory phenotypes and functions of virus-specific T cells was explored.

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ABBREVIATIONS

A	
Aa	Amino acid
Ad5	Adenovirus 5
Adpp65	Adenovirus encoding CMV pp65
AIDS	Acquired immunodeficiency syndrome
B	
BAX	Bcl-2 associated X protein
BCL2	B-cell lymphoma 2
BCLAF1	Bcl-2-associated factor 1
BEC	Blood endothelial cell
BLAST	Basic local alignment search tool
bZIP	Basic leucine zipper transcription factor
C	
CCL	Cellular chemokine ligand
cDNA	Complementary DNA
CDK-2, -4, -6	Cyclin dependent kinase -2, -4, -6
CFSE	Carboxy fluoroscein succinimidyl ester
CMV	Cytomegalovirus (also known as HHV5)
cPPT	Central polypurine tract from HIV-POL
CTL	Cytotoxic T lymphocyte
D	
DC	Dendritic cell
DNA	Deoxyribonucleic acid
E	
EBNA1	EBV nuclear antigen 1
EBV	Epstein-Barr virus (also known as HHV4)
EC	Endothelial cell
EF1α	Elongation factor 1-alpha
ELISA	Enzyme-linked immunofluorescence assay
ELISpot	Enzyme-linked immunosorbent spot assay
ERK	Extracellular signal-regulated kinase (also known as MAPK)
eYFP	Enhanced yellow fluorescence protein
F	
FADD	Fas-associated death domain
FCS	Foetal calf serum
FLICE	FADD-interleukin-1β-converting enzyme
G	
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
gB, gH, gp35/37	Glycoprotein B, H, 35/37
GFP	Green fluorescence protein

G continued	
GMCSF	Granulocyte/macrophage colony-stimulating factor
GorRHV1	Gorilla rhadinoherpesvirus 1
GPCR	G-protein coupled receptor
H	
HAART	Highly active antiretroviral therapy
HEK 293T	Transformed human embryonic kidney cell line
HHV4	Human herpesvirus 4 (also known as EBV)
HHV8	Human herpesvirus 8 (also known as KSHV)
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
I	
IAP	Inhibitor of apoptosis protein
ICAM	Inter-cellular adhesion molecule
IFA	Immunofluorescence assay
IFNγ	Interferon-gamma
IL -1β, -2, -4, -6, -12	Interleukin -1β, -2, -4, -6, or -12
IKK	IκB kinase complex
IRF	Interferon regulatory factor
J	
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
K	
KCP	KSHV complement control protein (also known as vCBP)
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus (also known as HHV8)
L	
LAMP	Lysosomal-associated membrane protein
LANA1	KSHV latent nuclear antigen 1
LB	Luria-Bertani
LCMV	Lymphocytic choriomeningitis virus
LEC	Lymphatic endothelial cell
LPS	Lipopolysaccharides
LTR	Long terminal repeat
M	
MAGE	Melanoma antigen-encoding gene
MAP(K)	Mitogen-activated protein (kinase) (also known as ERK)
MCD	Multicentric Castleman's disease
MCS	Multiple cloning site
mDC	Myeloid dendritic cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MHV68	Murine herpesvirus 68

M continued

MIP1β	Macrophage inflammatory protein 1 beta
MIR	Modulator of immune recognition
miRNA	MicroRNA
moDC	Monocyte-derived dendritic cell
MOI	Multiplicity of infection
mRNA	MessengerRNA
MTA	mRNA transcript accumulation
mTOR	Mammalian target of rapamycin

N

NCBI	National Center for Biotechnology Information
NFκb	Nuclear factor kappa-light-chain-enhancer of activated B cells

O

ORF	Open reading frame

P

PanRHV1	Pan-rhadinoherpesvirus 1
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell
PECAM	Platelet/endothelial cell adhesion molecule
PEL	Primary effusion lymphoma

P continued

PF8	Processivity factor 8
PGE2	Prostaglandin E2
PGK	Phosphoglycerate kinase promoter
pp65	Phosphoprotein 65 (from CMV)
pRB	Retinoblastoma protein
PTD	Protein transduction domain

Q

qPCR	Quantitative (real-time) polymerase chain reaction

R

RNA	Ribonucleic acid
Rta	Replication and transcription activator
RT-PCR	Reverse-transcription polymerase chain reaction

S

SFC	Spot-forming cells
SFFV	Spleen focus-forming virus
SMoC	Small-molecule carrier
SOX	SRY (sex determining region Y)-box
SSB	Single-strand binding (protein)
STAT	Signal transducers and activators of transcription

T

T_{CM}	Central memory T cell
T_{EM}	Effector memory T cell

T continued	
T_{EMRA}	Terminally-differentiated effector memory T cell
TCR	T-cell receptor
Th-1 or -2	T helper cell type-1 or -2
THBS1	Thrombospondin 1
TNFα	Tumour necrosis factor-alpha
TPA	12-*O*-tetradecanoylphorbol-13-acetate
Tx	Treatment
V	
vCBP	Viral complement binding protein (also known as KCP)
V continued	
VEGFR3	Vascular endothelial growth factor receptor 3
vFLIP	viral FLICE inhibitory protein
VSV-G	Vesicular stomatitis virus glycoprotein
W	
WPRE	Woodchuck hepatitis B post-transcriptional regulatory element

Amino Acids

A	Alanine	M	Methionine
C	Cysteine	N	Asparagine
D	Aspartic Acid	P	Proline
E	Glutamic Acid	Q	Glutamine
F	Phenylalanine	R	Arginine
G	Glycine	S	Serine
H	Histine	T	Threonine
I	Isoleucine	V	Valine
L	Leucine	W	Tryptophan
K	Lysine	Y	Tyrosine

DNA Bases

A	Adenine	G	Guanine
C	Cytosine	U	Thymine

Fluorochromes

APC	Allophycocyanin	PerCP	Peridinin chlorophyll protein complex
APCCy7	Allophycocyanin Cyanine 7		
FITC	Fluorescein isothiocyanate	PerCPCy5.5	Peridinin chlorophyll protein complex Cyanine 5.5
PE	Phycoerythrin		
PECy5	Phycoerythin Cyanine 5		
PECy7	Phycoerythrin Cyanine 7		

HIV Genes

ENV	Envelope gene	REV	Regulator of virion
GAG	Group-specific antigen	TAT	Transactivator of transcription
POL	Codes for reverse transcriptase, integrase and protease	VIF	Viral protein R
		VPR	Viral infectivity factor
		VPU	Viral protein U
NEF	Negative regulatory factor		

PUBLICATIONS

Papers

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CHAPTER 1: Introduction

1.1 Kaposi's Sarcoma-associated Herpesvirus (KSHV) and its Related Diseases

Kaposi's sarcoma-associated herpesvirus (KSHV) [or human herpesvirus 8 (HHV8)] is an oncogenic herpesvirus that is the aetiological agent of Kaposi's sarcoma (KS), a mesenchymal tumour characterised by *de novo* vascular formation and lymphocyte infiltration. KSHV is also involved in the pathogenesis of at least two lymphoproliferative disorders, primary effusion lymphoma and multicentric Castleman's disease.

1.1.a The Discovery of KSHV – a Causative Agent of KS?

As early as the 1960s, the uneven geographical distribution of KS observed in epidemiological studies gave rise to suspicion that KS was caused by an infectious agent (Oettle, 1962). This suspicion was compounded by the dramatic rise in KS incidence amongst immunosuppressed individuals (acquired or iatrogenic) in the 1980s (Beral, 1991). Moreover, the high prevalence and uneven distribution of KS observed among those with acquired immune deficiency syndrome (AIDS) pointed towards a sexually transmitted agent for KS. KS occurs predominantly amongst homosexual men with human immunodeficiency virus (HIV), with a lower incidence seen in those who acquired HIV through heterosexual contact and rare incidence amongst those with parenterally acquired HIV, for example, intravenous drug users and individuals with haemophilia (Beral et al., 1990).

In 1994, Chang *et al.* identified novel herpesvirus DNA sequences from KS lesions from individuals with AIDS (Chang et al., 1994). Molecular and serological epidemiology studies confirmed that this new herpesvirus (the eighth to be discovered, hence human herpesvirus 8 [HHV8]) was present in individuals with all forms of KS, but was not ubiquitous in the general population (Huang et al., 1995; Kedes et al., 1996; Gao et al., 1996). Furthermore, detection of high levels of HHV8 DNA in the peripheral blood of individuals with AIDS was found to predict subsequent development of KS (Whitby et al., 1995; Moore et al., 1996b). PCR *in situ* hybridisation demonstrated that HHV8 was present in endothelial cells and spindle (tumour) cells of KS lesions from all epidemiological forms of KS (Boshoff et al., 1995), and so the virus rapidly became known as KS-associated herpesvirus (KSHV). The observation that KSHV is latently expressed in more than 90% of tumour cells in advanced KS lesions (Dupin et al., 1999) provided further evidence that KSHV was indeed causally related to KS.

Significantly, a serological study by Gao *et al*. found no evidence of KSHV infection in healthy US blood donors, whereas they observed intermediate to high seroprevalence of KSHV amongst control populations from Italy (four percent) and Uganda (51%) – two countries where KS is endemic (Gao et al., 1996). Lennette *et al.* reported a similar pattern of KSHV seroprevalence, with dramatically higher incidence in the general population of Africa compared to the US, although the actual rates they observed were much higher than the previous study (80% in Uganda and between four percent and 28% in the US depending on the ages of the individuals being studied) (Lennette et al., 1996). These, and further studies into the epidemiology of KSHV infection, revealed that KSHV is a necessary but not

sufficient factor in KS pathogenesis, with immunosuppression representing another important co-factor.

Apparent disparities in the actual levels of KSHV prevalence reported by different groups likely arise from differences in the sensitivities of the assays used. Most serological studies use immunofluorescence assays (IFA) to determine the presence of KSHV-specific antibodies in peripheral blood serum samples. Early studies performed IFA using smears of Epstein-Barr virus (EBV)-negative KSHV-positive cell lines and analysed by microscopic examination (Lennette et al., 1996; Gao et al., 1996), a methodology that is susceptible to variations in interpretation between different laboratories. More latterly, enzyme-linked immunofluorescence assays (ELISAs) have been developed and those that detect both lytic- and latent-specific antibodies provide an arguably more accurate and less subjective method for detection of KSHV antibodies. Other studies use IFA in conjunction with a PCR-based test for the presence of KSHV DNA sequences in peripheral blood mononuclear cells (PBMCs) (Stebbing et al., 2003a).

1.1.b Transmission of KSHV

The exact mode of transmission of KSHV is not known. In the West, the high prevalence of KSHV amongst homosexual men and association with HIV infection indicates a role for sexual transmission (Martin et al., 1998). KSHV has been detected in semen (Howard et al., 1997) and risk of infection correlates with the number of sexual partners (Blackbourn et al., 1999). However, the route of transmission between sexual partners is not known. Strikingly, there is little

evidence of heterosexual transmission (Campbell et al., 2009; McDonald et al., 2009). An extensive study by Dukers *et al.* into the risk factors for contraction of KSHV amongst homosexual men found that orogenital sex was significantly associated with KSHV seroconversion, and that this practice was more important than just having a high number of sexual partners (Dukers et al., 2000). KSHV DNA is readily detectable in saliva and nasal secretions, and cell-free salivary fluid has been demonstrated to infect cell lines *in vitro* (Koelle et al., 1997; Vieira et al., 1997; Blackbourn et al., 1998). Together, this implicates the oropharynx as a possible site of KSHV replication and indicates a role for saliva in KSHV transmission. This is in keeping with the mode of transmission of Epstein-Barr virus [(EBV), the most closely related human herpesvirus to KSHV (Morgan et al., 1979)] but has not been definitively confirmed.

Within African, Middle Eastern and Mediterranean populations, seroepidemiological studies point towards horizontal, non-sexual transmission of KSHV, with seroconversion frequently occurring before puberty and seroprevalence increasing linearly with age (Bourboulia et al., 1998; Olsen et al., 1998; Mayama et al., 1998; Gessain et al., 1999; Plancoulaine et al., 2000; Davidovici et al., 2001; Cattani et al., 2003). As with transmission between sexual partners, oral routes of transmission (for example, kissing or 'licking' to ease mosquito bites) have been suggested and would explain both mother-to-child and sibling-to-sibling transmission. Alternatively, high mother-to-child transmission rates (Bourboulia et al., 1998) could indicate a role for breast milk, although there is as yet no evidence to support this.

1.1.c Kaposi's Sarcoma – Epidemiological Forms

Kaposi's sarcoma (KS) is named after Moritz Kaposi, an Austro-Hungarian dermatologist who was the first to describe the disease. In 1872 Kaposi published the case histories of five patients suffering from what he described as "idiopathic multiple pigmented sarcomas" of the skin. This form of the disease is now known as '**Classic KS**'. It is a relatively indolent disease, often confined to the extremities. It is generally a chronic condition persisting for several years before unrelated fatality. Classic KS is predominant in elderly patients, more frequently men, of Mediterranean, Eastern European or Middle Eastern origin (Hengge et al., 2002).

A second form of KS, known as '**Endemic KS**', was prevalent in Africa pre-dating the emergence of HIV (Cook-Mozaffari et al., 1998). It is a much more aggressive form of the disease, often spreading to the lymph nodes and affecting both adults and children (Olweny et al., 1976). Retrospective examination of medical records from the 1960s indicated that endemic KS accounted for between 4.5% and 7.0 % of tumours seen in Ugandan men (de Thé et al., 1999; Wabinga et al., 2000). However, until the 1980s, KS (endemic or classic) remained a relatively rare neoplasm, particularly in North America and Western Europe.

In 1981 an unusual epidemic of KS cases in young men from New York City and California was one of the first signs of the outbreak of AIDS (CDC Report, 1981; Jaffe, 2008). This new form of KS, dubbed '**AIDS-KS**', became an AIDS-defining disease and is now the most frequently arising malignancy in individuals with untreated HIV/AIDS (Boshoff and Weiss, 2002). Since the advent of highly active antiretroviral therapy (HAART), KS incidence has declined substantially in the

West (Ledergerber et al., 1999; Jones et al., 2000). However, AIDS-KS remains one of the most common cancers across Africa, with KS recently estimated to account for 12.9% of all cancers in African males and 5.1% of all cancers in African females (Parkin et al., 2008). AIDS-KS is an aggressive disease, often affecting the mouth, gastrointestinal tract or genitalia (Boshoff and Weiss, 2002).

The 1980s also saw the rise of a fourth epidemiological variety of KS. '**Iatrogenic KS**' occurs in transplant recipients, and other patients receiving immunosuppressive therapy. KS is up to 150 times more common in transplant recipients than the general Western population. Interestingly, transplant recipients in the West with origins (first or second generation) in countries where classic or endemic KS is prevalent are at greater risk of developing iatrogenic KS. This suggests an increased risk of developing KS amongst these populations even after geographical relocation (Boshoff and Weiss, 2001), which is likely to be simply explained by a higher prevalence of KSHV infection. There is, however, evidence that iatrogenic KS can arise from both pre-transplant KSHV infection and through receipt of a KSHV-infected graft (Regamey et al., 1998; Cattani et al., 2001).

1.1.d KS Tumour Biology

KS is a complex tumour with three distinct phases referred to as patch, plaque and nodular stages. Lesions are usually multi-focal, and can arise in the skin, viscera or mucosa (Hengge et al., 2002). The KS tumour cell is considered to be the spindle cell, which is distinguished by its spindle-shaped morphology and dominates final-stage nodular lesions (Gessain and Duprez, 2005). The exact origin of the spindle

cell is unclear. Early immunohistochemistry studies showed that most KS spindle cells express endothelial markers such as CD31 and CD34. However, expression of markers of macrophages, smooth muscle cells, fibroblasts and dendritic cells have also been demonstrated (Boshoff and Weiss, 2001; Gessain and Duprez, 2005), leading to speculation that spindle cells either represent a heterogenous population of cells or are derived from a multipotent mesenchymal precursor. More recently, evidence has pointed towards a lymphatic endothelial origin. Spindle cells were shown to express lymphatic markers including VEGFR3 and podoplanin (Dupin et al., 1999; Weninger et al., 1999). Gene expression microarrays revealed closer similarity between the expression profile of KS spindle cells and lymphatic endothelial cells (LECs) than between KS spindle cells and blood endothelial cells (BECs), although both LEC and BEC markers are present in the KS spindle cell expression signature (Wang et al., 2004). Further studies demonstrated that *in vitro* infection of BECs with KSHV leads to lymphatic reprogramming of these cells (Carroll et al., 2004; Hong et al., 2004), and so it has been suggested that *in vivo*, blood vessel endothelial cells are converted towards a lymphatic phenotype by latent KSHV infection, giving rise to the spindle cell (Gessain and Duprez, 2005).

Despite their different clinical manifestations, KS lesions observed in the four epidemiological forms of KS outlined above are histologically indistinguishable at comparative stages (Gessain and Duprez, 2005). At the earliest stage, the patch stage, KS lesions are macular and characterised by a proliferation of small, irregular endothelial-lined spaces surrounding normal dermal blood vessels with an accompanying infiltration of inflammatory lymphocytes. At the plaque stage, the lesions become palpable, and the abnormal vasculature spreads through the entire

dermis and sometimes into the subcutaneous fat. Clusters of spindle cells expand around the vascular spaces, and varying numbers of erythrocytes fill the channels. Nodular-stage KS lesions develop areas of pigmentation and are composed of sheets and large fascicles of uniform spindle cells, some of which are undergoing mitosis. Erythrocytes are trapped within an extensive network of slit-like vascular channels. The channels themselves, however, are not well defined, lacking pericytes in their walls and with fragmentation of their endothelial lining and basal lamina (Boshoff and Weiss, 2001; Hengge et al., 2002; Gessain and Duprez, 2005; Grayson and Pantanowitz, 2008). Figure 1.1 shows examples of KS lesions and histological sections from KS lesions of different stages.

An unusual feature of KS tumour biology is the important role played by inflammatory infiltrates, particularly at KS onset. Peripheral blood mononuclear cell (PBMC) infiltration at the patch stage precedes the formation of spindle cells and appears to be essential to tumour formation (Ensoli et al., 2001). KS onset is associated with a generalised disturbance of the immune system, with activation of CD8 cytotoxic T lymphocytes (CTLs) and increased production of T helper 1-type (Th1) cytokines, in particular interferon-γ (IFNγ), by circulating PBMCs (Ensoli et al., 2001). Early lesions are infiltrated by T cells (mostly CTLs), monocyte-macrophages and dendritic cells, and these cells also produce high levels of Th1 cytokines, including IFNγ; tumour necrosis factor-α (TNFα); interleukin (IL)1β; and IL6 (Sirianni et al., 1998; Fiorelli et al., 1998). These inflammatory cytokines could help to trigger the production of angiogenic factors, initiating angiogenesis and oedema and activating KSHV-infected endothelial cells (ECs) to form spindle

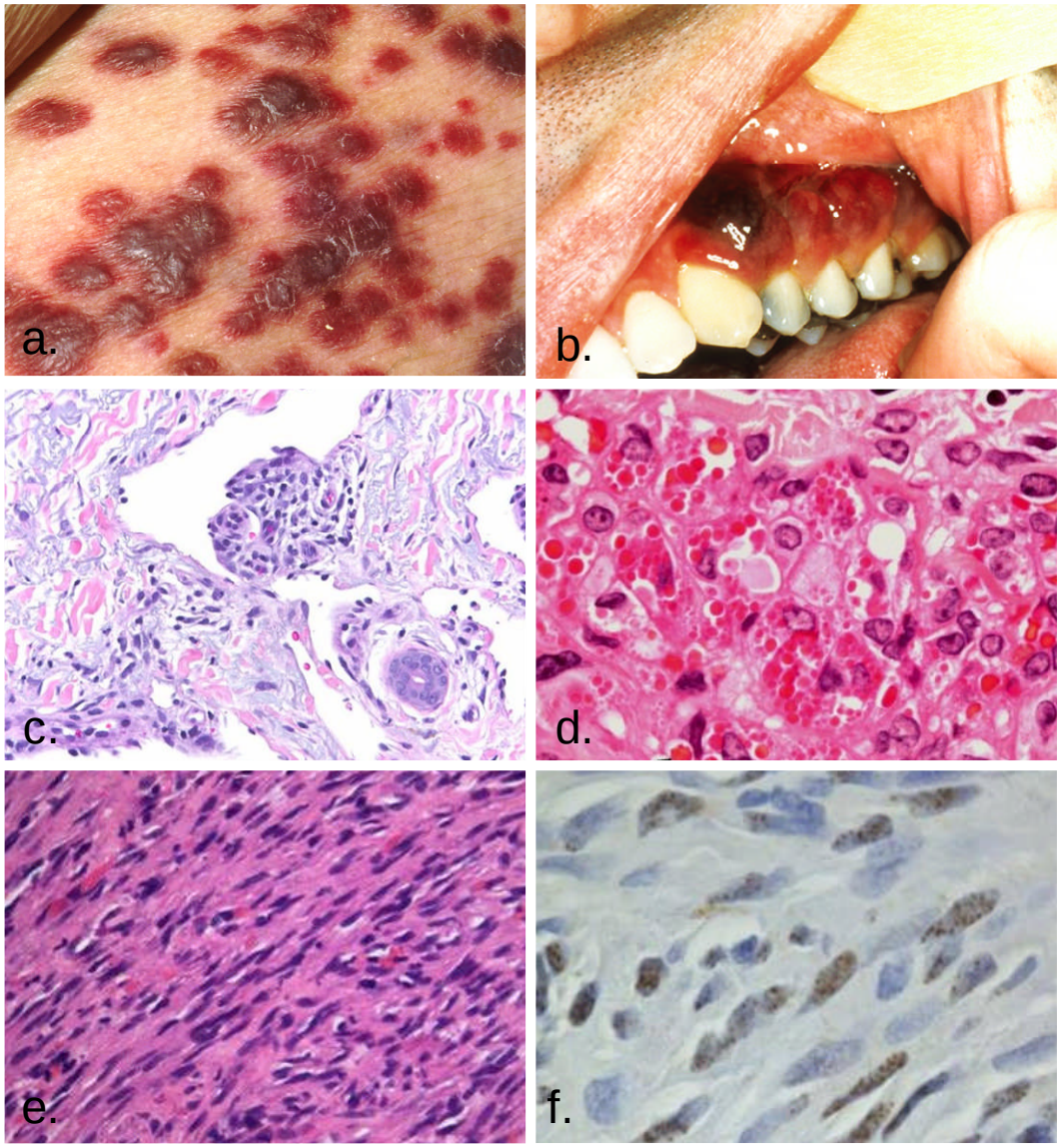


FIGURE 1.1: Kaposi's sarcoma lesions and their histopathology. a. Cutaneous KS lesion (nodular stage) from an individual with AIDS-KS. b. KS in the mouth of an individual with AIDS-KS (both a and b from National Cancer Institute Visuals Online). c – e. Haematoxylin and eosin stained sections of patch- (c), plaque- (d) and nodular- (e) stage KS lesions (from Grayson and Pantanowitz, 2008). At the patch stage, inflammatory cell infiltrates and newly formed slit-like vessels containing red blood cells are evident. At the plaque stage, the new vasculature is more extensive and erythrocytes can be seen in the channels. Spindle cells begin to form. By the nodular stage sheets of monomorphic spindle cells are evident and surround slit-like channels containing erythrocytes. f. Punctate LANA1 expression in the nucleus of spindle cells is revealed by immunohistochemistry staining (also from Grayson and Pantanowitz, 2008).

cells (Ensoli et al., 2001). Significantly, in early-stage lesions the proportion of KSHV-infected ECs is low, and these cells show markers of latent infection (Boshoff et al., 1995; Davis et al., 1997; Dupin et al., 1999). High levels of Th1 cytokines recruit KSHV-infected circulating macrophage cells to the site of the tumour. These cells, in contrast to the KSHV-infected ECs, are lytically infected and so produce mature KSHV viral particles (Blasig et al., 1997). They thus may be essential both for increasing the number of infected ECs, as indicated by evidence of polyclonal KSHV DNA within most KS lesions (Gill et al., 1998); and for maintaining infection of spindle cells, as indicated by the loss of KSHV infection by these cells upon culture (Lebbe et al., 1997; Grundhoff and Ganem, 2004). It is also possible that these KSHV-infected circulating macrophage cells are incorporated into KS lesions and differentiate into spindle cells, explaining the expression of macrophage markers by spindle cells (Ensoli et al., 2001; Gessain and Duprez, 2005).

The development of KS may thus be seen as a multi-factorial process in which infection by KSHV is a requirement; immunosuppression is an important co-factor; and, paradoxically, some level of systemic and localised immune activation in the form of increased Th1 cytokine production (induced either by KSHV infection, HIV infection or unknown causes) is also involved. The Th1 cytokine upregulation initiates an inflammatory-angiogenic process that leads to sites of activated tissue that are vulnerable to KS lesion formation (Ensoli et al., 2001). Recruitment of KSHV-infected cells and other PBMCs to these sites establishes a tissue microenvironment that has high levels of inflammatory cytokines and is increasingly rich in KSHV-infected cells of both macrophage and endothelial

origin. This positively reinforces cell recruitment, and promotes the formation and survival of spindle cells and the production of further cytokines, angiogenic factors and growth factors that contribute to the development of advanced KS lesions.

There has been much debate as to whether KS represents a true sarcoma or is more akin to a reactive hyperplasia as lesions are symmetrical; appear at several sites simultaneously; do not generally show signs of aneuploidy; and can regress spontaneously (Boshoff and Weiss, 2001; Ensoli et al., 2001). However, although generally diploid, some aneuploid spindle cells have been detected, and KS lesions from individuals with AIDS-KS have been shown to have high levels of microsatellite instability – a hallmark of tumour cells (Bedi et al., 1995; Ensoli et al., 2001). Several groups have examined the clonality of KS lesions in order to determine whether or not KS represents a disseminating monoclonal cancer in which cellular transformation occurs before the clonal expansion and metastasis of KS spindle cells. Early studies produced conflicting results. One study of the X-chromosome inactivation pattern of the human androgen receptor gene in cells from lesions from 12 women found that the majority of lesions were oligoclonal in nature, indicating that KS is not a clonal malignancy (Gill et al., 1998). Conversely, another study that used a very similar experimental procedure to examine clonality of lesions from eight women found evidence that spindle cells from multiple KS lesions from the same individual were predominately monoclonal (Rabkin et al., 1997). An alternative methodological approach examined the terminal repeat regions of KSHV episomes isolated from nodular KS lesions, and found that these displayed all patterns of clonality (Judde et al., 2000). In an attempt to clarify the clonality status of KS lesions, a recent extensive study examined KSHV clonality in

59 tumour samples (mostly nodular stage) taken from male and female individuals with all four epidemiological forms of KS, including 26 samples taken from multiple sites from six individuals with disseminated KS (Duprez et al., 2007). Eleven of the 59 samples were monoclonal, and 48 were oligoclonal. Samples from disseminated lesions were either monoclonal or oligoclonal. The authors concluded that whilst some KS lesions represent a true monoclonal expansion, the majority of advanced lesions are oligoclonal proliferations. Moreover, disseminated lesions represent distinct expansions of KSHV-infected spindle cells, indicating that most KS lesions are reactive proliferations rather than true metastatic malignancies.

1.1.e Primary Effusion Lymphoma

Primary effusion lymphoma (PEL) is a non-Hodgkin's B-cell lymphoma that usually occurs in the context of HIV-infection. It is clinically characterised by its unusual pathogenesis, commonly arising as lymphomatous effusions in body cavities such as the pleural, pericardial and peritoneal spaces in the absence of a solid tumour mass. This rare disorder (originally referred to as body cavity-based lymphoma) was first postulated to be a unique disease based on these peculiar clinical manifestations as well as its unusual morphologic and immunophenotypic characteristics (Knowles et al., 1989; Green et al., 1995). In 1995, Cesarman *et al.* demonstrated the presence of high levels of KSHV DNA in all cases of this atypical lymphoma, but not in any other HIV- or non-HIV-related non-Hodgkin's lymphoma (Cesarman et al., 1995). By definition, therefore, PEL patients must show evidence of KSHV infection although the mechanisms by which KSHV promotes oncogenesis in PEL are not fully understood. The vast majority of PEL

cells are also coinfected with EBV, making it difficult to determine the precise role of KSHV in the pathogenesis of this disease.

Recently, a number of other distinct KSHV-associated lymphomas have been defined (Carbone et al., 2009). In HIV-infected individuals these include solid lymphomas that either precede the development of, or follow the resolution of, PEL; and solid, extracavitary lymphomas that occur without serous effusions and are seen either within the lymph nodes or in extranodal tissue. In HIV-uninfected individuals, a unique KSHV-associated lymphoproliferative disorder that preferentially involves the germinal centres of lymph nodes has also been described.

1.1.f Multicentric Castleman's Disease

Multicentric Castleman's disease (MCD) is a rare, atypical lymphoproliferative disorder defined clinically by generalised polylymphadenopathy and evidence of multi-organ involvement (Oksenhendler et al., 1996). Its close association with KS, particularly in individuals with AIDS, prompted Soulier *et al*. to search for the presence of KSHV sequences in MCD samples (Soulier et al., 1995). They, and others (Parravinci et al., 1997), found KSHV DNA in MCD lesions from all HIV-related and about 40% of HIV-unrelated cases of MCD. It is therefore likely that there is more than one cause for this unusual disease. Nonetheless, a correlation between increased KSHV viral load in PBMCs and clinical exacerbations of MCD supports a role for KSHV in MCD pathogenesis (Grandadam et al., 1997).

There are two main pathological types of MCD – the hyaline-vascular and plasma-cell types, defined by the histological features of the affected lymph nodes. The former is more commonly seen in the localised, unicentric variant of Castleman's disease, whereas the latter is frequently multicentric. KSHV-positive MCD has been recognised as a distinct subset of MCD (predominately of the plasma-cell type) and designated plasmablastic MCD, since it is characterised by the presence of large plasmablastic cells all of which harbour KSHV (Dupin et al., 2000).

1.2 KSHV Molecular Pathology

KSHV belongs to the lymphotrophic herpesvirus subfamily, *gammaherpesviridae*. It is of the *Rhadinovirus* genus and, as such, is the first human gamma-2 herpesvirus to be identified. KSHV is most closely related to two old-world primate gamma-2 herpesvirus, the chimpanzee rhadinoherpesvirus 1a (PanRHV1a) and the gorilla rhadinoherpesvirus 1 (GorRHV1) (Lacoste et al., 2000). The most closely related human virus to KSHV is the human gamma-1 herpesvirus, Epstein-Barr virus (EBV) [or human herpesvirus 4 (HHV4)] (Moore et al., 1996a).

Like EBV, KSHV establishes latent infection in B cells (Decker et al., 1996). However, it has an unusually wide cellular tropism, and has been detected *in vivo* in endothelial cells (Boshoff et al., 1995), epithelial cells (Diamond et al., 1998), macrophages and monocytes (Blasig et al., 1997). *In vitro*, KSHV has been shown to infect a wide variety of human and animal cells, including human dendritic cells (Rappocciolo et al., 2006). This broad tropism seems to be attributable, at least in

part, to interaction between viral glycoproteins and the ubiquitous cell-surface molecule, heparin sulphate (Akula et al., 2001; Wang et al., 2001a).

1.2.a The KSHV Genome

KSHV is a large double-stranded DNA virus. The KSHV genome is approximately 165kb long, with a continuous 145kb-long unique coding region flanked by multiple GC-rich terminal repeat units approximately 800bp in length (Russo et al., 1996). Over 90 open reading frames (ORFs) have been identified within the KSHV genome, of which at least 81 ORFs are over 100 amino acids long. The genome consists of seven highly conserved gene blocks separated by short regions of unique or subfamily-specific genes. The conserved genes have homologues among other herpesviruses and include genes that code for structural proteins and genes involved in viral DNA replication and regulation of gene expression. A large number of the viral genes (both conserved and unique) encode homologues of cellular genes that have been captured from the host during the course of the virus's evolution (Neipel et al., 1997).

KSHV has developed several complex strategies for subverting the host's adaptive and innate immune system. Approximately one quarter of KSHV genes – both cellular homologues and unique viral genes – are involved in immune evasion. The roles of many viral genes in driving oncogenesis have also now been elucidated and, in many cases, these oncogenic activities result from the same molecular pathways employed by the virus in its immune-evasion activities (Moore and Chang, 2003). The wealth of cellular processes modulated by KSHV viral genes

includes the cell cycle; apoptosis; cytokine production; antigen presentation; cell signalling; and signal transduction. Thus KSHV infection can result in aberrant cell growth, proliferation, inflammation and angiogenesis, all of which contribute towards tumour development.

1.2.b Viral Gene Expression

The KSHV ORFs are differentially expressed during the course of the virus's life cycle. During latent infection, the virus exists in the nucleus as a closed circular episome and very few viral genes are expressed. During the lytic phase of the virus's life cycle (which is the replication phase) the virus linearises and most viral genes are expressed in a temporally regulated fashion (Sarid et al., 1998; Jenner et al., 2001). The constitutively expressed latent genes and lytically induced genes are referred to as class I and class III transcription units respectively. Genes displaying a third pattern of gene expression, designated class II transcription, are expressed at low levels during latency but are also induced during lytic replication (Sarid et al., 1998). Lytic genes are induced sequentially and can be further classified as immediate-early, early or late genes based on their temporal appearance during replication. The chronological expression of lytic genes correlates with their function. Genes involved in transcription regulation are induced first, followed by those involved in DNA replication and repair, and genes that are involved in virus assembly or code for structural proteins are induced last (Jenner et al., 2001). Viral homologues of cellular genes involved in immune evasion are predominately immediate-early or early genes, in keeping with their function to prevent the virus from attracting immune surveillance as it replicates in preparation for shedding of

virions (Jenner and Boshoff, 2002). Figure 1.2 shows a schematic illustration of the KSHV viral genome with the expression pattern of all known viral ORFs.

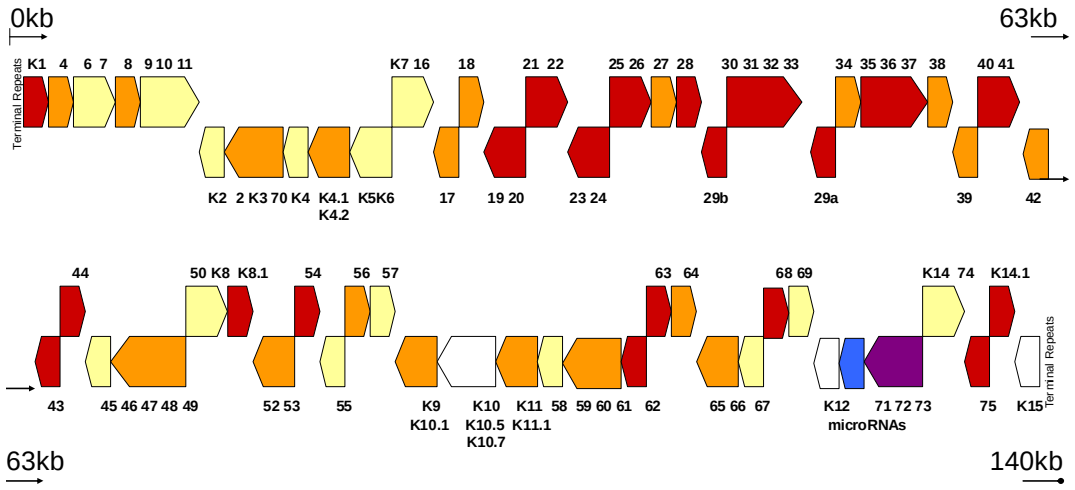


FIGURE 1.2: Schematic illustration of the KSHV genome showing viral gene expression. Adapted from Russo *et al*., 1996. Arrow direction indicates direction of transcription. Yellow, orange and red arrows indicate class III transcription units expressed during lytic replication: yellow arrows indicate immediate-early lytic ORFs; orange arrows indicate early lytic ORFs; red arrows indicate late lytic ORFs. White arrows indicate class II ORFs that are expressed at low levels in latency and are also induced during lytic replication. Purple arrow indicates the cluster of latently expressed (class I) ORFs. Blue arrow indicates the position of the KSHV microRNAs. NB: arrows are not drawn to scale with respect to the size of each ORF. ORFs are labelled with a 'K' suffix for the unique KSHV genes; where a suffix is absent this indicates an ORF with a cellular homologue or a homologue in another virus.

1.2.c Latent KSHV Genes

Latent infection itself is, arguably, the primary strategy in immune evasion, as the highly restricted pattern of viral gene expression that defines latency minimises the number of viral epitopes that are presented by infected cells to circulating cells of the immune system. Latency is also the state assumed to lead to proliferation of KSHV-infected cells, since lytic replication results in cell death and is therefore

anti-tumourigenic (as the host cell is lysed to release active virions). However, in early KS lesions, there is evidence that replicating latently infected KS spindle cells do not efficiently transfer viral DNA to all progeny and a low level of lytic infection is necessary to maintain infected cell numbers within the tumour (Grundhoff and Ganem, 2004). The vast majority of tumour cells in KS and PEL are latently infected, with only a few cells undergoing lytic replication. Tumour cells from MCD, by contrast, express both latent and lytic proteins (Parravicini et al., 2000; Katano et al., 2000).

Although latent infection is a characteristic of all herpesviruses, the latency genes themselves are not conserved between members of the family, but rather are highly evolved to adapt to their specific host-cell environment. The three class I KSHV latent genes are ORF73, ORF72 and ORF71, respectively coding for the latent nuclear antigen (LANA1); viral cyclin (v-cyclin); and vFLIP, which is the viral [Fas-associated death domain (FADD)]-interleukin-1β-converting enzyme (FLICE) inhibitory protein. These antigens are described in further detail below.

LANA1 is the major nuclear antigen of KSHV, the functional equivalent of the EBV nuclear antigen 1 (EBNA1) although there is no sequence similarity between the two genes. Like EBNA1, LANA1 is responsible for maintenance of the episome. LANA1 binds histone H1, which tethers the viral episome to host chromosomes during host-cell replication and so ensures persistence of the viral genome in successive daughter cells (Ballestas et al., 1999; Cotter and Robertson, 1999). In addition to this, LANA1, like EBNA1, is a major regulator of gene expression, interacting with a variety of both viral and cellular proteins to both

transactivate and repress transcription (Friborg, Jr. et al., 1999; Lim et al., 2000; Radkov et al., 2000). LANA1 maintains latency by transactivating expression from the viral latency genes' promoter (Renne et al., 2001) and by repressing the promoter of ORF50 (Rta, the viral activator of the latency to lytic switch) thus inhibiting the induction of lytic replication (Lan et al., 2004).

Recently, it was reported that LANA1, again like EBNA1, also acts to inhibit antigen presentation, enabling the virus to evade the host's immune system during latency despite the expression of a long protein that could potentially yield a number of viral epitopes (Zaldumbide et al., 2007). EBNA1 codes for a long glycine- and alanine-rich repeat that inhibits its own proteasomal degradation thereby preventing the generation of antigenic peptides through the action of an acidic-repeat region of amino acids (Levitskaya et al., 1995). LANA1 contains a central acidic-rich repeat region of 580 amino acids consisting almost exclusively of aspartic acid, glutamine and glutamic acid residues that was reported to interfere *in cis* with antigen processing (Zaldumbide et al., 2007). However, data presented by Sabbah and Hislop at the 11th International Workshop on KSHV & Related Agents, Birmingham, UK (2008) demonstrated no inhibition of antigen presentation by this repeat region, and thus the ability of LANA1 to evade immune recognition in this way remains open to debate.

Viral cyclin (v-cyclin) is a homologue of cellular cyclin D (Chang et al., 1996). Cyclin D binds the cyclin-dependent kinases CDK4 and CDK6 to create an active complex that phosphorylates pRb, releasing the transcription factor E2F. E2F activates the transcription of S-phase genes including cyclin E and so drives the G1-

to S-phase transition. V-cyclin also forms an active complex with CDK6 and, to a lesser extent, CDK4 that phosphorylates pRB to stimulate S-phase entry (Li et al., 1997; Godden-Kent et al., 1997). Significantly, the v-cyclin-CDK complex is not only less sensitive to the CDK inhibitors $p16^{INK4A}$, $p21^{CIP1}$ and $p27^{KIP1}$ than the cyclin D-CDK complex (Swanton et al., 1997), it also phosphorylates $p27^{KIP1}$, marking it for proteosomal degradation and thereby escaping its control (Ellis et al., 1999; Mann et al., 1999). Thus v-cyclin expression blocks CDK inhibitor-imposed G1 arrest, driving entry into S phase and deregulating the cell cycle. In addition to this, the v-cyclin-CDK6 complex phosphorylates a much wider array of substrates than the cyclin D-CDK complex, including cellular CDK2 targets, and thus causes further deregulation throughout the cell-cycle (Li et al., 1997; Godden-Kent et al., 1997; Jenner and Boshoff, 2002). A paradoxical feature of v-cyclin is that its activity on pRB triggers S-phase entry, but for unknown reasons is often associated with defective mitosis and so results in polyploidy. This in turn triggers p53 stabilisation, which induces growth arrest and apoptosis (Verschuren et al., 2002). Thus v-cyclin can promote oncogenesis only in the absence of p53, when v-cyclin-expressing cells can survive and expand as an aneuploid population.

Viral FLIP (vFLIP) is a homologue of cellular FLIP. vFLIP, like cellular FLIP, sequesters procaspase 8 and prevents its cleavage to the active form, thereby inhibiting the activity of the Fas death receptor and protecting cells from Fas-mediated apoptosis (Belanger et al., 2001). Furthermore, murine B cells transduced to express vFLIP are resistant to death receptor-induced apoptosis triggered by CTLs (Djerbi et al., 1999). vFLIP also inhibits apoptosis by activating the NFκB pathway, primarily through binding and activating the IκB kinase complex (IKK)

(Chaudhary et al., 1999; Liu et al., 2002; Field et al., 2003). Constitutive activation of NFκB by vFLIP has been shown to be essential for PEL cell survival (Guasparri et al., 2004) and also for cellular transformation by vFLIP (Sun et al., 2003), making this pathway an attractive therapeutic target for treatment of KSHV-associated lymphomas (Direkze and Laman, 2004; Godfrey et al., 2005).

1.2.d Lytic KSHV genes

As mentioned above, the temporal pattern of lytic gene expression correlates with gene function. The first viral gene to be expressed on the initiation of lytic replication is the transactivator ORF50, which encodes Rta and is both necessary and sufficient for lytic cycle induction (Lukac et al., 1998; Gradoville et al., 2000). This is closely followed by other genes involved in the regulation of gene expression, notably, K8 (which encodes K-bZIP, another transactivator and a homologue of EBV Zta,) and ORF57, a post-transcriptional regulator of gene expression. Next to be expressed are genes involved in viral DNA replication and repair, such as the viral DNA polymerase (ORF9) and its processivity factor (ORF59), and the single strand DNA binding protein (ORF6). The genes that are induced last are those that encode structural proteins or those involved in virus assembly, such as the glycoproteins gB (ORF8), gH (ORF22) and gp35/37 (K8.1); the major and minor capsid proteins (ORF25 and ORF26, respectively); and the capsid-interacting protein ORF65 (Nealon et al., 2001). Viral homologues of cellular genes involved in immune evasion are generally immediate-early or early genes, aiding the virus to avoid immune system recognition as the full complement of its genes are expressed during the course of the lytic cycle and ensuring that viral

replication is successful. Table 1.1 briefly summarises some of the most well characterised KSHV genes.

1.2.e KSHV microRNAs

KSHV encodes a cluster of microRNAs (miRNAs) within the latency-associated region of its genome (Samols et al., 2005; Cai et al., 2005). miRNAs are short (19 to 24 nucleotides long), non-coding RNAs that post-transcriptionally regulate gene expression by binding messenger RNAs (mRNAs), inhibiting their translation and promoting their degradation (Nilsen, 2007). They have been implicated in a host of cellular and biological functions, including development, oncogenesis and immune system function. A total of 17 KSHV miRNAs have been identified, derived from 12 pre-miRNAs. They are detectable at high levels in latently infected cells (Cai et al., 2005) and are postulated to modulate host gene expression in order to create a favourable environment for latent infection.

The cellular targets of the KSHV miRNAs are not yet fully understood. One group used microarray expression profiling to identify changes in gene expression in cells ectopically expressing ten of the known KSHV miRNAs (Samols et al., 2007). They identified 81 genes whose expression was significantly regulated by the KSHV miRNAs. Out of these 81 genes, eight showed a downregulation of more than four-fold, and of these eight, five were genes with roles important to immune evasion or tumourigenesis, such as proliferation, angiogenesis, apoptosis and immune modulation. Interestingly, one of these genes, Thrombospondin 1 (THBS1)

KSHV ORF	Gene Product	Function(s)	Reference(s)
Latent			
ORF71*	vFLIP	Anti-apoptotic	(Belanger et al., 2001)
ORF72*	v-cyclin	Homologue of cellular cyclin D	(Li et al., 1997)
ORF73*	LANA1	Maintenance of the episome, gene expression Immune evasion – inhibition of antigen presentation?	(Ballestas et al., 1999; Renne et al., 2001)
Expressed at low-levels in latency and induced during lytic cycle			
K11.1*	vIRF2	Immune evasion – disruption of host anti-viral interferon response	(Burysek et al., 1999)
K12*	Kaposin	Oncogenic, promotes cell proliferation	(Muralidhar et al., 2000)
K15-P*	LAMP	Immune evasion – down regulates B-cell receptor signal transduction Activates NFκB, ERK2 and JNK1 pathways	(Choi et al., 2000)
Immediate-Early Lytic			
ORF50*	Rta	Gene expression – transactivator of lytic replication; both necessary and sufficient for lytic cycle induction	(Lukac et al., 1998; Gradoville et al., 2000)
K8*	K-bZIP	Gene expression – transactivation (homologue of EBV Zta)	
ORF57**	MTA	Gene expression – post-transcriptional regulation (homologue of EBV SM protein)	(Gupta et al., 2000)
ORF9		DNA replication – viral DNA polymerase	(Lin et al., 1998)
ORF6	SSB	DNA replication – single strand DNA binding protein	(Wu et al., 2001)
ORF16	vBCL2	Anti-apoptotic – inhibits BAX-mediated and virus-induced apoptosis	(Sarid et al., 1997)
K7	vIAP	Anti-apoptotic – protects against apoptosis induced by various stimuli	(Wang et al., 2002a)
K2*	vIL6	Binds gp130 directly to activate the JAK/STAT signalling pathway Anti-apoptotic – inhibits IFN-mediated apoptosis Immune evasion – inhibition of chemokine-driven recruitment of neutrophils	(Fielding et al., 2005)
K6* K4*	vCCL1 vCCL2	Immune evasion – skewing of CD4 T-cell response to Th2-type (less effective against intracellular pathogens)	(Kledal et al., 1997; Dairaghi et al., 1999; Weber et al., 2001)
K5*	MIR2	Immune evasion – downregulates expression of MHC-I, CD86, ICAM1, PECAM1 and CD1d	(Coscoy and Ganem, 2000)
K14*	vCD200 or vOX2	Immune evasion – inhibits myeloid cell activation, reduces production of Th1-attracting cytokines, inhibits neutrophil function	(Foster-Cuevas et al., 2004; Rezaee et al., 2005)

TABLE 1.1: KSHV ORFs. * indicates genes that were contained in the KSHV lentiviral library prior to October 2006**;** **indicates known or putative immunogenic KSHV genes cloned into lentiviral expression vectors and added to the lentiviral library in the course of this research (see section 3.1.a).

KSHV ORF	Gene Product	Function(s)	Reference(s)
ORF45*		Immune evasion – disruption of host anti-viral interferon response by interfering with IRF-7 activity	(Zhu et al., 2002)
ORF74*	vGPCR	Binds IL8; constitutively active GPCR Immune evasion – downregulates Toll-like receptor 4	(Couty and Gershengorn, 2004) (Lagos et al., 2008)
ORF58*		Unknown function, homologue of EBV envelope protein BMRF2	(Russo et al., 1996)
Early Lytic			
ORF59**	PF8	DNA replication –processivity factor, needed for efficient extension by the viral DNA polymerase	(Lin et al., 1998)
ORF61**		DNA replication – large ribonucleotide reductase	
K4.1*	vCCL3	Immune evasion – skewing of CD4 T-cell response to Th2-type	(Stine et al., 2000)
K3*	MIR1	Immune evasion – downregulates expression of MHC-I and CD1d	(Coscoy and Ganem, 2000)
K9*	vIRF1	Immune evasion – disruption of host anti-viral interferon response; downregulates MHC-I; downregulates Toll-like receptor 4	(Gao et al., 1997; Lagos et al., 2007; Lagos et al., 2008)
ORF4	CBP or KCP	Immune evasion – inhibition of complement	(Spiller et al., 2003)
ORF49*		Activates the JNK and p38 MAP kinase pathways	(Gonzalez et al., 2006)
ORF8**	gB	Structural – glycoprotein B	
ORF65**		Structural – capsid-interacting protein	(Nealon et al., 2001)
Late Lytic			
K1*		Immune evasion – downregulates B-cell receptor surface expression	(Lee et al., 2000)
ORF28*		Structural - envelope glycoprotein	(Zhu et al., 2005)
ORF33*		Structural? – tegument protein, homologue of EBV BGLF2	(Russo et al., 1996)
ORF36*		Serine protein kinase - activates the JNK pathway	(Hamza et al., 2004)
ORF37*	SOX	Immune evasion – host mRNA shut off	(Glaunsinger and Ganem, 2004)
K8.1*	gp35/37	Structural – glycoprotein 35/37	
ORF22	gH	Structural – glycoprotein H	
ORF25		Structural – major capsid protein	(Nealon et al., 2001)
ORF26		Structural – minor capsid protein	(Nealon et al., 2001)

TABLE 1.1 continued: KSHV ORFs. * indicates genes that were contained in the KSHV lentiviral library prior to October 2006**;** **indicates known or putative immunogenic KSHV genes cloned into lentiviral expression vectors and added to the lentiviral library in the course of this research (see section 3.1.a).

is a potent inhibitor of angiogenesis and was previously known to be downregulated in KS lesions although no KSHV protein had been identified that mediated this suppression (Taraboletti et al., 1999). Another study used multiple screens to identify changes in mRNA transcript levels under different conditions of miRNA expression or inhibition in a variety of different cell types (Ziegelbauer et al., 2009). They identified a BCL2-associated factor, BCLAF1, as a target for the KSHV miRNAs, in particular mir-K5. BCLAF1 inhibition sensitised latently infected cells to stimuli inducing lytic replication, and the authors suggested that this activity thus plays an important role in the KSHV life cycle, since latent infection alone does not permit viral replication or the dispersal of virions to new cells.

1.3 The T-cell Immune Response Against KSHV

In immunocompetent hosts, KSHV can establish persistent, asymptomatic infection. KSHV infection has been reported in three to 20 percent of US blood donors (Lennette et al., 1996; Pellett et al., 2003) and 40 to 80 percent of the general population of East African countries (Lennette et al., 1996; Gao et al., 1996; Hladik et al., 2003). However, upon immunosuppression (acquired or iatrogenic) both the seroprevalence of KSHV, and the incidence of KS in KSHV carriers, significantly increase. KS is more than 100 times more common in immunosuppressed individuals such as HIV-infected individuals and transplant recipients compared to immunocompetent individuals (Boshoff and Weiss, 2002). This indicates that loss of immune control allows KSHV-infected cells to proliferate unchecked and KSHV-related tumours to develop. Furthermore, a dramatic clinical improvement is seen in iatrogenic KS patients when general immunity is restored following the

withdrawal of immunosuppressive drugs (Nagy et al., 2000), and spontaneous tumour regression has been reported in individuals with AIDS-KS after starting anti-HIV therapy (Cattelan et al., 1999). Recent evidence indicates that a positive clinical outcome is associated with the restoration of KSHV-specific immunity (Bourboulia et al., 2004; Bihl et al., 2007a). Thus, successful immunity targeted against KSHV plays a key role in containing KSHV infection, enabling the virus to establish controlled life-long infection and to co-exist with its host.

Establishing such an equilibrium with the host's immune system is a common biological feature of human herpesviruses, with T cells mediating chronic infection by recognising and eliminating infected cells (Rickinson and Moss, 1997; Komanduri et al., 1998). Identifying the targets of the host's cellular immune responses is important to our understanding of how herpesvirus infections are controlled in immunocompetent individuals and is a crucial step towards developing treatments such as immunotherapies, or even vaccines, against herpesvirus-related diseases. The adaptive T-cell immune responses against many human herpesviruses are well characterised. For example, Epstein Barr virus [(EBV), a γ-herpesvirus closely related to KSHV] infects 80-90% of the adult population and several EBV genes that elicit both CD4 and CD8 T-cell responses have been described (Landais et al., 2005a). The CD8 responses to EBV are heavily skewed towards EBV genes expressed early in the lytic cycle (Pudney et al., 2005). However, the KSHV-specific T-cell immune responses remain poorly described.

T-cell responses to KSHV have been studied mostly in KS patients and asymptomatic carriers of KSHV. Responses have been detected against several lytic

and latent proteins (Osman et al., 1999; Brander et al., 2001; Wang et al., 2001b; Wilkinson et al., 2002; Micheletti et al., 2002; Stebbing et al., 2003b; Woodberry et al., 2005; Lambert et al., 2006; Ribechini et al., 2006; Guihot et al., 2006; Bihl et al., 2007b). Some of these responses have been demonstrated to be functionally cytotoxic *in vitro* (by the specific lysis of target cells expressing KSHV ORFs by cytotoxic T lymphocytes (CTLs) from KSHV-seropositive individuals) (Osman et al., 1999; Wang et al., 2000); and there is some evidence they exert evolutionary pressure on the virus *in vivo* (Stebbing et al., 2003b). A few HLA-restricted KSHV-specific T-cell epitopes have been identified [(Brander et al., 2001; Wilkinson et al., 2002; Micheletti et al., 2002; Wang et al., 2002b; Stebbing et al., 2003b; Lambert et al., 2006; Ribechini et al., 2006; Guihot et al., 2006; Bihl et al., 2007b); summarised in Table 1.2]. However, these are almost exclusively CD8 epitopes and they elicit weak T-cell responses compared to epitopes from other viruses such as HIV-1 and EBV (Brander et al., 2001; Bihl et al., 2007b). Neither the breadth of the antigenic repertoire of the KSHV-specific T-cell immune response, nor its immunodominant targets are fully understood.

1.3.a The CD8 T-cell Response Against KSHV

i. CD8 T-cell Responses to Primary Infection

Unlike primary EBV infection, which in adolescents and adults often results in acute infectious mononucleosis, primary KSHV infection is not apparently associated with any specific severe illness. Studies of cohorts at risk of KSHV infection have identified adults and children undergoing KSHV seroconversion and

Expression	KSHV ORF	Epitope Position	Epitope Sequence	HLA Restriction	Other Responsive HLA Types	Reference
T-cell subtype not determined						
Latent(?) and Lytic	K10.5	Not determined	Not determined	N/A	N/A	Bihl et al., 2007b
CD4						
Latent and induced in Lytic Cycle	*K12	aa46-60	RGPVAFRTRVATGAH	Not determined	N/A	Guihot et al., 2006
	*K15	aa171-185	GNIKLVSSVSFICAG	Not determined	N/A	Guihot et al., 2006
Late Lytic	*K8.1	Not determined	Not determined	N/A	N/A	Barozzi et al., 2008
CD8						
Latent	*ORF 73	aa71-79 aa238-246 aa1116-1124	FTSGLPAFV WATESPIYV QMARLAWEA	A2 A2 A2	A26/A29 A26/A29; A39/A69 A26/A29; A2/A69	Guihot et al., 2006 Guihot et al., 2006 Guihot et al., 2006
Latent and induced in Lytic Cycle	*K12	aa17-25 aa16-25 aa31-45	LLNGWRWRL VLLNGWRWRL LVCLLAISVVPPSGQ	A*0201 A2 B7	None identified None identified B7/B53; B7/B14; B35/B49; B7/B51	Brander et al., 2001 Micheletti et al., 2002 Guihot et al., 2006
	*K15	aa123-131 aa155-163 aa166-175	ILFTSTFAV FTLSLPFLY ATVKTGNIKL	A2 B44 B7	A2/A24 B44/B49 None identified	Guihot et al., 2006 Guihot et al., 2006 Guihot et al., 2008
Immediate-Early Lytic	*K5	aa154-163	ALYAANNTRV	A2	None identified	Ribechini et al., 2006
	ORF 6	aa1050-1058	VLGDEVLSL	A*0201	None identified	Lambert et al., 2006
	*ORF 57	aa212-220	ISARGQELF	B57	B58?	Bihl et al., 2007b

TABLE 1.2: Known immunogenic KSHV ORFs and CD8 and CD4 epitopes. * indicates ORFs included in the KSHV lentiviral library.

Expression	KSHV ORF	Epitope Position	Epitope Sequence	HLA Restriction	Other Responsive HLA Types	Reference
CD8						
Early Lytic	*K3	aa72-81	LPRLTYQEGL	B7	None identified	Ribechini et al., 2006
		aa96-105	GLAAATWVWL	A2	None identified	Ribechini et al., 2006
		aa127-137	FVFYQLFVV	A2	None identified	Ribechini et al., 2006
	*ORF 8	aa492-500	LMWYELSKI	A2	None identified	Wang et al., 2002b
	*ORF 61	aa505-513	GLADVFAEL	A*0201	None identified	Lambert et al., 2006
	*ORF 65	aa35-43	NMSQAEYLV	A*0201	None identified	Lambert et al., 2006
	ORF 70	aa108-116	VVQELLWFL	A2	None identified	Ribechini et al., 2006
		aa255-264	SLLTYMLAHV	A2	None identified	Ribechini et al., 2006
		aa259-267	YMLAHVTGL	A2	None identified	Ribechini et al., 2006
		aa236-245	LYQRSGDMGL	A24	None identified	Ribechini et al., 2006
		aa253-261	SYSLLTYML	A24	None identified	Ribechini et al., 2006
		aa296-305	TPRPFPRLEI	B7	None identified	Ribechini et al., 2006
Late Lytic	*K1	aa58-66	FRLTERTLF	Cw3	None identified	Stebbing et al., 2003b
		aa82-90	HRQSIWITW	B*2702	None identified	Stebbing et al., 2003b
		aa91-99	YPQPVLQTL	B51	None identified	Stebbing et al., 2003b
		aa93-101	QPVLQTLCA	B55	None identified	Stebbing et al., 2003b
	*K8.1	aa131-145	ELTDALISAFSGSYS	A24	None identified	Wilkinson et al., 2002
		aa211-225	LILYLCVPRCRRKKP	Not determined	N/A	Wilkinson et al., 2002
		aa209-217	LVLILYLCV	A2	None identified	Bourboulia et al., 2004
	ORF 22	aa59-68	FLNWQNLLNV	A2	None identified	Micheletti et al., 2002
	ORF 25	Not determined	Not determined	N/A	N/A	Wang et al., 2000
	ORF 26	aa103-111	FQWDSNTQL	A2	None identified	Ribechini et al., 2006
		aa125-134	IVLESNGFDL	A2	None identified	Ribechini et al., 2006
		aa203-211	VLDDLSMYL	A2	None identified	Ribechini et al., 2006

TABLE 1.2 continued: Known immunogenic KSHV ORFs and CD8 and CD4 epitopes. * indicates ORFs included in the KSHV lentiviral library.

report occasional mild symptoms of fatigue, diarrhoea, fever and localised rash (Wang et al., 2001b; Andreoni et al., 2002). This lack of notable symptoms makes identifying primary KSHV infection a challenge, and thus little is known about the T-cell response to KSHV at acquisition. One 15-year longitudinal study of HIV-negative adults at risk of KSHV infection identified five individuals who had seroconverted during the study period and retrospectively analysed cryopreserved PBMCs from blood samples taken from these individuals for several years before and after KSHV seroconversion (Wang et al., 2001b). The authors first looked for global changes in T-cell phenotype around the time of KSHV seroconversion, as expansion of activated CD8 T cells is a classic finding in EBV mononucleosis in adults (Callan et al., 1996; Lynne et al., 1998). They observed no generalised expansion of CD8 or CD4 T-cell populations after primary KSHV infection, and no changes in the expression of naïve and memory markers CD45RA and CD45RO, or of activation and costimulatory markers CD38, CD28 and HLA-DR (Wang et al., 2001b). They additionally observed no immunosuppressive effects of primary KSHV infection as measured by responses to mitogen and a nominal recall antigen, in contrast to the transient immunosuppression associated with primary symptomatic EBV infection (Mangi et al., 1974; Perez-Blas et al., 1992). The authors were, however, able to detect KSHV-specific CD8 T-cell responses (measuring CTL precursor frequency by limiting dilution and IFNγ production) directed against a broad spectrum of immediate-early, early and late lytic KSHV ORFs (cloned into recombinant vaccinia viruses). Such responses were observed at the time of primary KSHV infection in all KSHV seroconverters. The authors observed no dominant CTL target between the study subjects (Wang et al., 2001b).

A second study examined two HLA-A2-positive KSHV-negative recipients of kidneys from KSHV-positive donors. Both recipients remained consistently clear of detectable KSHV infection (by serology and PCR) for 24 and 11 months after transplantation respectively. Strikingly, CD8 T cells specific for both lytic and latent KSHV ORFs were detected in one of these recipients (by staining with HLA-A2-restricted KSHV-specific tetramers based on peptides shown in Table 1.2) whose donor was also HLA-A2-positive. The second recipient, whose donor was HLA-A2-negative, showed no detectable response. The authors therefore suggest that KSHV-specific CTLs are restricted by shared donor/recipient HLA alleles, in this case HLA-A2. In the first recipient, the majority of tetramer-positive CD8 T cells were of a terminally differentiated effector memory phenotype (CD45RA+CCR7-) and expressed perforin, indicating that the generation of a functional KSHV-specific CTL response can lead to abortive infection (Lambert et al., 2006). In keeping with this, KSHV-specific T-cell responses have also been detected in KSHV-seronegative individuals defined as being at high risk of KSHV infection [responses measured by IFNγ release by whole PBMCs after stimulation with overlapping peptides spanning the entire length of a latent (ORF73) and a lytic (ORF65) gene product] (Woodberry et al., 2005). The authors argued that overall evidence from this study indicated that those individuals who did not show a detectable KSHV serologic response but who showed positive KSHV-specific T-cell responses were indeed KSHV-infected. It is possible, therefore, that a very low level of viral replication may be sufficient to prime a KSHV-specific T-cell immune response that may confer protection against chronic KSHV infection. However, it is perhaps more likely that the current serological and PCR-based methods for

detecting KSHV infection are not sensitive enough to identify low-level latent infection.

ii. Frequency and Diversity of CD8 T-cell Responses in Asymptomatic Infection and Disease

Following seroconversion, the frequencies of both CD8 CTL precursors and CD8 IFNγ-producing cells directed against lytic KSHV antigens increase to a peak one to two years after primary infection, after which they decrease in correlation with declines in antibody titres, possibly due to decreased viral replication and lower antigenic burden (Wang et al., 2001b). One study found that T-cell responses to KSHV increased with viral load in the peripheral blood and were more readily detectable in individuals with active KS than those who did not present with active KS (Woodberry et al., 2005). However, other groups have been unable to confirm this and there is otherwise strong evidence (discussed below) that high levels of KSHV-specific CD8 T-cell responses confer protection against KS oncogenesis. Responses to KSHV CD8 peptides have been found to be of higher frequency and of greater diversity in their antigenic repertoire in asymptomatic carriers of KSHV compared to those with either AIDS-related, classic or iatrogenic KS (Lambert et al., 2006; Guihot et al., 2006; Barozzi et al., 2008). KSHV-specific T-cell responses (measured by IFNγ release by whole PBMCs in response to pools of nine-, 10-, and 20-mer peptides and recombinant proteins from lytic and latent gene products) appeared concurrent with clinical improvement in iatrogenic KS patients after a reduction of their immunosuppressive therapy or a conversion from calcineurin inhibitors (which block calcineurin-activated transcription of IL2) to sirolimus (also

known as rapamycin, which acts through mTOR to inhibit responses to IL2) (Barozzi et al., 2008). A longitudinal study of an iatrogenic KS patient who presented with recurrent episodes of remission and relapse of KS lesions found a correlation between reduced frequency of KSHV-specific CD8 T cells and recurrence of active KS (Lambert et al., 2006). Furthermore, both the magnitude and the frequency of responses to KSHV CD8 peptides increase with immune reconstitution through HAART, which apparently correlates with spontaneous KS regression (Wilkinson et al., 2002; Bourboulia et al., 2004).

To address whether low frequencies of KSHV-specific CD8 T cells in the peripheral blood of KS patients is due to recruitment of these cells to the site of the tumour, one group performed *in situ* tetramer staining and confocal laser scanning microscopy on KS biopsy specimens from two patients who had detectable circulating KSHV-specific CD8 T cells at the time of biopsy. They found large numbers of KSHV-tetramer-negative CD8 T-cell infiltrates in the vicinity of LANA1-positive spindle cells, but observed very few CD8 T cells that costained with KSHV tetramers. The few tetramer-positive CD8 infiltrates that were seen were mainly found in LANA1-negative tissue (Lambert et al., 2006). Thus, in this one small study, KSHV-tetramer-specific CD8 T cells do not appear to be preferentially recruited to inflammed tumour tissue. Further investigation is warranted to confirm these findings and to understand their biological relevance.

Together, the above data indicate that KS oncogenesis is associated with loss of CD8 T cell-mediated control of KSHV-infected cells. Interestingly, a recent study investigating KSHV-specific CD8 T-cell responses in multicentric Castleman's

disease (MCD) found that individuals with MCD had similar frequencies of KSHV-specific CD8 T-cell responses, and these were directed against a similar antigenic repertoire, as compared to asymptomatic KSHV carriers (Guihot et al., 2008). These findings were corroborated by a second, smaller study which reported high numbers of IFNγ-secreting KSHV-specific CD8 T-cells in two individuals with MCD (Barozzi et al., 2009). This is in direct contrast to what is observed in KS, and indicates that whilst KSHV-specific CD8 T cells may confer protection against the emergence of KS, they do not apparently protect against the development of MCD.

iii. Functional Properties and Phenotypes of KSHV-specific CD8 T Cells in Asymptomatic Infection and Disease

The precise role of KSHV-specific CD8 T cells in the pathogenesis or control of KSHV-related diseases may additionally depend on the functional properties and differentiation phenotypes of these cells. EBV-specific and HIV-specific CD8 T cells have been shown to produce a range of cytokines besides interferon IFNγ (Lichterfeld et al., 2004), and there is evidence that polyfunctional T-cell responses may be a correlate of control of HIV-infection (Betts et al., 2006). However, little is known about the functionality of KSHV-specific CD8 T cells in asymptomatic infection and disease. One study compared cytokine release by CD8 T cells from four individuals with AIDS-KS that spontaneously regressed after initiation of HAART ('KS non-progressors') with cytokine release by CD8 T cells from three individuals with AIDS-KS that progressed and required additional chemotherapy despite initiation of HAART ('KS progressors') (Bihl et al., 2009). They found that KSHV-specific CTL responses from KS non-progressors were more frequently

polyfunctional (production of both IFNγ and TNFα) than CTL responses from KS progressors. By contrast, KSHV-specific CD8 T cells from both individuals with MCD and asymptomatic carriers of KSHV were demonstrated to be polyfunctional (secretion of two or more of IFNγ, IL2, TNFα, MIP1ß or mobilisation of CD107a) after stimulation with pools of both lytic and latent peptides (Guihot et al., 2008). Another study compared the functionality of KSHV-specific CD8 T cells directed against lytic or latent antigens in HIV-positive asymptomatic carriers of KSHV. They assessed T-cell release of IFNγ and TNFα, as well as the ability to degranulate (as measured by mobilisation of CD107a to the cell surface) after antigen contact. Multifunctional KSHV-specific T cells were more frequently detected in CD8 T-cell populations that responded to latent antigens than those targeted to lytic antigens, in keeping with observations in EBV-specific T cells (Bihl et al., 2007b).

Human memory CD8 and CD4 T cells can be divided into phenotypic subsets based on their functions and expression of certain cell surface markers. These subsets are broadly defined as central memory (T_{CM}), effector memory (T_{EM}) and terminally differentiated effector memory ($T_{EMRA;}$ a subset of CD8 T cells only) (Sallusto et al., 2004). Effector memory T cells can be further subdivided into early-, intermediate- and late- effector memory cells (van Baarle et al., 2002). T_{CM} cells have little or no effector function but migrate to lymph nodes and readily proliferate and differentiate into T_{EM} cells in response to stimulation by their specific antigen (Sallusto et al., 1999). T_{EM} cells migrate to inflamed tissues and are characterised by rapid effector function upon antigenic stimulation. Early and intermediate T_{EM} cells have low cytolytic activity and maintain high proliferative capacity. Late T_{EM} cells have less proliferative capacity but strong effector cytolytic function (van

Baarle et al., 2002; Appay et al., 2002). T_{EMRA} cells are a unique subclass of CD8 T_{EM} cells that are fully differentiated, strongly cytolytic, carry large amounts of perforin, and are non-replicative. They are a hallmark of a prolonged immune response (Sallusto et al., 2004). Very few CD4 T_{EM} cells reexpress CD45RA and so T_{EMRA} cells are generally not defined as a separate subset of CD4 T cells.

The expression of several cell-surface molecules can be used to define memory subsets. Among the most commonly used of these are CD45RO, CD45RA, CCR7, CD27 and CD28. CD45RO is expressed in response to antigen stimulation. It is therefore absent in naïve T cells, but present in both T_{CM} and T_{EM}, but lost again on differentiation to T_{EMRA}. CD45RA is expressed by naïve cells, absent in T_{CM} and T_{EM}, and reexpressed by T_{EMRA} as their defining feature, signified by the 'RA' annotation. CCR7 is a lymphoid-homing receptor, and thus is expressed by naïve and T_{CM} cells, but lost by T_{EM} and T_{EMRA} cells. CD27 and CD28 are costimulatory molecules that are expressed by naïve and T_{CM} cells and gradually lost by T_{EM} cells, providing markers for early (CD27+CD28+), intermediate (CD27+CD28-) and late (CD27-CD28-) T_{EM} subdivisions (Sallusto et al., 1999; van Baarle et al., 2002; Appay et al., 2002; Sallusto et al., 2004). CD8 T-cell marker expression is summarised in Figure 1.3. Different viral infections are associated with varying representation of memory subsets by both the circulating T-cell population as a whole and by the virus-specific T cells. In certain viral infections, the representation of memory subsets further varies in chronic infection and disease (Appay et al., 2002). For example, HIV infection is associated with dramatically decreased proportions of naïve and central memory cells within the total circulating CD8 T-cell population and a correspondingly increased proportion of effector memory and

terminally differentiated effector memory cells (Chen et al., 2001). Within the HIV-specific CD8 population, cells are predominately CD45RA-CCR7- effector memory cells, and show defective differentiation towards CD45RA+CCR7- terminally differentiated effector cells (Champagne et al., 2001).

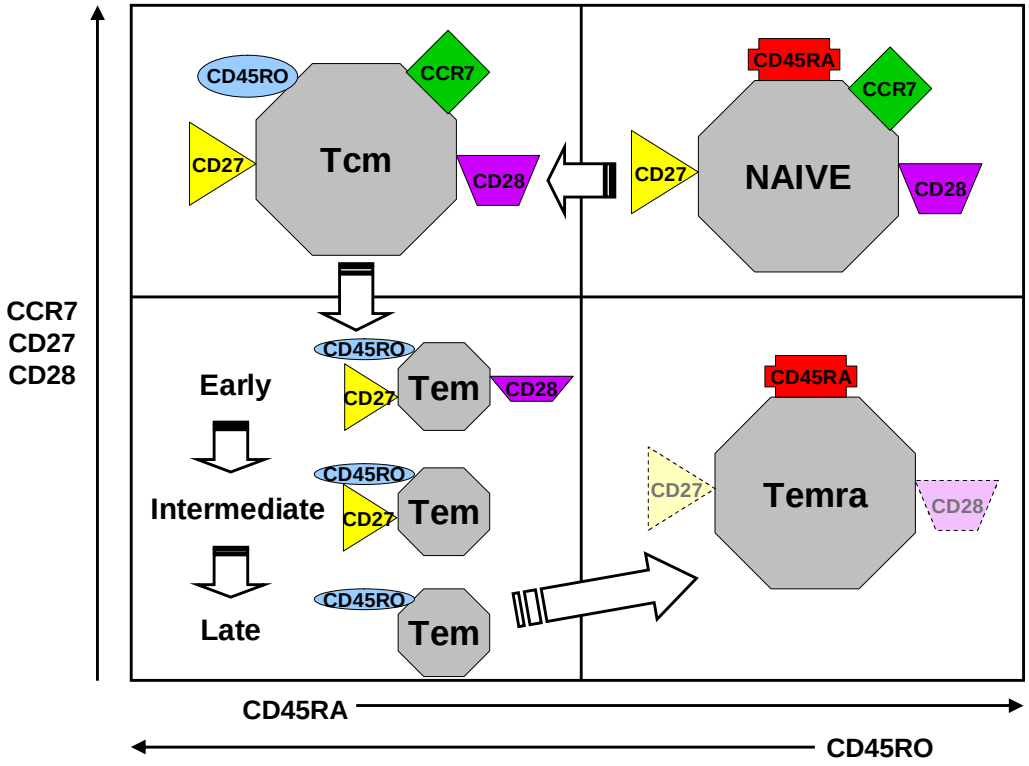


FIGURE 1.3: Schematic illustration of a model for the differentiation of CD8 T cells showing cell-surface marker expression. Open arrows indicate the path of differentiation upon antigen exposure (naïve > T_{CM}) and re-exposure to antigen (T_{CM} > Early T_{EM} > Intermediate T_{EM} > Late T_{EM} > T_{EMRA}). Naïve CD8 T cells lose expression of CD45RA and express CD45RO on differentiation to T_{CM}. Differentiation from T_{CM} to effector memory T_{EM} is characterised by sequential loss of expression of CCR7, CD28 and CD27. Finally, terminally differentiated effector memory CD8 T cells lose expression of CD45RO and reexpress CD45RA, and are thus known as T_{EMRA} cells. Some T_{EMRA} cells may express CD27 and CD28, indicated by the dashed lines.

In a study of three iatrogenic KS patients, conversion of their immunosuppressive drug regime from calcineurin inhibitors to sirolimus (also known as rapamycin, as described on pages 52-53) led to an increase in the frequency of naïve and central

memory T cells in the general population of circulating CD8 T cells in conjunction with KS regression (Barozzi et al., 2008). A very recent study found, unexpectedly, that treating mice with rapamycin (previously classified as an immunosuppressive as it inhibits responses to IL2) following infection with lymphocytic choriomeningitis virus (LCMV) enhanced both the quality and quantity of central memory virus-specific T cells (Araki et al., 2009). Thus converting these iatrogenic KS patients to sirolimus may be acting to promote an enrichment of their central memory CD8 T cell pool, and these cells may be playing an important role in keeping KSHV-infected cells in check and promoting regression of KS lesions.

There are few studies of the differentiation phenotype of KSHV-specific CD8 T cells. The few reports to date are somewhat contradictory and on the whole rather limited, particularly in terms of sample numbers. This, coupled with the complication of the background of immunosuppression (acquired or iatrogenic) in which the studies are conducted, means that a definitive understanding of the differentiation phenotype of KSHV-specific T cells remains elusive.

Six transplant recipients who spontaneously controlled KSHV infection had significantly higher proportions of CD45RA+CCR7- T_{EMRA} KSHV-specific cells and significantly lower proportions of CD45RA-CCR7- T_{EM} KSHV-specific cells compared to seven patients with active KS or five patients with KS in remission (Lambert et al., 2006). In six MCD patients, the majority of KSHV-specific CD8 T cells were also CD45RA-CCR7- T_{EM} cells. However, in this study the dominance of this phenotype did not differ from that of seven HIV-positive asymptomatic carriers of KSHV. Furthermore, in the MCD patients, a significantly higher

proportion of KSHV-specific CD8 T cells were found to be CD45RA-CCR7-CD27- late effector memory T cells and a correspondingly lower proportion were CD45RA-CCR7-CD27+ early and intermediate effector memory T cells, as compared to asymptomatic carriers (Guihot et al., 2008). This more differentiated phenotype correlated with increased viral load, and was postulated to result from high antigenic burden.

In primary symptomatic EBV infection (infectious mononucleosis), EBV-specific CD8 T cells show a uniform CD45RA-CCR7- T_{EM} phenotype. However, with the regression of infectious mononucleosis and the establishment of chronic asymptomatic infection, the phenotype of EBV-specific CD8 T cells depends upon whether the cell targets a latent or a lytic EBV antigen (Hislop et al., 2002). CD8 T cells directed against latent EBV antigens are predominately of a CD45RA-CCR7- T_{EM} phenotype with a low proportion of cells of a CD45RA+CCR7- T_{EMRA} phenotype. By contrast, CD8 T cells directed against lytic EBV antigens are predominately CD4RA+CCR7- T_{EMRA} cells, with a lower proportion of cells that are CD45RA-CCR7- T_{EM} cells (Hislop et al., 2001; Hislop et al., 2002; Catalina et al., 2002).

Two studies have compared the phenotypes of KSHV-specific T cells targeting either lytic or latent antigens. One group reportedly observed no difference between the differentiation phenotype of KSHV-specific CD8 T cells targeted against latent or lytic antigens (Lambert et al., 2006). However, it was not made clear whether or not they compared T cells specific for different antigens from within the same individual, or if they only compared lytic-antigen-specific T cells from one

individual with latent-antigen-specific T cells from another individual. Neither was it clear how many responses were investigated or in how many individuals.

Another group reported that the phenotype of KSHV-specific T cells targeting lytic or latent antigens concurs with the differences seen in EBV-specific T cells directed against latent or lytic antigens (Bihl et al., 2007b). They compared five KSHV lytic-antigen-specific T-cell populations to four KSHV latent-antigen-specific T-cell populations. Again, it was not made clear if these were from within the same individual or from different individuals. The authors reported that CD8 T cells that recognised latent KSHV antigens were predominately of a CD45RA-CCR7- T_{EM} phenotype with a low proportion of cells of a CD45RA+CCR7- T_{EMRA} phenotype, whereas a much higher proportion of CD8 T cells directed against lytic KSHV antigens were CD45RA+CCR7-. Interestingly, KSHV-specific T cells were overall more differentiated (higher proportions of CD45RA+CCR7- cells) than EBV-specific T cells (Bihl et al., 2007b).

1.3.b The CD4 T-cell Response Against KSHV

The CD4 T-cell response against KSHV remains largely unexplored. Although some studies have looked at responses to KSHV by mixed CD8 and CD4 T-cell populations (Woodberry et al., 2005; Bihl et al., 2007a; Bihl et al., 2007b), there has been almost no investigation specifically into the CD4 T-cell response against KSHV. One of the studies with mixed T cells used intracellular cytokine staining to determine whether the observed responses were mediated by CD4 or CD8 T cells. However, they found that the responses were mostly CD8 T-cell mediated with only

two samples out of 11 showing borderline CD4 T-cell reactivity (Bihl et al., 2007b). They did not state which of the two antigens they were testing [ORF57 (lytic) and ORF73 (latent)] initiated these CD4 responses. Another group reported the identification of two CD4 T-cell epitopes (the only ones described to date) from within the latent antigens K12 and K15 in one individual with AIDS-KS using an IFNγ enzyme-linked immunospot (ELIspot) assay (Guihot et al., 2006). The longitudinal study of three iatrogenic KS patients described in the CD8 response section above (pages 52 and 57) reported the emergence of CD4 responses to K12 (latent) and K8.1 (lytic) in conjunction with KS regression in two of these three individuals (Barozzi et al., 2008). The one individual in which no KSHV-specific CD4 responses were observed was the only one out of the three that did not achieve full remission of their KS. The authors suggested that this was indicative of the importance of KSHV-specific CD4 responses in controlling KSHV infection. Although the small sample number and limited number of antigens make it difficult to reach a firm conclusion from this study, it seems likely that CD4 T cells do play a key role in the immune response against KSHV.

The final phases of KSHV virion assembly occur in the endosomal cellular compartments with extensive targeting of viral proteins to endosomes. Thus viral proteins can be efficiently processed through the intracellular endosome pathway, resulting in the presentation of CD4-specific viral epitopes through MHC-II to helper T cells. Processing of the EBV antigen EBNA1 for presentation in the context of MHC-II is also known to occur through the autophagy pathway (Paludan et al., 2005). Furthermore, the presentation of the EBV antigens EBNA2, EBNA3C and BHRF1 through MHC-II occurs by intercellular transfer of an antigenic moiety

(Landais et al., 2005b; Taylor et al., 2006; Taylor and Rickinson, 2007). This process does not require cell contact, and the antigenic particle is taken up by neighbouring cells and processed as exogenous antigen for MHC-II-mediated presentation. It seems reasonable that one or all of these pathways may also be used for the presentation of KSHV antigens through MHC-II.

The lack of known CD4 epitopes or antigens has limited studies into the association between KSHV-specific CD4 responses and the control of KSHV or the development (and subsequent resolution) of KSHV-related neoplasms. Low CD4 counts are associated with the incidence of KS, and KS can spontaneously regress with immune reconstitution through HAART. A weak association was reported between increased CD4 counts after starting HAART and reconstitution of KSHV-specific immune responses (by PBMCs to overlapping peptides from lytic and latent antigens) (Bihl et al., 2007a). Interestingly, however, clinical improvement of KS after initiation of HAART was not found to be associated with increased CD4 count although it was significantly related to decreased HIV viral load (Gill et al., 2002). In primary symptomatic EBV infection (infectious mononucleosis) there is a significant, early CD4 T-cell response which, although lower in size than the primary CD8 response to EBV, is comparable to other early CD4 responses to viral infections such as lymphocytic choriomeningitis virus (LCMV) infection in mice and HIV in humans (Amyes et al., 2003). After this primary burst, the frequency of EBV-specific CD4 T cells drops off significantly, leaving a small population of EBV-specific CD4 T cells that persists during chronic infection (Amyes et al., 2003). Peripheral blood EBV viral load correlates with the frequency of EBV-specific CD4 T cells, both at presentation with infectious mononucleosis and on

establishment of chronic infection, indicating the EBV-specific CD4 responses are antigen driven (Precopio et al., 2003). Immunocompetent individuals with EBV-positive lymphomas have decreased EBV-specific CD4 responses compared to both healthy EBV carriers and EBV carriers with EBV-negative lymphomas (Heller et al., 2008).

The absence of known targets of the KSHV-specific CD4 response has also restricted the investigation of the functionality and the differentiation phenotypes of KSHV-specific CD4 T cells in chronic infection and disease. In the three iatrogenic KS patients mentioned above (pages 52, 57, and 61), complete KS regression in two of the patients was associated with an expansion of the naïve and central memory compartments of the total circulating CD4 T-cell population. In the third patient, who did not achieve complete resolution of their KS, there was no enrichment of their central memory CD4 T-cell compartment, consistent with these cells playing a role in KS control. EBV-specific CD4 T cells are predominately CD45RA-CCR7-CD28+CD27+ (early effector memory phenotype) (Amyes et al., 2003). By contrast, CMV-specific CD4 T cells are predominately of a later effector memory phenotype, CCR7-CD27-, with heterogenous expression of CD28 and CD45RA (Amyes et al., 2003; Gamadia et al., 2004). The majority of both EBV- and CMV-specific CD4 T cells are Th1-polarised and produce the cytokines IFNγ and TNFα (Bickham et al., 2001; Gamadia et al., 2004). Limited production of Th2-type cytokines IL4, IL5 and IL13 by EBV EBNA1-specific T cells has also been reported in a of minority EBV-positive donors (Steigerwald-Mullen et al., 2000; Bickham et al., 2001). Of note, the sensitivity of Th1 versus Th2 cytokine production by helper T cells to subtle variations in culture conditions must always

be considered in such investigations, particular when comparing results from different studies.

1.3.c γδ T cells in the Control of KSHV

CD4 and CD8 T cells make up the majority of CD3 T cells found in the body and are both characterised by T-cell receptors comprised of an α-chain and a β-chain. A small proportion of CD3 T cells have T-cell receptors made up of a γ-chain and a δ-chain and are thus known as γδ T cells. γδ T cells typically account for less than five percent of circulating T cells, but are enriched in epithelial-rich tissues such as the skin and intestines (Carding and Egan, 2002). There are two main sub-types of γδ T cells designated Vδ1 and Vδ2. Vδ2 are the majority of γδ T cells found in healthy adults. They are found in the peripheral blood and thought to play a role in defence against intracellular pathogens and haematological malignancies (Fisch et al., 1997). Vδ1 are the minority overall in adults, but predominate at epithelial sites such as the intestine and skin where they are suggested to provide first-line defence against viral infections (Deusch et al., 1991; Maeurer et al., 1996). In certain disease states, the representation of Vδ1 and Vδ2 shifts dramatically, for example in HIV-1 infection Vδ2 cells are lost and Vδ1 cells expand (De Paoli et al., 1991; De Maria et al., 1992). Although the significance of such changes is not understood, they imply a role for γδ T cells in anti-viral immune responses (Carding and Egan, 2002). Unlike αβ T cells, γδ T cells do not recognise MHC-bound peptides but instead bind their respective antigens in a manner more similar to antibodies, independent of MHC presentation and more dependent on the conformational shape of the antigen (Girardi, 2006). They recognise a range of both protein- and non-

protein-derived antigens (Carding and Egan, 2002), including microbial products and stress-induced self antigens, and thus are suggested to represent a bridge between innate and adaptive immunity (Holtmeier and Kabelitz, 2005).

One group has examined the involvement of γδ T cells in the control of chronic KSHV infection (Barcy et al., 2008). The authors observed a significant expansion of γδ T cells of the Vδ1 subtype in the peripheral blood of HIV-negative asymptomatic carriers of KSHV as compared to age-matched, HIV-negative, KSHV-negative healthy controls. Vδ1 T-cell expansion has been previously described in two instances: in all stages of HIV infection and in transplant recipients with active CMV infection (Dechanet et al., 1999). Asymptomatic KSHV infection is, therefore, the second viral infection (after HIV) in which specific, long-lasting Vδ1 T-cell expansion is observed during chronic stages of infection, and the only viral infection in which Vδ1 expansion has been documented in immunocompetent individuals. Barcy and colleagues further found that in asymptomatic carriers of KSHV, the γδ Vδ1 T-cell subpopulation displayed an increase in the relative frequency of cells expressing an effector phenotype compared to KSHV-negative controls. The same trend was seen in both the αβ CD4 and the αβ CD8 T-cell subpopulations in the KSHV carriers in keeping with what has been observed in other persistent viral infections (Brenchley et al., 2003; Palmer et al., 2005). *In vitro* experiments demonstrated Vδ1 T-cell activation in response to infectious KSHV particles; KSHV-infected cell lines; and the KSHV viral proteins glycoprotein B (encoded by ORF8), K8.1 and ORF65 (Barcy et al., 2008). Moreover, Vδ1 T cells prevented the release of infectious KSHV virions from KSHV-infected cell lines following the induction of lytic replication (Barcy et al., 2008).

1.3.d The T-cell Response Against KSHV – Remaining Questions

There is still much to learn about the adaptive T-cell responses against KSHV. Although some CD8 epitopes have been identified, the evidence discussed above indicates that there may be immunodominant epitopes yet to be determined. It seems clear that the targets of the KSHV-specific CD4 response remain poorly understood. Further characterisation of the functionality and differentiation phenotypes of both CD8 and CD4 KSHV-specific T cells will be greatly aided by first achieving a better understanding of the targets of these cells.

In the studies completed so far into the targets of the KSHV-specific CD8 T-cell response a necessary limiting factor has been the size of the KSHV genome. Each study has been confined to a handful of genes, selected either due to their homology with immunogenic genes from other γ-herpesviruses (Lambert et al., 2006), their expression profile (Micheletti et al., 2002; Ribechini et al., 2006), or evidence of sequence variation arising from immunological pressure (Stebbing et al., 2003b). Epitope identification has been performed using overlapping peptides for smaller genes (Wilkinson et al., 2002; Stebbing et al., 2003b; Guihot et al., 2006; Bihl et al., 2007b) or predictive algorithms for peptide HLA-binding affinity to identify potential epitopes for larger genes (Brander et al., 2001; Micheletti et al., 2002; Wang et al., 2002b; Ribechini et al., 2006; Guihot et al., 2006).

The work described in this thesis investigates further the immunogenic profile of KSHV. Lentiviral-transduced dendritic cells were used to express a significant number of the genes contained within the KSHV genome to perform a large-scale

screen for immunogenic KSHV genes that elicit both CD8 and CD4 T-cell responses.

1.4 The Use of Lentiviral-transduced Dendritic Cells to Investigate T-cell Responses

1.4.a Dendritic Cells

Dendritic cells (DCs) are potent antigen presenting cells that act as the 'sentinels' of the immune system. They constantly sample their microenvironment, taking up antigens and presenting them through their class I and class II major histocompatibility (MHC) complexes to CD8 and CD4 T cells respectively. They express costimulation and adhesion molecules involved in T-cell activation and are capable of stimulating both memory and naïve T cells (Banchereau and Steinman, 1998).

DCs arise from bone marrow-derived haematopoietic mononuclear progenitors and are initially described as being in an 'immature' state. Immature DCs are found throughout the body and are extremely efficient at taking up antigens, but have low T-cell stimulatory capacity and are unable to prime a naïve T-cell response. In response to stimuli alerting them to inflammation, tissue injury or pathogen attack, immature DCs undergo a maturation process, whereby they lose their ability to capture antigens but become potent stimulators of both memory and naïve T cells. Mature DCs migrate to the T-cell areas of the lymph nodes, where they can recruit

T cells and promote an immune response (Banchereau and Steinman, 1998). In humans, two main subtypes of DCs can be identified *in vivo* based on their expression of the cell-surface markers CD11c and CD123 (interleukin 3 receptor) – myeloid DCs (mDCs) are CD11c-positive and $CD123^{lo}$ and plasmacytoid DCs (pDCs) are CD11c-negative and $CD123^{hi}$. However, both these DC subtypes are difficult to isolate in sufficient numbers for meaningful *ex vivo* investigations. Therefore, most DC research has been conducted on DCs grown *in vitro* from precursor cells retrieved from the body.

DCs can be derived *in vitro* from CD14+ monocytes isolated from peripheral blood (Sallusto and Lanzavecchia, 1994). After seven days' culture in the presence of the cytokines granulocyte/macrophage colony-stimulating factor (GMCSF) and interleukin 4 (IL4), the monocytes take on a typical DC morphology and have the antigen-capturing, processing and presenting capacities of immature DCs. They no longer express CD14 and instead express cell-surface markers of immature DCs such as the antigen-presenting protein complexes major histocompatibility complex -1 (MHC-I) and -2 (HLA-DR); the costimulatory molecules CD40, CD80 and CD86, which bind the T-cell molecules CD40L, CD28 and CTLA-4 respectively; and the myeloid DC marker CD11c. From an immature state, these cells undergo a maturation process in response to stimulation by proinflammatory cytokines such as tumour necrosis factor α (TNFα) or microbial products such as lipopolysaccharides (LPS) to become characteristic mature DCs, expressing CD83 and high levels of cell-surface HLA-DR and with high T-cell stimulatory capacity (Sallusto and Lanzavecchia, 1994; Romani et al., 1996; Shortman and Liu, 2002). They are thus

closely related to myeloid DCs in terms of both morphology and function and are known as monocyte-derived DCs (moDCs).

1.4.b Antigen Delivery to Dendritic Cells

As potent antigen-presenting cells, DCs loaded with antigens of interest have many potential applications in basic immunology research. Moreover, the pivotal role played by DCs in initiating T-cell immune responses *in vivo* has made them attractive targets for novel immunotherapy strategies, particularly for the treatment of cancer, due to their ability to generate primary anti-tumour T-cell responses (Kirk and Mule, 2000). This has led to extensive investigations into methods of efficient antigen delivery into DCs. DCs can be directly loaded with peptides or antigens, but genetically modifying DCs to introduce an immunogenic gene is an attractive alternative (Jenne et al., 2001). Not only does this technique potentially enhance delivery, it also allows natural processing of the immunogenic protein by the DC and thus presentation of the naturally occurring optimal epitopes, thereby avoiding the limitations associated with the use of artificial peptides. Most importantly, no prior knowledge of the optimal peptide is required and each individual's DCs can process the antigenic protein to produce the best-suited, HLA-restricted peptide for that individual, regardless of their HLA type. In addition, endogenous expression of the antigen should result in more sustained antigen presentation than is achieved by introducing the antigen by pulsing DCs (Dyall et al., 2001; Jenne et al., 2001; Esslinger et al., 2002; Collins and Cerundolo, 2004).

Monocyte-derived DCs (moDCs) are quiescent in culture and therefore difficult to introduce foreign genetic material into using non-viral methods of gene transfer (Arthur et al., 1997). Likewise, vectors derived from oncogenic retroviruses, which cannot deliver into non-dividing cells, show low transduction efficiency into moDCs (Chinnasamy et al., 2000). Adenovirus- and vaccinia-based vectors efficiently transduce non-dividing cells. However, a major disadvantage of using vectors based on these viruses is that they are both highly immunogenic and are prevalent in all human populations and have the potential, therefore, to elicit a notable T-cell response to viral proteins encoded in the vector construct itself. Strong anti-vector T-cell responses could interfere with any target transgene-specific immune responses, making these vectors less attractive for use in both immunotherapies and basic research. The introduction of HIV-1- and HIV-2-derived lentiviral vectors and their demonstrated ability to transduce non-dividing cells (Miyake et al., 1998) thus offered a promising alternative vehicle for moDC gene delivery. For safety reasons, lentiviral vectors based on HIV-1 are designed as so-called 'gutless vectors' – although they are constructed from HIV viral proteins, they do not express any HIV viral proteins when in their target cell (see section 1.4.d., *Construction of Lentiviral Vectors*). This minimises the potential problem of pre-existing immunity to viral vector proteins interfering with antigen-specific responses as a result of stimulating anti-vector over anti-transgene CTLs (Palmowski et al., 2004; Collins and Cerundolo, 2004). Furthermore, lentiviral vectors, unlike adenovirus- and vaccinia-based vectors, integrate into the cellular genome resulting in stable gene expression.

1.4.c Lentiviral Transduction of Dendritic Cells

The first report of successful transduction of moDCs by an HIV-1-based lentiviral vector system was by Unutmuz *et al.* (Unutmaz et al., 1999). However, the first extensive study in this area was by Schroers *et al.* (Schroers et al., 2000). The authors showed efficient transduction of moDCs by a lentiviral vector encoding the enhanced yellow fluorescence protein (eYFP) reporter gene. Interestingly, they and others found that immature moDCs are more efficiently transduced than mature moDCs (Schroers et al., 2000; Gruber et al., 2000; Dyall et al., 2001). Esslinger *et al.* made a direct comparison of the transduction efficiency of adenoviral and lentiviral vectors encoding the green fluorescence protein (GFP) and found the lentiviral vectors were twice as efficient at transducing human moDCs and ten times more efficient at transducing murine moDCs (Esslinger et al., 2002). Kinetic analysis by Schroers *et al.* revealed that maximum transgene (eYFP) expression, in terms of both number of cells transduced and fluorescence intensity, was reached four to five days post-transduction, but no decrease in fluorescence activity was seen up to 10 days post-transduction. A similar pattern was observed by others (Dyall et al., 2001) and one group reported stable transgene expression for up to 28 days (Rouas et al., 2002). Several studies have demonstrated that lentiviral vectors integrate into the moDC cellular genome thus providing for stable gene expression (Schroers et al., 2000; Chinnasamy et al., 2000; Dyall et al., 2001).

Lentiviral-transduced moDCs have a normal morphology (Gruber et al., 2000; Esslinger et al., 2002) and their viability is not affected at low multiplicities of infection (MOI) (less than ten) (Gruber et al., 2000; Dyall et al., 2001). Some

groups report no loss of viability at higher MOIs (Esslinger et al., 2002). At low MOIs, lentiviral-transduced moDCs maintain their immunophenotype, as characterised by their expression of DC cell-surface markers CD11c, CD40, CD80, CD83, CD86 and HLA-DR (Koya et al., 2003; Dullaers et al., 2004). These markers are upregulated appropriately by lentiviral-transduced moDCs in response to maturation stimuli such as CD40 ligand, LPS and a 'cocktail' of inflammatory cytokines (Gruber et al., 2000; Chinnasamy et al., 2000; Jenne et al., 2001; Dullaers et al., 2004). Lentiviral transduction at very high MOIs, however, seems to have some effect on immunophenotype and maturation. One group reported that at a high MOI of 50 to 100, lentiviral-transduced moDCs showed reduced expression of DC cell-surface markers after maturation (Chen et al., 2004). Conversely, another group reported activation of moDCs and upregulation of cell-surface markers after lentiviral transduction at high MOIs (Tan et al., 2005). Importantly, however, in allogenic mixed lymphocyte reactions, lentiviral-transduced moDCs show no decrease in their T-cell stimulation capacity as compared to non-transduced moDCs (Schroers et al., 2000; Chinnasamy et al., 2000; Dyall et al., 2001; Chen et al., 2004) even after transduction at high MOIs (Chen et al., 2004; Tan et al., 2005). In addition, Gruber *et al*. demonstrated peptide-specific lysis of moDCs lentivirally transduced with an empty vector and subsequently loaded with the melanoma-associated tyrosinase CTL epitope by a CD8 tyrosinase peptide-specific cell line (Gruber et al., 2000). Together, these findings indicate that lentiviral transduction does not affect the antigen-presenting function of moDCs.

There is limited knowledge regarding the effects of lentiviral transduction on moDC cytokine secretion and their induction of Th1- or Th2-type immune responses. Two

studies using high MOIs reported conflicting results. Chen *et al*. found that lentiviral transduction impaired moDC-mediated Th1 immunity. They saw a dramatic reduction in the proportion of IFNγ-producing Th1 cells in a T-cell population that had been incubated with lentiviral-transduced moDCs as compared to a T-cell population that had been incubated with non-transduced moDCs. The proportion of Th2 cells in each population was essentially the same (Chen et al., 2004). In contrast, Tan *et al*. found that lentiviral transduction promoted increased secretion of the Th1 cytokines IL1β, IL6 and IL12 by immature moDCs. This upregulation of Th1 cytokines was less than was seen after adenoviral transduction, and was also only seen in immature moDCs. Mature moDCs showed no change in their production of Th1 cytokines after lentiviral transduction (Tan et al., 2005). At MOIs of less than 20, Rouas *et al*. saw no difference in the amount of the bioactive IL12 (an important Th1 cytokine) secreted by lentiviral-transduced or non-transduced moDCs (Rouas et al., 2002).

Dyall *et al*. were the first to demonstrate that moDCs lentivirally transduced to express a foreign peptide can activate a strong peptide-specific cytotoxic T-cell (CTL) response (Dyall et al., 2001). They compared the T-cell stimulatory capacity of moDCs transduced with a lentiviral vector encoding the optimal HLA-A*0201 influenza matrix protein peptide with that of non-transduced moDCs pulsed with the same peptide. By using a lentiviral vector encoding the influenza peptide they were able to make a direct comparison with pulsed moDCs, as in this case the antigen does not require processing by the transduced moDCs and is not part of a larger protein that could encode additional T-cell epitopes including T-helper epitopes. The lentiviral-transduced moDCs were able to stimulate an influenza-

specific primary CTL response in a mixed T-cell population that was comparable to stimulation by the peptide-pulsed moDCs. This was despite the transduced moDCs displaying a relatively low transduction efficiency with only 15-18% of cells expressing the antigen. Furthermore, the lentiviral-transduced moDCs were able to restimulate primed CTLs and could also stimulate a CTL response in a pure CD8 CTL population without CD4 T-cell help or the addition of endogenous cytokines.

Further *in vitro* studies demonstrated that moDCs lentivirally transduced with melanoma-associated antigens stimulated both primary and recall anti-cancer T-cell responses. Lentiviral-transduced moDCs expressing the melanoma-associated antigens tyrosinase or Melan-A stimulated antigen-specific CD8 T-cell lines or T-cell clones derived from tumour-infiltrating lymphocytes from melanoma patients (Esslinger et al., 2002; Firat et al., 2002; Lizee et al., 2004). moDCs lentivirally transduced to express the HLA-A2 restricted Melan-A_{27-35} epitope were also shown to stimulate the expansion of naïve T cells from HLA-A2 blood donors (Lopes et al., 2006). Furthermore, and very importantly for the work described in this thesis, lentiviral-transduced DCs were demonstrated to efficiently process and present both MHC class I- and II-restricted epitopes and so prime both CD8 and CD4 T-cell responses (Dullaers et al., 2004; He et al., 2005).

Several studies in mice have shown that intravenous injection of murine DCs lentivirally transduced with an antigenic epitope can induce antigen-specific T-cell responses *in vivo* (Esslinger et al., 2002; Zarei et al., 2004; Palmowski et al., 2004; He et al., 2005). These responses are stronger and longer-lasting than those elicited by peptide- or protein-pulsed or mRNA-electroporated DCs (Dullaers et al., 2004;

He et al., 2005). In a murine model of aggressive melanoma, immunisation with DCs lentivirally transduced with a tumour antigen significantly inhibited growth of established tumours and resulted in prolonged survival (He et al., 2005). In a murine model of lymphocytic choriomeningitis virus (LCMV), immunisation with DCs lentivirally transduced with an LCMV epitope protected against primary LCMV infection (Zarei et al., 2004). Together, these studies indicate the initiation of functional antigen-specific T-cell responses as a result of *in vivo* priming with lentiviral-transduced DCs. Significantly, immunisation with lentiviral-transduced DCs resulted in less activation of non-specific CD8 T cells than immunisation with DCs transduced with adenovirus-based vectors, possibly indicating less of an anti-vector immune response (Esslinger et al., 2002).

In addition to their potential use in immunotherapies, lentiviral-transduced DCs have the capacity to be a useful tool in immunology research. Breckpot *et al.* used moDCs lentivirally transduced with the melanoma-specific antigenic protein MAGE-3 to identify a new antigenic peptide (Breckpot et al., 2004). T cells from a blood donor without cancer were stimulated with the lentiviral-transduced moDCs and a responding anti-MAGE-3 CTL was isolated that recognised a previously unknown peptide.

1.4.d Construction of Lentiviral Vectors

Lentiviral gene-delivery vectors based on HIV-1 were developed as an improvement on existing retroviral vectors (Naldini et al., 1996). They have the major advantage of being able to mediate stable gene delivery in to non-dividing

cells, and so have many potential applications in basic, translational and even clinical research.

For safety reasons, lentiviral vectors are separated into cis- and trans- acting elements encoded on separate plasmids – a transfer plasmid and a packaging plasmid, respectively (Dullaers and Thielemans, 2006; Breckpot et al., 2007). The ENV gene is deleted from the packaging plasmid and a third plasmid encodes the envelope (Delenda, 2004). Active virions are produced through the transfection of a packaging cell line such as HEK 293T cells with all three plasmids (see Figure 1.4). The transfer plasmid contains the expression cassette encoding the transgene of interest, along with cis-acting HIV factors required for packaging, reverse transcription, integration and transcription. Self-inactivating vectors were developed by deleting the U3 region of the 3' long terminal repeat (LTR) of the transfer plasmid in order to further reduce the risk of generating a replication-competent virus (Miyoshi et al., 1998; Zufferey et al., 1998). The following additional elements have been added to the transfer plasmid to improve transgene transfer and expression (Delenda, 2004). The HIV POL gene's central polypurine tract (cPPT) enhances nuclear import of pre-integration complexes thereby enhancing vector efficiency in both dividing and non-dividing cells (Follenzi et al., 2000). The woodchuck hepatitis B post-transcriptional regulatory element (WPRE) improves transgene expression through its post-transcriptional activities, modifying polyadenylation, RNA export or translation (Zufferey et al., 1999).

In the original lentiviral vectors (so-called first-generation vectors) the packaging plasmid encoding the trans-acting HIV factors contained GAG, POL, the regulatory

genes REV and TAT, and the accessory genes VIF, VPR, VPU and NEF. Safety was further improved in second-generation vectors by deleting the four accessory genes from the packaging plasmid – these are required for viral pathogenesis *in vivo* but were found to be unnecessary for gene transfer. In third-generation lentiviral vectors a split-genome packaging system was introduced, whereby the REV gene is expressed from a separate, non-overlapping plasmid and the TAT gene is deleted and replaced by a strong constitutional promoter in the 5' LTR of the transfer plasmid (Delenda, 2004).

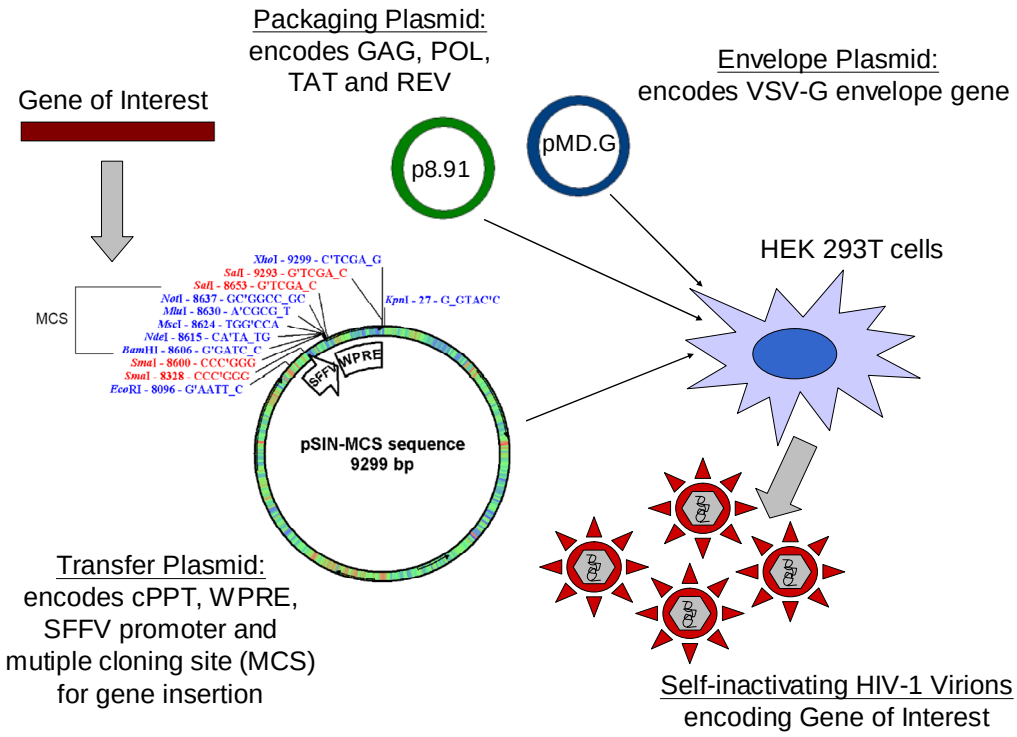


FIGURE 1.4: Schematic illustration of the lentiviral packaging system for the pSIN vector. The gene of interest is cloned into the transfer plasmid at the multiple cloning site (MCS). VSV-G-pseudotyped virions are produced by co-transfection of a packaging cell line of transformed human embryonic kidney cells (HEK 293T) with the transfer plasmid, the packaging plasmid (encoding the HIV-1 genes GAG, POL, TAT and REV) and the envelope plasmid (encoding VSV-G-derived envelope gene).

The envelope plasmid can be pseudotyped to provide optimal delivery to the target cell type. The most widely used heterologous envelope is the vesicular stomatitis virus glycoprotein (VSV-G), as this envelope has a broad host cell tropism (Breckpot et al., 2007). By acting through a ubiquitous cellular receptor, VSV-G facilitates efficient virion entry into a wide range of cell types (Cronin et al., 2005).

Several studies have compared the moDC transduction efficiency of lentiviral vectors carrying the different modifications outlined above (Dullaers and Thielemans, 2006). Self-inactivating vectors resulted in comparable, or even higher, transduction efficiencies than vectors without this modification (Breckpot et al., 2003; Koya et al., 2003). The inclusion of cPPT is universally agreed to enhance transduction efficiency into DCs (Firat et al., 2002; Rouas et al., 2002; Breckpot et al., 2003). There are some reports that the addition of WPRE does not improve transduction efficiency of lentiviral vectors in moDCs and that its presence may result in increased cell death (Breckpot et al., 2003), however, many groups have used vectors with this inclusion in moDCs with success (Koya et al., 2003; Lizee et al., 2004; Dupuy et al., 2005).

Some comparisons of different transgene promoters have been made. The most commonly used are constitutively active promoters such as the phosphoglycerate kinase promoter (PGK), the promoter of elongation factor 1-α (EF1α) and the cytomegalovirus (CMV) immediate early promoter (Dullaers and Thielemans, 2006). Dupuy *et al*. made a direct comparison of transgene expression from either an EF1α or a CMV promoter in different vectors and different cell types. In moDCs, HIV-1-based vectors with the EF1α promoter resulted in significantly higher levels of transgene expression than those with the CMV promoter (Dupuy et

al., 2005). This was in contrast to DCs derived from CD34-positive progenitors, which showed better transgene expression from the CMV promoter. To the best of my knowledge, there is no reported use of the spleen focus-forming virus promoter (SFFV; the promoter in the vector used in the work described in this thesis) in moDCs. However, the SFFV promoter results in improved transgene expression as compared to the CMV promoter in CD34+ haematopoietic stem cells and other cells of the myeloid lineage (Demaison et al., 2002; Yam et al., 2002).

1.5 Aims and Hypothesis

There are several lines of evidence to indicate that KS oncogenesis is associated with the loss of KSHV-specific T cells. Although some KSHV epitopes have been identified, neither the breadth of the KSHV-specific T-cell response, nor its immunodominant targets, are fully understood. Furthermore, the phenotype and function of KSHV-specific T cells remain largely unexplored. The aim of this PhD thesis was to characterise further the T-cell response against KSHV. My initial hypothesis was that the KSHV gene products that elicit the strongest T-cell responses are those that are expressed early in the viral lytic cycle, as this is the pattern observed in the T-cell response against EBV (the most closely related human herpesvirus to KSHV) (Rickinson and Moss, 1997; Pudney et al., 2005; Landais et al., 2005a). Early in the lytic cycle is also the time when it is most important for the host to keep the virus in check in order to prevent uncontrolled replication.

Lentiviral-transduced moDCs have proved to be powerful tools for priming immune responses both *in vitro* and *in vivo*, and have the advantage of providing an autologous system for optimal antigen presentation without requiring prior knowledge of HLA-restriction or the immunodominant epitopes from within an ORF. Members of the Cancer Research UK Viral Oncology Group at University College London previously constructed a library of lentiviral expression vectors, each coding for one of 26 key KSHV ORFs for use in experiments investigating the precise effects of different KSHV ORFs on the host cell (Vart et al., 2007). These 26 genes are indicated by a single asterisk in Table 1.1. It was thus proposed to utilise this lentiviral library to transduce moDCs and then use these transduced moDCs (which present KSHV antigens) in culture with autologous T cells to investigate further the targets of the KSHV-specific CD8 and CD4 T-cell responses.

The first aim of this thesis was, therefore, to perform preliminary experiments to prepare for and design a protocol to use lentiviral-transduced moDCs to screen for immunogenic KSHV ORFs. The KSHV lentiviral library was first expanded to include five further KSHV ORFs that are known or putative T-cell immunogens (also indicated on Table 1.1). This expanded library was then used to transduce moDCs and expression of each of the KSHV ORFs within the library by the moDCs was demonstrated. A GFP-encoding lentiviral construct was used to optimise transduction conditions for moDCs (multiplicity of infection and time-point) and to ensure that lentiviral transduction did not affect the antigen-presenting surface phenotype of moDCs or their maturation. moDCs transduced with an adenovirus expressing the immunodominant CMV gene phosphoprotein 65 (CMVpp65) were used to determine the best culture conditions (culture medium

serum supplement and maturation stimulus) for moDCs for use in such assays, and to refine a CFSE dye-based assay for measuring T-cell responses. Based on the results of these experiments, a protocol for the use of the KSHV lentiviral library to screen for immunogenic KSHV ORFs was developed.

The second aim of this thesis was to use the KSHV lentiviral library to perform a large-scale screen to identify the targets of the KSHV-specific CD4 and CD8 T-cell responses. moDCs from 14 KSHV-seropositive individuals (12 HIV-positive, two HIV-negative) were transduced with KSHV-ORF-encoding lentiviral vectors and then cocultured with autologous T cells. A CFSE dye-based T-cell response assay was used to assess which of the KSHV ORFs yield antigens that are recognised by CD8 and CD4 KSHV-specific T cells.

The third and final aim of this thesis was to identify potential new KSHV CD8 epitopes, and to compare recognition of these epitopes with recognition of nine previously published epitopes in order to determine patterns of immunodominance. Pentamer staining was proposed for the investigation of the memory phenotype and function of KSHV-specific CD8 T cells upon the identification of an immunodominant epitope to target in such studies. Potential new epitopes were identified from within KSHV ORFs indicated as strongly immunogenic by our lentiviral-based experiments. The MHC-binding properties of these peptides were investigated and the peptides that formed the strongest complexes with HLA-A*0201 were identified. Responses to these peptides were tested in 18 A*0201-positive KSHV-seropositive individuals, and compared to responses to nine previously published A*0201-restricted KSHV epitopes. The memory phenotype of

the whole population of circulating CD8 T cells in KSHV-infected individuals in chronic infection and disease was also investigated. Finally, the use of pentamers in conjunction with staining for cell-surface markers and the production of intracellular cytokines to investigate the memory phenotypes functional properties of virus-specific T cells was investigated.

CHAPTER 2: Materials and Methods

2.1 Lentivirus Production

2.1.a Cloning KSHV ORFs into the Lentiviral Vector

i. Preparation of Plasmids pSIN-MCS, p8.91 and pMD.G

The lentiviral vector (pSIN-MCS) was previously derived by members of the Cancer Research UK Viral Oncology Group at University College London from the green fluorescence protein (GFP)-encoding vector pCSGW (a kind gift from Professor Adrian Thrasher, Institute of Child Health, University College London). The two packaging plasmids (p8.91 and pMD.G) were a kind gift from Professor Didier Trono, l'École Polytechnique Fédérale de Lausanne, Switzerland).

The three plasmids were prepared from glycerol stocks as follows. Luria-Bertani (LB) Broth (Sigma, Poole, UK) and plates of LB Agar (Sigma) were prepared according to the manufacturer's instructions and supplemented with 100µg/ml ampicillin (Amp, Sigma). Glycerol stocks of transformed bacteria containing each of the plasmids were streaked onto LB Amp Agar plates and incubated overnight at 37ºC. The following morning single colonies were picked and used to inoculate starter cultures of 5ml of LB Amp Broth which were shaken at 200rpm and 37ºC for 8 hours (hr) and then used to inoculate 250ml LB Amp Broth in conical flasks. These large cultures were shaken at 200rpm and 37ºC overnight. The following

morning, a Maxiprep Kit (Qiagen, Hilden, Germany) was used following the manufacturer's instructions to isolate plasmid DNA. At the end of the procedure the DNA pellet was resuspended in 500µl double-distilled (dd) H_2O.

ii. PCR Amplification of KSHV ORFs of Interest

For each of the novel KSHV ORFs of interest (ORF8, ORF57, ORF59, ORF61 and ORF65), primers were designed for the 5' and 3' ends of the gene according to the National Center for Biotechnology Information (NCBI)'s published KSHV genome sequence (NC 003409). Restriction sites complementary to those present in the multiple cloning site (MCS) of the pSIN-MCS vector were added to each primer to allow for directional cloning of each KSHV ORF into pSIN. In addition, "junk DNA" was added to each primer to enhance restriction site recognition by restriction enzymes, as recommended by New England Biolabs. Table 2.1 shows the primers designed for each KSHV ORF.

In order to minimise the introduction of point mutations in the PCR product, the proof-reading enzyme *Pfu* Ultra (Stratagene, La Jolla, CA, USA) was used to amplify KSHV ORFs from genomic DNA from BC-3 cells (a KSHV-positive, EBV-positive cell line). The basic PCR reaction mix (total volume 50.0µl) was: 5.0µl *Pfu* Ultra PCR Buffer (10x); 38.5µl ddH_2O; 2.0µl forward primer (10µM); 2.0µl reverse primer (10µM); 1.0µl dNTPs (10mM, Invitrogen); 1.0µl BC-3 DNA template; and 0.5µl *Pfu* Ultra Enzyme. A DNA Engine Dyad™ Peltier thermal cycler was used to perform the PCR amplifications. The basic PCR programme used for each amplification was: 95ºC for 2 minutes (mins); 35 cycles of

denaturation at 95ºC for 1 min, annealing at XºC for 1 min and extension at 72ºC for Y mins; and 72ºC for 5 mins. A heated lid (110ºC) was used to prevent reaction evaporation. X and Y were variable parameters dependent on the annealing temperature of the primers (X) and the length of the KSHV ORF (Y). For each KSHV ORF, the parameters used were: ORF 8: X = 56, Y = 2.5; ORF57: X = 60, Y = 0.75; ORF59: X = 58, Y = 1.25; ORF61: X = 58, Y = 2.5; ORF65: X = 60, Y = 0.5.

KSHV ORF	Forward Primer (Top) and Reverse Primer (Bottom) (5'……..3')
ORF 8	**CGC<u>GGATCC</u>**ATGACTCCCAGGTCTAGATTG **ATAAGAAT<u>GCGGCCGC</u>**TCACTCCCCCGTTTCCGG
ORF 57	**CGC<u>GGATCC</u>**ATGATAATTGACGGTGAGAG **ATAAGAAT<u>GCGGCCGC</u>**TTAGAAAGTGGATAAAAGAATAAAC
ORF 59	**CGC<u>GGATCC</u>**ATGCCTGTGGATTTTCACTATG **ATAAGAAT<u>GCGGCCGC</u>**TCAAATCAGGGGGTTAAATGTG
ORF 61	**CGC<u>GGATCC</u>**ATGTCTGTCCGGACATTTTG **ATAAGAAT<u>GCGGCCGC</u>**CTACTGACAGACCAGGCACTCG
ORF 65	**CGC<u>GGATCC</u>**ATGTCCAACTTTAAGGTGAG **ATAAGAAT<u>GCGGCCGC</u>**CTATTTCTTTTTGCCAGAGG

TABLE 2.1: Primers used to amplify KSHV genes from BC-3 DNA. BOLD text indicates 'junk' DNA used to enhance restriction site recognition**. <u>BOLD UNDERLINED</u>** text indicates restriction site**.** REGULAR text indicates gene-specific primer sequence. **<u>GGATCC</u>** is the restriction site for BamHI and **CGC** is the 'junk' DNA sequence appropriate for improved BamHI restriction site recognition. **<u>GCGGCCGC</u>** is the restriction site for NotI and **ATAAGAAT** is the 'junk' DNA sequence appropriate for improved NotI restriction site recognition.

After amplification, the PCR products were visualised and purified by gel electrophoresis followed by gel extraction. One percent weight/volume agarose (Sigma) gels were prepared in TAE buffer (tris-acetate 0.4M, ethylene diamino tetraacetic acid [EDTA] 0.01M). PCR products were diluted 5:1 with loading dye

(Fermentas, York, UK) and run in parallel with a 1kb ladder (New England Biolabs, Ipswich MA, USA). Electrophoresis was performed at 100 volts for approximately 1 hr. Gels were visualised with a UV transilluminator and photographed using a VersaDoc imaging system (Bio-Rad). Bands were excised using a UV light box (Anderman), and DNA was purified using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. At the end of the protocol, DNA was eluted in 30µl ddH_2O and the concentration of DNA in the elutant was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

iii. Restriction Enzyme Digestion of the PCR Product and the pSIN-MCS Vector

The purified PCR product and pSIN plasmid were digested with the appropriate restriction enzymes in preparation for ligation. For all five KSHV ORFs, the restriction sites BamHI and NotI were used. PCR products were digested in a 24.0µl reaction as follows: 20.5µl purified PCR product; 2.5µl Restriction Enzyme Buffer D (New England Biolabs); 1.0µl Not I (New England Biolabs). The reaction was incubated at 37ºC overnight. The following morning, 1.0µl BamHI was added to the reaction mix (to make 25.0µl volume) and incubated for a further 2 hr at 37ºC.

Digested PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen). Digested PCR products were not run on an agarose gel prior to purification. Instead, 300µl of Buffer QG was added directly to the digestion

mixture and then the protocol was followed according to the manufacturer's instructions omitting the incubation to dissolve the gel slice. At the end of the protocol the digested PCR products were eluted in 25µl ddH_2O and quantified using a NanoDrop spectrophotometer (NanoDrop Technologies). Digested PCR products were stored at -20ºC.

The pSIN vector was digested in a 20.0µl reaction as follows: Xµl (volume calculated to be equivalent to 1-2µg) pSIN vector; 2.0µl Buffer D (New England Biolabs); 1.0µl NotI (New England Biolabs); 1.0µl BamHI (New England Biolabs); (20.0 – X) µl ddH_20. The reaction mix was incubated at 37°C for 2 hr. Due to the presence of the MCS in the reaction mix after digestion, the digested vector was purified by running the reaction mix on a 1% agarose gel (Sigma, prepared as above) at 100V for 1 hr. The digested vector was then excised from the gel and purified using a QIAquick Gel Extraction Kit (Qiagen) as above.

iv. Ligation of the KSHV Gene Insert into the pSIN-MCS Vector

After digestion and purification of the inserts and vector, each KSHV ORF was ligated into pSIN using T4 DNA ligase (New England Biolabs). A ligation reaction mix of a total volume of 20.0µl was used, containing insert and vector to a molar ratio of 8:1. Ligation reactions (total volume 20.0µl) were set up as follows: Xµl (=100ng) digested pSIN vector; Yµl (8:1 molar ratio to vector) digested KSHV gene insert; 2.0µl Ligation Buffer (10x; New England Biolabs); 1.0µl T4 Ligase (New England Biolabs); (17.0 – X – Y) µl ddH_2O. Ligation reactions were

incubated at room temperature for 1 hr, with the exception of the ORF8 ligation reaction, which was incubated at 16ºC overnight.

v. Plasmid Amplification by Bacterial Transformation

After ligation, plasmids containing the KSHV ORF inserts were amplified by transformation of chemically competent bacteria. Two microlitres of ligation mix was used to transform one vial of One Shot® TOP10 Chemically Competent *E. coli* cells (Invitrogen, Paisley, UK) following the manufacturer's instructions. At the end of the protocol, the transformation mix was plated on LB Amp plates and incubated overnight at 37ºC. Transformed bacteria carrying the ampicillin-resistance gene encoded by pSIN formed colonies on the LB Amp plates. Eight single colonies were picked and used to inoculate individual 5ml cultures of LB Amp Broth. Inoculated cultures were left shaking overnight at 37ºC and 200 rpm. The following morning the bacterial suspension was spun at 3000 rpm for 15 mins. The supernatant was discarded, and plasmid DNA was isolated from the bacterial pellet using a QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. At the end of the protocol, plasmid DNA was eluted in 50µl ddH_2O. Plasmid DNA was then stored at -20ºC.

vi. Confirming the Presence of a KSHV ORF Insert in the pSIN-MCS Vector

To screen each plasmid DNA solution for the presence of each KSHV ORF insert in the pSIN vector, plasmids were digested with the same restriction enzymes used

to insert the KSHV ORF into the MCS of the pSIN vector. Restriction digestions (total volume 15.0µl) were set up as follows: 5.0µl plasmid DNA solution; 1.5µl Buffer D (New England Biolabs); 1.0µl NotI (New England Biolabs); 1.0µl BamHI (New England Biolabs); 6.5µl ddH_2O.

Restriction digestions were incubated at 37ºC for 1 hr and then visualised by gel electrophoresis. Three microlitres of loading dye (Fermentas) was added to 15µl restriction digestion mix and samples were loaded on to a 1% agarose gel (prepared as above) alongside a 1kb ladder (New England Biolabs). Electrophoresis was performed at 100v for approximately 1 hr. Gels were visualised with a UV transilluminator and photographed using a VersaDoc imaging system (BioRad). Plasmid DNA solutions were assessed for presence of the linearised vector (10kb) and a second DNA fragment of the correct length for the inserted KSHV ORF.

vii. Sequencing the KSHV ORF Inserts

Plasmids containing the desired KSHV ORF were sequenced to ensure that there were no mutations in the insert that may have occurred during PCR or due to recombination during transformation. For all sequencing, the SFFV_MOD forward primer and pSIN_rev primer were used, as these primers bind to pSIN at either side of the MCS. For inserts larger than 500bps, internal primers were also used in order to sequence the entire length of the insert. Sequencing was carried out by the Scientific Support Services at the Wolfson Institute for Biomedical Research, UCL, on a Beckman Coulter CEQ 8000 using WellRed dye-terminator chemistry (Beckman Coulter, Fullerton, California, USA). Sequence data was analysed using

Bioedit software and insert sequences were compared to the published KSHV gene sequences (NC 003409; NCBI) using the NCBI's Basic Local Alignment Tool (BLAST) software.

2.1.b Lentivirus Production: 293T Transfection

Thirty millilitres of each KSHV ORF-encoding lentivirus (including the five ORFs cloned into the vector as outlined above as well as the 26 ORFs already present in the lentiviral library, listed in Table 2.3 and indicated in Table 1.1), plus the empty lentiviral vector and the GFP-encoding lentivirus, were produced by transfection of 293T cells. Twenty-four hours before transfection, confluent 10cm culture dishes (Corning Incorporated, New York, USA) of 293T cells were split 1:7, and replated on 10cm culture dishes. For each lentivirus, three 10cm plates were transfected using the transfection reagent Fugene (Roche, Basel, Switzerland) following the manufacturer's instructions.

One hour prior to transfection, 293T cells were washed once with sterile phosphate buffered saline (PBS; Sigma), and culture media was replaced with 8ml Optimem (Gibco, Invitrogen, Paisley, UK). Cells were returned to a 37ºC, 5% CO_2 incubator. For each plate of cells to be transfected, a Fugene master mix was prepared from 15µl Fugene and 35µl Optimem and incubated at room temperature for 5 mins. DNA mixes were prepared as follows. For each plate, a total of 5µg of DNA was used, consisting of 1.5µg p8.91, 1.5µg of pMD.G and 2µg of pSIN (either empty vector, or encoding GFP or KSHV ORF of interest). Each DNA mix was prepared to a final volume of 50µl per plate in Optimem. Fugene mix was then added directly

to the DNA mix, and incubated for 20 mins at room temperature. 100µl Fugene/DNA mix was then added drop-wise to each plate of 293T cells, and plates were returned to the incubator for 5 hr. After this incubation, cells were washed once in PBS, and Optimem was replaced with 10ml DMEM (Gibco) supplemented with 10% FCS (Sigma, Poole, UK) and penicillin-streptomycin (Sigma). Plates were then returned to the incubator. After 48 hr, virion-containing supernatant was harvested by careful aspiration, filtered through a 45µm filter (Nalgene, Rochester, NY, USA), divided into 1ml aliquots, and immediately stored at minus 80ºC.

2.2 Culture and Transduction of Monocyte-derived Dendritic Cells

2.2.a PBMC Isolation

Peripheral blood samples were collected from consenting volunteers into lithium heparin tubes (BD Biosciences, Oxford, UK). Monocyte-derived dendritic cells (moDCs) were isolated from peripheral blood following an established protocol (Sallusto and Lanzavecchia, 1994). Briefly, whole blood was diluted 1:2 with PBS. Four parts blood/PBS was layered over one part Histopaque-1077 (Sigma) and spun in a Mistral 3000E centrifuge (MSE, London, UK) at 2000rpm for 30 mins at room temperature with the brake off. Peripheral blood mononuclear cells (PBMCs) were collected from the interface and washed three times in PBS + 1% foetal calf serum (FCS; Sigma).

2.2.b moDC Isolation, Culture and Maturation

PBMCs were resuspended in 80µl MACs Buffer [2mM EDTA (Gibco) in PBS/2%FCS] per 10^7 cells and 20µl CD14+ MACs Beads (Miltenyi Biotech, Bergisch Gladbach, Germany) per 10^7 cells, and incubated at 4°C for 15 mins. PBMCs were then washed in 2ml MACs Buffer per 10^7 cells, resuspended in 500µl MACs Buffer and passed through an equilibrated MS column (Miltenyi Biotech). The MS column was washed three times with 500µl MACs Buffer and CD14-negative cells were collected in the flow-through. CD14-positive cells were eluted in 1ml MACs Buffer. Both CD14-negative and CD14-positive monocytes were washed twice in PBS/1%FCS. CD14-negative cells were stored at -80ºC in a freezer mix made up from 50% RPMI 1640 (Sigma), 40% FCS and 10% dimethyl sulfoxide (DMSO; Sigma), and reserved for later isolation of T cells (see section 2.3.a).

CD14-positive monocytes were plated on 24-well plates (Cellstar, Greiner Bio-one, Gloucestershire, UK) at 3 x 10^5 cells per well and cultured in RPMI 1640 with Hepes modification (Sigma) supplemented with l-glutamine (Sigma), penicillin-streptomycin (Sigma) and either Human AB Serum (Lot Number 027K0432; Sigma) at a final concentration of either 2% or 5%, or FCS (Sigma) at a final concentration of 10%. The cells were further supplemented with 75ng/ml interleukin 4 (IL4; R and D Systems, Oxford, UK) and 75ng/ml granulocyte/macrophage colony-stimulating factor (GMCSF; R and D Systems) in order to drive differentiation into immature moDCs. Cells were fed with fresh media and cytokines every second day. Immature moDCs were matured by

stimulation with either 20ng/ml lipopolysaccharides (LPS; Sigma) for 24 hr or a "cytokine cocktail" made up from 1µg/ml prostaglandin E2 (PGE2; Sigma), 5ng/ml TNFα (R and D Systems), 5ng/ml IL1ß (R and D Systems) and 150ng/ml IL6 (R and D Systems) for 48 hr.

2.2.c Flow Cytometric Analysis of Cell-surface Marker Expression by moDCs

moDCs were analysed for expression of cell-surface markers by multi-parameter flow cytometry. All antibodies and isotype controls used were purchased from BD Biosciences and are shown in Table 2.2. GFP-transduced moDCs were not stained with antibodies conjugated to FITC, as GFP expression is measured through fluorochrome 1. GFP-transduced moDCs were therefore only stained for expression of the cell-surface markers MHC-I, CD80, CD83 and CD40.

moDC cell-surface molecule	Fluoro-chrome	Clone	Antibody Catalogue Number	Isotype	Isotype Control Clone	Isotype Catalogue Number
MHC-I	APC	G46-2.6	555555	IgG1κ	MOPC-21	555751
HLA-DR	FITC	TU36	555560	IgG2bκ	27-35	555742
CD80	PE	L307.4	340294	IgG1κ	MOPC-21	555749
CD86	FITC	2331	555657	IgG1κ	MOPC-21	555748
CD40	PECy5	5C3	555590	IgG1κ	MOPC-21	555750
CD83	PE	HB15e	556855	IgG1κ	MOPC-21	555749

TABLE 2.2: Antibodies and isotype controls used for characterisation of moDCs by immunostaining and flow cytometry. All antibodies were mouse anti-human, and all isotype controls were from mice.

For all staining, moDCs were harvested by aspiration, spun at 1400 rpm for 8 mins and resuspended in 95µl PBS/1%FCS. Five microlitres of antibody or isotype control was added to give a final antibody dilution of 1 in 20. Cells were stained on ice for 30 mins, protected from light. Cells were then washed once in PBS/1%FCS, and resuspended in 250µl PBS/1%FCS. Four-colour flow cytometric analysis was performed on a FACSCalibur (BD Biosciences) and analysed with CELLQuest (BD Biosciences) software. Ten thousand events were collected for each sample. moDCs were gated on the live-cell population according to the expected forward scatter and side scatter. Isotype controls were used to set gates for positive staining for each of the antibodies used to detect cell-surface marker expression.

2.2.d Transduction of moDCs with a GFP-encoding Lentivirus

On day four in culture, immature moDCs were transduced with a pSIN lentivirus encoding GFP (pCSGW). pCSGW lentivirus was thawed on ice for 30 mins, and then warmed to 37ºC in a water bath directly prior to use. Lentivirus was added directly to the cells in their culture dishes. After transduction, moDCs were fed every second day with fresh media and cytokines.

i. Time-point Optimisation

Immature moDCs were transduced with 1ml pCSGW per well. On days four, five, six and seven post-transduction, moDCs were harvested by aspiration, spun at 1400rpm for 8 mins, resuspended in 250µl PBS/1%FCS and analysed for GFP expression by flow cytometry performed on a FACSCalibur (BD Biosciences).

Non-transduced moDCs were used as a negative control and 10, 000 events were collected for each sample.

ii. Multiplicity of Infection

Immature moDCs were transduced with 50µl, 100µl, 300µl, 1ml or 1.5ml pCSGW per well. On day six post-transduction, moDCs were harvested by aspiration for either flow cytometric analysis of GFP expression (section 2.2.d.i) or DNA extraction to determine the multiplicity of infection (MOI) by quantitative real-time PCR (qPCR). For DNA extraction, cells were pelleted and washed once in PBS. DNA extraction was performed using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. At the end of the protocol, DNA was eluted in 70µl AE Buffer and four aliquots were prepared for future use to avoid sample contamination. DNA solutions were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies).

qPCR was performed in sterile conditions in an isolated, DNA-free laboratory to avoid sample contamination. To determine the number of lentiviral copies per cell, qPCR was performed for the lentiviral packaging signal using the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as the reference signal. GAPDH primers and probes were used as previously described (Bourboulia et al., 2004) and as follows: GAPDH forward primer 5'-GGAGTCAACGGATTTGGTCGTA-3'; GAPDH reverse primer 5'-GGCAACAATATCCACTTTACCAGAGT-3'; GAPDH TaqMan probe 5'-FAM-CGCCTGGTCACCAGGGCTGC-3'-TAMRA. Primers were used at a final concentration of 0.7µM and probe at 0.15 µM. The

primers and probe for the lentiviral packaging signal and their concentrations were: Lenti forward primer 5'-GCACGGCAAGAGCGA-3' (0.3μM); Lenti reverse primer 5'-CGCACCCATCTCTCTCCTTCTA-3' (0.3μM); Lenti TaqMan probe 5'-FAM-CGGCGACTGGTGATACGCCAAAAAT-3'-TAMRA (0.15μM). Each reaction contained 25.0μl 2x Absolute QPCR ROX and dUTP mix (ABgene Limited, Epsom, UK) and 10.0μl genomic DNA (concentration 50-100 ng/μl) and were made up to a total final volume of 50.0μl with ddH_2O. Reactions were performed in duplicate and run alongside: standard DNA mixes containing a known number of copies of either linearised pcDNA3.1/V5-His-TOPO GAPDH (GAPDH standard) or linearised pSIN plasmid vector (Lenti standard); non-transduced moDC DNA samples; and ddH_2O negative controls. qPCR was carried out on a Perkin-Elmer 7700 sequence detector (Perkin-Elmer Applied Biosystems, Waltham, Massachusetts, USA) using the following conditions: 50ºC for 2 mins; 95ºC for 15 mins; 40 cycles of 95ºC for 15 seconds (secs) followed by 60ºC for 1 min. Lentiviral copy number per cell was determined by adjusting the number of lentiviral particles per sample to the number of cells present in that sample (as determined by the number of copies of GAPDH present).

2.2.e Transduction of moDCs with the KSHV Lentiviral Library

moDCs were cultured in RPMI supplemented with 5% human AB serum. On day four in culture, moDCs were singly transduced with 300μl of each of the lentiviruses encoding KSHV ORFs from the lentiviral library. Lentiviral preparations were thawed on ice for 30 mins then warmed to 37ºC in a water bath directly prior to use. Lentivirus was added directly to cells in their culture dishes.

After transduction, moDCs were fed with fresh media and cytokines every second day. On day eight in culture (day four post-transduction), moDCs were matured by stimulation with a "cytokine cocktail" (see section 2.2.b). On day 10 in culture (day six post-transduction), moDCs were harvested by aspiration, pelleted and stored at -80ºC. Cell pellets were thawed on ice, and either RNA was extracted to confirm viral gene expression through the creation of cDNA libraries, or DNA was extracted in order to determine lentiviral copy number per cell for moDCs transduced with each of the different lentiviruses.

i. Confirmation of Viral Gene Expression by RT-PCR

RNA was extracted from cell pellets using an RNeasy kit (Qiagen) according to the manufacturer's instructions including the optional DNase steps to ensure no contamination from plasmid DNA. At the end of the protocol, RNA was eluted in 25.0µl RNase-free water. The RNA was used as a template to synthesise cDNA by reverse transcription using SuperScript® II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was used as a template to confirm viral gene expression by reverse transcription (RT)-PCR. PCR primers were designed for each ORF according to the published KSHV genome sequence (NC 003409; NCBI). Table 2.3 shows the primers used for each ORF. PCR products were visualised by gel electrophoresis as described above. PCR reactions (total volume 50.0µl) were set up as follows: 38.5µl ddH_2O; 5.0µl Taq 10x Buffer (Invitrogen); 2.0µl forward primer (10µM); 2.0µl reverse primer (10µM); 1.0µl DNTPs (10Mm; Invitrogen); 1.0µl cDNA or no reverse-transcriptase control; 0.5µl Taq Polymerase (Invitrogen). PCR was performed using a DNA

KSHV ORF	Forward Primer (5'……..3')	Reverse Primer (5'……..3')
K1	GCAACGATACTCGGCTTCTC	TGATGGTTGTCCACACAAGG
K2	ATGGGTGATCGATGAATGCT	ATCGGCGAGCTTTTTAAGG
K3	GAGAGCTCGAGAACGTCCAT	TTCCAGACCCTCCTGGTAAG
K4	ATGGACACCAAGGGCATCCTG	TCAGCGAGCAGTGACTGGTAAT
K4.1	GAGAATTCCCTGTCCAGTGC	ATCTCCGTGTGCTTCTCCAT
K5	GGTGACCGTACTGCCATACC	CGTCACGTTCTTTGTCTCCA
K6	CTGCGTTAGCGTACGCTTG	TCAGCTGCCTAACCCAGTTT
K8	AAGCTCGCTGTTGTCAACCT	ATCTGCGAGTTGGAAGCTGT
K8.1	CACCACAGAACTGACCGATG	GTAGTGCGCGTCTCTCTTCCTC
K9	GCGTCAATCAAGGATTGGAT	CTTGCAAGAGACGTGCCATA
K11.1	GGAATGGCTCACGGACTTTA	CTCAGTCTCCGGGATTTCTG
K12	AGGCTTAACGGTGTTTGTGG	CTCGTGTCCTGAATGCTACG
K14	CCAGGAGCAGTTCACTGACA	TAGGCCCACCAGAGTAATGG
K15-P	CCTATGCTTGCTTAATCACCAC	GGACCAGCATGTTTGTCATC
ORF8	ATGACTCCCAGGTCTAGATTG	TCACTCCCCCGTTTCCGG
ORF28	CTGCGTCTACTGCTGCATTC	AGGGCTCCTGGGTAGCTATG
ORF33	GTCGCCGGGTCTATCTAACA	GGGGTTGGGTGGCTAGTTAT
ORF36	TGGGAGCAAGTGGACTAACC	GTGTAGCCCAACGAGGACAT
ORF37	ATGGAGGCCACCCCCACACC	TTCCACGTCTACGGGCTGTGAG
ORF45	CATGGGATGGGTTAGTCAGG	GGGTCGCTGTATGGTGAACT
ORF49	GCACGTCCCTAACTCTCCTG	AATGGTGTAGGTGGGAGCAG
ORF50	CAAGGTGTGCCGTGTAGAGA	TCCCAAAGAGGTACCAGGTG
ORF57	ATGATAATTGACGGTGAGAG	TTAGAAAGTGGATAAAAGAATAAAC
ORF58	GCCGCCAATAGTACACAGGT	TGCCTAAATGCAAAAGTCC
ORF59	ATGCCTGTGGATTTTCACTATG	TCAAATCAGGGGGTTAAATGTG
ORF61	ATGTCTGTCCGGACATTTTG	CTACTGACAGACCAGGCACTCG
ORF65	ATGTCCAACTTTAAGGTGAG	CTATTTCTTTTTGCCAGAGG
ORF71	AGCTGTGTGCGAGGGATATT	GGCGATAGTGTTGGGAGTGT
ORF72	ACGAGGTCAACACCCTGATT	CGCCTGTAGAACGGAAACAT
ORF73	TTGCCACCCACGCAGTCT	GGACGCATAGGTGTTGAAGAGTCT
ORF74	CGCTGCACTGTTAATTGCAT	GTCGCCTTAGCAGAGTGTCC

TABLE 2.3: Primers used to detect expression of KSHV ORFs by transduced moDCs using reverse transcription PCR.

Engine Dyad™ Peltier thermal cycler. The typical PCR programme used for each ORF's amplification was 95ºC for 15 mins; 40 cycles of denaturation at 95ºC for 1 min, annealing at 56ºC for 1 min and extension at 72ºC for 1 min; and 72ºC for 5 mins. Exceptions to this were ORF8, ORF57, ORF59, ORF61 and ORF65, for which the PCR programmes used were the same as described in section 2.1.a.ii; and K4, ORF 37 and ORF 73, for which the PCR programmes used are shown in Table 2.4.

Step	Process	Temperature and Time		
		K4	ORF 37	ORF 73
1	Start	95ºC, 15 mins	95ºC, 15 mins	95ºC, 15 mins
2	Denaturation	95ºC, 1 min	95ºC, 1 min	95ºC, 30 secs
3	Annealing	54ºC, 1 min	64ºC, 1 min	60ºC, 30 secs
4	Extension	72ºC, 45 secs	72ºC, 2 mins	72ºC, 15 secs
5	Repeat steps 2-4 for 35 cycles			
6	Finish	72ºC, 5 mins	72ºC, 5 mins	72ºC, 5 mins
7	End	4ºC Forever	4ºC Forever	4ºC Forever

TABLE 2.4: PCR programmes used to confirm KSHV gene expression by transduced moDCs using reverse transcription PCR.

ii. Titre of Lentivirus by qPCR.

DNA was extracted from cell pellets using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. qPCR was performed in sterile conditions as described in section 2.2.d to determine the MOI of each lentiviral preparation.

iii. Multiple Transductions

Seven wells of moDCs were transduced simultaneously with one, two or three different KSHV-ORF-encoding lentiviral constructs (see section 3.2.d; Figure 3.12). Transductions were normalised to an MOI of approximately 6 for each construct. On day six post-transduction, RNA was extracted from which cDNA was synthesised and RT-PCR performed as outlined in section 2.2.e.i.

2.2.f Transduction of moDCs with an Adenovirus Encoding the CMV Gene Phosphoprotein 65

moDCs transduced to express the immunodominant CMV gene phosphoprotein 65 (CMVpp65) were used to refine and establish protocols for T-cell response assays, and later as a positive control when investigating the immunogenic profile of KSHV. Unfortunately, all attempts at cloning the CMV gene phosphoprotein 65 (CMVpp65) into the lentiviral vector using the methods outlined in Materials and Methods section 2.1.a were unsuccessful (discussed in more detail in section 3.1.b). Therefore, an adenovirus encoding CMVpp65 (Adpp65) was used to transduce moDCs.

Adpp65 was a kind gift from Dr. Magnus Essand of Uppsala University and used as previously described (Carlsson et al., 2003). Briefly, on day eight in culture, immature moDCs were harvested by aspiration, spun at 1400rpm for 8 mins, washed once in PBS, counted using a disposable Glasstic® haemocytometer (Hycor Biomedical Ltd., Pencuik, UK) and resuspended in 250µl media. moDCs were

transduced with Adpp65 (thawed on ice for 30 mins prior to use) to a final MOI of 300, at 37ºC for 2 hr. moDCs were then plated on 24-well plates (Cellstar) in medium to a final volume of 1ml per well. moDCs were supplemented with 75ng/ml IL4 (R and D Systems) and 75ng/ml GMCSF (R and D Systems) in order to maintain an immature phenotype or stimulated with a "cytokine cocktail" to induce maturation (see section 2.2.b). On day two post-transduction (day 10 in culture), moDCs were harvested by aspiration and cultured with autologous T cells (see section 2.3.d). Expression of pp65 by moDCs was confirmed by RT-PCR using the same protocol as for the KSHV ORFs (section 2.2.e.i). The primers for pp65 were: Forward, 5' ATGATATCCGTACTGGGTCCC 3'; and reverse, 5' CGGGTCTTCGTGGGAGGTC 3'. The PCR programme was 95ºC for 2 mins; 40 cycles of 95ºC for 1 min, 60ºC for 1 min, 72ºC for 2 mins; and 72ºC for 7 mins.

2.3 T-cell Response Assays

2.3.a T-cell Isolation

T cells were isolated from fresh or cryogenically preserved CD14-negative PBMCs using a standard protocol for immunodepletion to avoid activating the cells. A Pan T-cell Isolation Kit (magnetic beads labelled with monoclonal antibodies directed against CD14, CD16, CD19, CD36, CD56, CD123 and Glycophorin A; Miltenyi Biotec) was used according to the manufacturer's instructions. Briefly, cells were resuspended in 40µl MACs Buffer per 10^7 cells and 10µl Biotin Antibody Cocktail per 10^7 cells, and incubated at 4ºC for 10 mins. 30µl of MACs Buffer per 10^7 cells

and 20µl Anti Biotin Beads per 10^7 cells was then added to the cells, and the cells were incubated at 4ºC for a further 15 mins. The cells were then washed once in 5ml MACs Buffer, resuspended in 500µl MACs Buffer and passed through an equilibrated LS column (Miltenyi Biotech). The LS column was washed three times with 3ml MACs Buffer, and T cells were collected in the flow-through. The non-T-cell fraction was discarded. T cells were washed three times in PBS/1%FCS, and then stained with CFSE (section 2.3.b)

2.3.b CFSE Staining

T cells were resuspended in 5µM carboxy fluoroscein succinimidyl ester (CFSE; Molecular Probes, Invitrogen) solution and incubated at room temperature for 5 mins, then blocked with FCS and incubated at room temperature for a further 15 mins. CFSE-stained cells were washed three times in PBS/1%FCS, and then plated for culture.

2.3.c Allogenic Mixed Lymphocyte Assays

CFSE-stained T cells were cultured in round-bottomed 96-well plates (Cellstar) at 100,000 cells per well in 200µl T-cell media (RPMI supplemented with 10% Human AB Serum, l-glutamine and penicillin-streptomycin). T cells were cultured with allogenic moDCs at four different ratios of moDCs to T cells: 1 to 160, 1 to 80, 1 to 40 and 1 to 20. After six days in culture, T cells were harvested by aspiration and their CFSE-fluorescence was measured by flow cytometry performed on a

FACSCalibur (BD Biosciences). Fifteen thousand events were collected for each sample. The data were analysed with CELLQuest software (BD Biosciences).

2.3.d Autologous Memory T-cell Responses Assays

CFSE-stained T cells were cultured with moDCs as for allogenic mixed lymphocyte reactions (section 2.3.c) but with either transduced or non-transduced autologous moDCs. After six days in culture with moDCs, T cells were harvested by aspiration, T-cell proliferation was assessed by CFSE fluorescence (section 2.3.b), and T cells were stained for cell-surface markers and intracellular cytokine production (section 2.3.e). The maximum number of events possible (10 000 – 30 000) were collected per sample. The data were analysed with CELLQuest software (BD Biosciences). Gating and controls were performed as described in section 2.3.e.

2.3.e Flow Cytometric Analysis of Cell-surface Marker Expression and Th1/Th2 Intracellular Cytokine Production by T cells

For intracellular staining for the production of Th1 and Th2 cytokines, T cells were cultured with moDCs at a ratio of 20 to 1. After six days in culture, T cells were stimulated by the addition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA; Sigma) to 50ng/ml and ionomycin (Sigma) to 500ng/ml. T cells were then incubated at 37ºC for 1 hr then blocked with 10µg/ml Brefeldin A (BFA; Sigma). T cells were incubated at 37ºC for a further 4 hr, and then harvested and stained for cell-surface

marker expression and intracellular cytokine production. All antibodies used were purchased from BD Biosciences along with their appropriate isotype control and are shown in Table 2.5.

Cells were washed once in PBS and stained for cell-surface marker expression by resuspension in 95µl PBS/1%FCS and 5µl of antibody or isotype control to give a final antibody dilution of 1 in 20. Cells were stained on ice, protected from light for 30 mins. Cells were then washed once in PBS/1%FCS and fixed and permeabilised by resuspension in 250µl Cytofix™ Fixation Buffer (BD Biosciences) and incubation on ice for 20 mins. After permeabilisation, cells were washed once in Cell Permwash (BD Biosciences), and then stained for intracellular cytokine production by resuspension in 100µl Cell Permwash and 2µl of IFNγ-PE or 1µl IL4-APC (diluted1:1 in Cell Permwash). Cells were stained on ice for 30 mins, protected from light. Finally, cells were washed once in Cell Permwash and resuspended in 250µl Cell Permwash for analysis.

T-cell - surface marker or intracellular cytokine	Fluoro-chrome	Clone	Antibody Catalogue Number	Isotype	Isotype Control Clone	Isotype Catalogue Number
CD8	PECy5	RPA-T8	555368	IgG1κ	MOPC-21	555750
CD4	PECy5	RPA-T4	555348	IgG1κ	MOPC-21	555750
IFNγ	PE	4S.B3	554552	IgG1κ	MOPC-21	554680
IL4	APC	MP4-25D2	554486	IgG1κ	R3-34	554686

TABLE 2.5: Antibodies and isotype controls used for characterisation of T cells by cell-surface marker expression and intracellular cytokine production. All antibodies were mouse anti-human and all isotype controls were from mice with the exception of IL4-APC, which was rat anti-human, and its corresponding isotype control IgG1κ-APC, which was from rat.

Four-colour flow cytometric analysis was performed on a FACSCalibur (BD Biosciences) and 15 000 events were collected for each sample. Data were analysed with CELLQuest software (BD Biosciences). The live lymphocyte gate was set according to expected forward scatter and side scatter. Isotype controls were used to set gates for positive staining for each of the antibodies. CFSE fluorescence was used to gate for proliferating or non-proliferating cells.

2.4 Protocol for a Screen for Immunogenic KSHV ORFs Using Lentiviral-Transduced moDCs

2.4.a Study Participants

Fourteen KSHV-seropositive (12 HIV-seropositive) and seven KSHV-seronegative individuals (four HIV positive) were recruited from Chelsea and Westminster Hospital, London, UK. All participants provided written, informed consent. All participants were male, apart from one HIV-seropositive KSHV-seronegative female. Study participants' age on date of venesection ranged from 30 to 74 years. All HIV-seropositive individuals were on HAART and had an undetectable HIV viral load (<50 copies per ml). CD4 counts of HIV-seropositive individuals ranged from 171 to 785 cells/mm^3. Study participant characteristics are discussed in more detail in section 4.1.a and summarised in Table 4.1. Individual characteristics of all HIV-seropositive study participants are shown in Table 2.6.

Group	Participant ID	Age	CD4 Count	HIV Viral Load	Receiving HAART?	KSHV-related Disease History	KSHV-related Disease Status	Cancer-specific Treatment (Tx)	Yrs since KS/MCD/Tx
2	C122	70	144	<50	Y	None	NA	NA	NA
	D403	40	411	<50	Y	None	NA	NA	NA
	D919	49	174	<50	Y	None	NA	NA	NA
	S810	32	652	<50	Y	None	NA	NA	NA
3	H419	56	373	<50	Y	None	NA	NA	NA
4	A556	49	226	<50	Y	KS	Remission	Chemotherapy	3.5
	B196	49	209	<50	Y	KS	Remission	Chemotherapy	<1
	B625	39	406	<50	Y	KS	Remission	None	<1
	D208	46	785	<50	Y	KS	Remission	Chemo/radiotherapy	<1
	K058	46	565	<50	Y	PEL	Remission	Chemotherapy	3.5
	M620	38	496	<50	Y	KS	Remission	Chemo/radiotherapy	8.5
	P940	36	645	<50	Y	KS and MCD	Remission	Chemotherapy	<1
	S079	74	183	<50	Y	KS	Remission	None	2
	T328	40	308	<50	Y	KS	Remission	None	<1
	T541	51	171	<50	Y	KS	Remission	Chemotherapy	5
	V689	53	281	<50	Y	KS and MCD	Remission	Chemotherapy	1.5

TABLE 2.6: Individual characteristics of HIV-positive study participants in the screen for immunogenic KSHV ORFs. Y = yes; NA = not applicable.

2.4.b moDC Isolation, Culture and Transduction with the KSHV Lentiviral Library

Sixty millilitres of peripheral blood was collected in to lithium heparin tubes, and moDCs were isolated and cultured as outlined in sections 2.2.a and 2.2.b. moDCs were cultured in media supplemented with 5% human AB serum. On day four in culture, moDCs were transduced by incubation with lentiviral preparations (MOI = 3 to 8). moDCs were transduced with up to three KSHV ORFs grouped according to each ORF's expression during the viral life cycle in PEL cells (Jenner et al., 2001; Jenner and Boshoff, 2002) (Table 2.7). KSHV ORFs known to affect the expression of MHC-I (K3, K5, K9 and ORF 71) were used to singly transduce moDCs, since these genes' functions may affect T-cell priming by moDCs thus skewing the results.

Controls		**Latent**			**Immediate-early Lytic**		
No lenti	pSIN	ORF71	ORF72 ORF73	K11.1 K12 K15	ORF45 ORF50 ORF58	ORF74 K2 K4	K6 K8 K14
Immediate-early Lytic		**Early Lytic**				**Late Lytic**	
ORF57	K5	ORF8 ORF49 ORF61	ORF59 ORF65 K4.1	K3	K9	ORF28 ORF36 ORF37	ORF33 K1 K8.1

TABLE 2.7: Plate map for transduction of moDCs. moDCs were transduced with up to three KSHV ORFs simultaneously. KSHV ORFs were grouped according to their expression profile in the KSHV viral life cycle. ORFs known to affect the expression of MHC-I (K3, K5, K9 and ORF71) were used to singly transduce moDCs since these genes' functions may affect the T-cell response. ORF57 was simply the remainder after the other immediate-early ORFs had been grouped in threes.

After transduction, moDCs were fed with fresh media and cytokines every second day. On day eight in culture (day four post-transduction) moDCs were matured by stimulation with a "cytokine cocktail". On day 10 in culture (day six post-transduction) moDCs were harvested for use in T-cell response assays (section 2.5.c). Any surplus transduced moDCs were stained with antibodies against CD80 and HLA-DR and analysed by flow cytometry (as described in section 2.2.c).

2.4.c T-cell Responses to Lentiviral-transduced moDCs Expressing KSHV ORFs

After the culture and transduction of moDCs (section 2.5.b), autologous T cells were isolated from cryogenically-preserved CD14-negative PBMCs (section 2.3.a) and stained with CFSE (section 2.3.b). CFSE-stained T cells (100 000) were plated for culture in proliferation reactions with 2 500 autologous transduced or non-transduced moDCs (i.e. 40 T cells to 1 moDC) in round-bottomed 96-well plates (Cellstar) in a total volume of 200µl T-cell media (section 2.3.c). In addition, for each experiment the following control wells were set up: 1) T cells only; 2) T cells + non-transduced moDCs + 5µg/ml phytohemagglutinin (PHA; Sigma); 3) T cells + moDCs transduced with Adpp65. All conditions were performed in triplicate.

After six days of culture, cells were harvested, stained with a monoclonal antibody against CD8 conjugated to PECy5 (mouse anti-human, clone RPA-T8, IgG1κ; BD Biosciences) or the appropriate isotype control at a final antibody dilution of 1 in 20, for 30 mins on ice, protected from light. Cells were then washed twice in PBS/1%FCS, resuspended in 250µl Cytofix (BD Biosciences) and incubated for 20

mins on ice protected from light. Cells were washed once more in PBS/5%FCS, and then resuspended in 300µl PBS/5%FCS for flow cytometric analysis. Flow cytometry was performed on a FACSCalibur (BD Biosciences). The maximum possible events (typically between 10 000 and 30 000) were collected for each sample. Data were analysed with CELLQuest software (BD Biosciences). T cells were gated on the live-lymphocyte population according to the expected forward scatter and side scatter, and then on either CD8-positive or CD8-negative (CD4) T-cell populations.

2.4.d Statistical Analysis

For T-cell responses to transduced moDCs, a positive response was designated as a response that fitted three criteria of significance above the background response to moDCs transduced with the empty lentiviral vector: 1) $p<0.05$; unpaired student T test; 2) response > 3.5 standard deviations above background; and 3) response > 10% above background. A borderline response was designated as $p<0.05$ and response > 3 standard deviations above background and > 9% above background.

2.5 Identification of Potential New Late Lytic KSHV CD8 Epitopes

2.5.a ProImmune REVEAL™ Binding Assay

Ninety-four overlapping nine-mer peptides (off-set by one amino acid) were generated to span the entire ORF28 gene sequence using PEPscreen® custom peptide library synthesis and designated P1 to P94. Peptide sequences are shown in Table 2.8. The binding affinity of each peptide to HLA-A*0201 was assessed in a REVEAL™ MHC-peptide binding assay (ProImmune Ltd., Oxford, UK) as previously described (Westrop et al., 2009). Briefly, binding was detected using an antibody specific for the HLA-A*0201-peptide complex in a conformational ELISA. Samples were taken at defined time-points and snap-frozen in liquid nitrogen prior to analysis. Binding affinity was given as a percentage score relative to the binding affinity of a known high-affinity A*0201-restricted T-cell epitope, GILGFVFTL, from the Influenza Matrix protein. A known intermediate-affinity A*0201-restricted T-cell epitopes acted as an additional control. The identity of this epitope was undisclosed by the manufacturer, but was described as: ‘a known T-cell epitope of marginal binding’. One of the best documented A*0201-restricted KSHV CD8 epitope from ORF8 (aa492-500, LMWYELSKI; designated P95) was also included in the assay for comparison. Peptides with a score of greater than 45% were referred to as ‘passed’ peptides and included in future assays (P2, P12, P21, P29, P30, P34, P36, P38, P45, P51, P55, P95; indicated on Table 2.8).

Peptide ID	Peptide Sequence	Peptide ID	Peptide Sequence
1	MSMTSPSPV	49	RVFLAARLW
2*	SMTSPSPVT*	50	VFLAARLWR
3	MTSPSPVTG	51*	FLAARLWRA*
4	TSPSPVTGG	52	LAARLWRAT
5	SPSPVTGGM	53	AARLWRATP
6	PSPVTGGMV	54	ARLWRATPL
7	SPVTGGMVD	55*	RLWRATPLG*
8	PVTGGMVDG	56	LWRATPLGR
9	VTGGMVDGS	57	WRATPLGRA
10	TGGMVDGSV	58	RATPLGRAT
11	GGMVDGSVL	59	ATPLGRATV
12*	GMVDGSVLV*	60	TPLGRATVA
13	MVDGSVLVR	61	PLGRATVAY
14	VDGSVLVRM	62	LGRATVAYQ
15	DGSVLVRMA	63	GRATVAYQV
16	GSVLVRMAT	64	RATVAYQVL
17	SVLVRMATK	65	ATVAYQVLR
18	VLVRMATKP	66	TVAYQVLRT
19	LVRMATKPP	67	VAYQVLRTL
20	VRMATKPPV	68	AYQVLRTLG
21*	RMATKPPVI*	69	YQVLRTLGP
22	MATKPPVIG	70	QVLRTLGPQ
23	ATKPPVIGL	71	VLRTLGPQA
24	TKPPVIGLI	72	LRTLGPQAG
25	KPPVIGLIT	73	RTLGPQAGS
26	PPVIGLITV	74	TLGPQAGSH
27	PVIGLITVL	75	LGPQAGSHA
28	VIGLITVLF	76	*Failed in Synthesis*
29*	IGLITVLFL*	77	PQAGSHAPP
30*	GLITVLFLL*	78	QAGSHAPPT
31	LITVLFLLV	79	AGSHAPPTV
32	ITVLFLLVI	80	GSHAPPTVG
33	TVLFLLVIG	81	SHAPPTVGI
34*	VLFLLVIGA*	82	HAPPTVGIA
35	LFLLVIGAC	83	APPTVGIAT
36*	FLLVIGACV*	84	PPTVGIATQ
37	LLVIGACVY	85	PTVGIATQE
38*	LVIGACVYC*	86	TVGIATQEP
39	VIGACVYCC	87	VGIATQEPY
40	IGACVYCCI	88	GIATQEPYR
41	GACVYCCIR	89	IATQEPYRT
42	ACVYCCIRV	90	ATQEPYRTI
43	CVYCCIRVF	91	TQEPYRTIY
44	VYCCIRVFL	92	QEPYRTIYM
45*	YCCIRVFLA*	93	EPYRTIYMP
46	CCIRVFLAA	94	PYRTIYMPD
47	CIRVFLAAR		
48	IRVFLAARL	95**	LMWYELSKI**

TABLE 2.8: Overlapping nine-mer peptides (off-set by one amino acid) spanning the entire KSHV ORF28 gene sequence. * indicates peptides that scored highest in the REVEAL™ Binding Assay and were included in later assays for further analysis. ** indicates known KSHV control peptide from within ORF8 (aa492 – 500).

2.5.b ProImmune REVEAL™ Off-rate Assay

The twelve highest scoring peptides in the REVEAL™ Binding Assay (P2, P12, P21, P29, P30, P34, P36, P38, P45, P51, P55, P95; indicated on Table 2.8) were further analysed for the stability of their resulting peptide-MHC complexes in a REVEAL™ Off-rate Assay (ProImmune Ltd.). The rate of dissociation for each peptide-A*0201 complex was measured at 0 hr, 2 hr and 24 hr at 37ºC. At each time-point, samples were snap-frozen in liquid nitrogen prior to analysis by conformational ELISA. The percentage denaturation at each time-point was used in the following equation to calculate K (the rate of dissociation):

$$Y = (Y_0 - \text{Plateau})*\exp(-K*X) + \text{Plateau}$$

Where: X = time; Y = % denaturation; Plateau = 0; Y_0 and Plateau are the same units as Y; and $Y = Y_0$ when X = 0.

Half-life values [$t_{1/2}$ (h)] were then calculated for each peptide-A*0201 complex according to Y = 50, therefore X = ln (2)/K.

2.5.c *In Silico* Analysis of Potential Epitopes

Three different epitope-prediction computer algorithms were used to analyse potential epitopes from the late lytic gene pool. These were Immune Epitope Database (IEDB; www.immuneepitope.org); SYFPEITHI (www.syfpeithi.de); and HLA_BIND (www-bimas.cit.nih.gov/molbio/hla_bind/). Each of these algorithms has a different method for scoring potential peptides and different cut-off points are

used to distinguish peptides that are good candidate epitopes. With IEDB, a low score is best and a cut-off score of greater than 5000 was used to eliminate peptides that are not considered likely to be epitopes. With SYFPEITHI, a high score is best, and a cut-off score of less than 15 was used. With HLA_BIND a high score is best and a cut-off score of less than 10 was used.

*2.6 Recognition of Peptide Epitopes by T cells from A*0201-positive KSHV-positive Individuals*

2.6.a Study Participants

Eighteen KSHV-seropositive, HIV-seropositive, HLA-A*0201-positive individuals were recruited from Chelsea and Westminster Hospital, London, UK. All participants provided written, informed consent. Individual characteristics of participants can be seen in Table 2.9. All participants were male, and their age at venesection ranged from 29 to 67 years. CD4 counts ranged from 48 to 1467 cells/mm^3. All participants were on HAART except one (M907, who had previously received HAART but had terminated treatment) and HIV viral loads ranged from undetectable (<50 copies per ml) to 11164. Three individuals had active KS at the time of venesection (B257, B792 and S314), the rest were either in remission from KSHV-related neoplasia, or had quiescent (regressing) KS.

Peripheral blood samples were collected into lithium heparin tubes (BD Biosciences) and PBMCs were isolated as in section 2.2.a.

Participant ID	Age	CD4 Count	HIV Viral Load	Receiving HAART?	KSHV-related Disease History	KSHV-related Disease Status	Cancer-specific Treatment (Tx)	Yrs since KS/MCD/Tx
B042	29	504	<50	Y	KS	Remission	Chemotherapy	<0.5
B066	45	114	<50	Y	KS	Remission	Chemotherapy	<0.5
B257	67	546	<50	Y	KS	Active	None	0
B792	66	300	<50	Y	KS	Active	None	0
C448	37	468	205	Y	KS	Remission	Excision	<0.5
D592	60	1467	<50	Y	MCD	Remission	Chemotherapy	1
D850	48	761	250	Y	MCD	Remission	Chemotherapy	2
G386	55	603	100	Y	MCD	Remission	Chemotherapy	3
H501	43	707	<50	Y	KS and MCD	Remission	Chemotherapy	<1
H980	42	321	<50	Y	KS	Remission	Unknown	6
J228	30	1001	<50	Y	MCD	Remission	Chemotherapy	1
K331	51	302	<50	Y	KS	Remission	Chemotherapy	<1
M907	56	285	11164	N*	KS	Quiescent	Chemotherapy	3
P896	40	500	<50	Y	KS and MCD	Remission	Chemotherapy	<0.5
P940	38	751	<50	Y	KS and MCD	Remission	Chemotherapy	2
S314	42	48	<50	Y	KS	Active	None	0
S929	56	873	<50	Y	KS	Remission	Unknown	9
T541	53	262	<50	Y	KS	Remission	Chemotherapy	7

TABLE 2.9: Characteristics of HIV-positive study participants used for testing HLA-A*0201-restricted peptides. Y = yes; N = no; * = individual was not receiving HAART at the time of venesection for this experiment, but had done previously.

2.6.b Measurement of IFNγ Production in Response to Peptides

Customised peptides were synthesised (ProImmune Ltd.) corresponding to the 12 highest scoring peptides in the REVEAL™ binding assay (P2, P12, P21, P29, P30, P34, P36, P38, P45, P51, P55, P95; indicated on Table 2.8) and eight additional previously published HLA-A*0201-restricted KSHV CD8 epitopes (sequences shown in Table 2.10; more details and references shown in Table 1.2 and Table 5.1).

Expression	KSHV ORF	Epitope Position	Epitope Sequence
Latent	K12	aa17-25	LLNGWRWRL
Immediate-early Lytic	K5	aa154-163	ALYAANNTRV
	ORF6	aa1050-1058	VLGDEVLSL
Early Lytic	ORF61	aa505-513	GLADVFAEL
	ORF65	aa35-43	NMSQAEYLV
	ORF70	aa259-267	YMLAHVTGL
Late Lytic	K8.1	aa209-217	LVLILYLCV
	ORF22	aa59-68	FLNWQNLLNV

TABLE 2.10: Sequences of previously published HLA-A*0201-restricted KSHV epitopes tested for recognition by T cells from KSHV-positive individuals.

IFNγ production by CD8 T cells in response to stimulation with these peptides was measured in ELISpot assays according to the manufacturer's recommendations (Mabtech, Stockholm, Sweden) and as described previously (Burton et al., 2006).

Briefly, 96-well polyvinylidene difluoride-backed plates (Millipore, Watford, United Kingdom) were coated with 100µl/well anti-IFNγ monoclonal antibody (diluted to 5µg/mL in sterile PBS; Sigma) for 1 hr at room temperature or overnight at 4ºC. Plates were washed six times and then blocked with culture media (RPMI 1640 supplemented with penicillin-streptomycin, 2mM l-glutamine and 10% heat inactivated Human AB Serum; all Sigma) for 1 hr at room temperature. PBMCs (2 x 10^5) and 5 or 20µg/ml peptide were added to each well in culture media to a final volume of 200µl/well. Plates were incubated for 18 – 24 hr at 37ºC and 5% CO_2. Plates were then washed six times in PBS, 100µl of a biotinylated anti-cytokine detection antibody (0.5µg/mL in sterile PBS) was added to each well and plates were incubated for 24 hr at 4ºC. Plates were washed again six times with PBS and treated with 100µl/well streptavidine-alkaline phosphatase conjugate (diluted 1:2000 in PBS) for 1 hr at room temperature. Plates were washed a further six times with PBS and then developed with 100µl/well chromogen (AP Conjugate Substrate Kit; Bio-Rad, Hemel Hempstead, UK; prepared according to manufacturer's instructions) for 10 to 15 mins at room temperature, away from intense light. The chromogen was then discarded and plates were washed three times under slow running tap water and then left to dry for 24 hr before analysis. Plates were analysed using a Compact ELISpot Reader (Zeiss, Munich, Germany). The following were included in each assay as controls: PHA (5µg/ml); a pool of Influenza, EBV, and CMV peptides (FEC; 5µg/ml; National Institute for Biological Standards and Control [NIBSC], Hertfordshire, UK); the immunodominant HLA-A*0201-restricted epitopes from HIV GAG (SLYNTVATL; NIBSC; 5µg/ml) and CMV pp65 (NLVPMVATV; ProImmune Ltd.; 5µg/ml); and T-cell media alone. A positive response was designated as a response that was greater than 20 spot-

forming cells (SFC) per million PBMCs above the background response to media alone and greater than five times the background response.

2.6.c Enhancing Peptide Recognition by Autologous moDC Presentation

moDCs were isolated as outlined above (section 2.2.a and 2.2.b). After maturation, moDCs were pulsed with 50μg/ml peptide for 2 hr at 37ºC. Pulsed moDCs were then washed three times, and used as stimuli in the place of peptide solutions in an ELISpot assay with autologous PBMCs. ELISpots were performed as described in section 2.6.b.

2.7 Flow Cytometric Analysis of the Memory Phenotypes and Functions of Virus-specific T cells

Pentamers (ProImmune Ltd) were used to identify virus-specific CD8 T cells by flow cytometry. These pentamers were used in conjunction with immunostaining for cell-surface markers to investigate the phenotype of virus-specific T cells. The use of pentamers in conjunction with intracellular cytokine production to determine the functions of virus-specific T cells was also investigated. Pentamers directed against T-cell receptors specific for the immunodominant HLA-A*0201-restricted epitopes from HIV GAG (SLYNTVATL) and CMV pp65 (NLVPMVATV) were

used to establish assays. Unfortunately no suitable KSHV peptide to target a pentamer against was identified during the course of this work.

2.7.a Memory Phenotypes of Virus-specific CD8 T cells

The pentamer-specific PE fluorotag was spun in a chilled centrifuge (14 000g) for 5 mins prior to starting and then stored on ice, protected from light until use. Two to three million PBMCs per staining condition were aliquoted into FACS tubes (Greiner Bio-one) and washed with 2ml BSA Stain Buffer (BD Biosciences) and spun at 1000g and 4ºC for 5 mins. The supernatant was discarded and cells were resuspended in the residual liquid (~50µl). One test of unlabelled pentamer (2µl for SLYNTVATL- and NLVPMVATV-specific pentamers) was added per tube and mixed by pipetting. Cells were incubated on ice for 40 mins, then washed once as before, the supernatant was discarded and cells were resuspended in the residual liquid. Optimal amounts of monoclonal antibodies for T-cell-surface markers or isotype controls (Table 2.11) were added to cells along with 8µl of the chilled fluorotag and mixed by pipetting. Cells were incubated on ice, protected from light for 20 mins. Cells were washed twice as above and then resuspended in 300µl in Stabilising Fixative (BD Biosciences) before analysis on an LSR II flow cytometer (BD Biosciences). One hundred thousand CD8 events were acquired for each sample and data were analysed using BD FACSDiva software (BD Biosciences). The live lymphocyte gate was set were according to expected forward scatter and side scatter. Isotype controls were used to set gates for positive staining for each of the antibodies. A sample labelled with the fluorotag alone without a pentamer was used as a negative control.

T-cell - surface molecule	Antibody- or Isotype-Fluorochrome	Clone	Isotype	Antibody Catalogue Number	Volume per Test (µl)
CD3	CD3-PerCP	SK7	IgG1κ	345766	3
CD8	CD8-FITC	LT8	IgG1	A003-3B-G	1
CD45RA	CD45RA-PECy7	L48	IgG1κ	337186	3
	IgG1κ-PECy7	MOPC-21	IgG1κ	557872	3
CCR7	CCR7-APC	150503	IgG_{2A}	FAB197A	10
	IgG_{2A}-APC	20102	IgG_{2A}	IC003A	10

TABLE 2.11: Antibodies used to distinguish T-cell memory subsets of pentamer-positive virus-specific T cells. All antibodies and isotypes were purchased from BD Biosciences apart from CCR7 and its isotype, which were from R and D systems and CD8 which was from ProImmune Ltd. All antibodies were mouse anti-human and all isotype controls were from mice.

2.7.b Functions of Virus-specific CD8 T cells

PE-labelled NLVPMVATV-specific pentamer (ProImmune Ltd) was spun in a chilled centrifuge at 14 000g for 5 mins prior to starting and then stored on ice, protected from light until use. Two to three million PBMCs per staining condition were aliquoted into polypropylene culture tubes (BD Biosciences), washed with 2ml BSA Stain Buffer (BD Biosciences) and spun at 1000g and 4ºC for 5 mins. The supernatant was discarded and cells were resuspended in the residual liquid (~50µl). One test of pentamer (10µl) was added per tube and mixed by pipetting. Cells were incubated on ice for 40 mins, then washed once as before, the supernatant was discarded and cells were resuspended in 500µl RPMI 1640 media supplemented with penicillin-streptomycin, l-glutamine and 10% heat-inactivated human AB serum (all Sigma). Cells were stimulated with 1µl Leukocyte Activation cocktail (LAC; Sigma) and placed in a 37ºC humidified CO_2 incubator. After 1 hr, 10µg/ml

Brefeldin A was added to the appropriate tubes (LAC contains Brefeldin A) and then cells were returned to the incubator for 15 hr. Tubes were then centrifuged at 1000g for 5 mins at 4ºC, the supernatant was aspirated, and cell pellets were resuspended in 50µl BSA Stain Buffer containing anti-CD3 and anti-CD8 antibodies (Table 2.12). Cells were incubated on ice, protected from light for 20 mins, then washed in 500µl BSA Stain Buffer and spun as before. The supernatant was aspirated, and cell pellets were resuspended in 200µl 4% paraformaldehyde and incubated on ice for 20 mins. Cells were then washed in 200µl permeabilisation buffer (0.1% saponin, 1% FCS, 0.1% sodium azide in PBS; all Sigma) and spun as before. Supernatant was aspirated and pellets resuspended in 100µl permeabilisation buffer and incubated at room temperature. Antibodies against intracellular cytokines (Table 2.12) were added to tubes and cells were incubated for a further 20 mins at room temperature. Cells were washed once in permeabilisation buffer as before, supernatant aspirated, then resuspended in 300µl Stabilising Fixative (BD

T-cell - surface molecule or intracellular cytokine	Antibody-Fluorochrome	Clone	Isotype	Antibody Catalogue Number	Volume per Test (µl)
CD3	CD3-APCCy7	SK7	IgG1κ	341110	5
CD8	CD8-PerCP	MEM-31	IgG_{2A}	ab65949	25
IFNγ	IFNγ-FITC	4S.B3	IgG1κ	554551	1
TNFα	TNFα-PECy7	MAb11	IgG1κ	557647	3
IL2	IL2-APC	5344.111	IgG1κ	341116	3

TABLE 2.12: Antibodies used to determine functions of pentamer-positive virus-specific T cells. All antibodies and isotypes were purchased from BD Biosciences apart from CD8, which was from Abcam, Cambridge, UK. All antibodies were mouse anti-human.

Biosciences), and transferred to FACS tubes (Greiner Bio-one) before analysis on an LSR II flow cytometer (BD Biosciences). One hundred thousand CD8 events were acquired for each sample and data were analysed using BD FACSDiva software (BD Biosciences). The live lymphocyte gate was set were according to expected forward scatter and side scatter. Un-stimulated controls were used to set gates for positive staining for each of the intracellular antibodies.

2.8 Flow Cytometric Analysis of the Representation of T-cell Memory Subsets in KSHV-infected Individuals

2.8.a Study Participants

Eleven HIV-negative, KSHV-negative and 25 HIV-seropositive, KSHV-positive individuals (all male) were recruited at the Chelsea and Westminster Hospital, London, UK. All participants provided written, informed consent. Of the 25 HIV-seropositive, KSHV-positive individuals, nine had active KS and 15 were in remission from a KSHV-related neoplasm (eight KS and seven MCD). Participants' ages ranged from 24 to 73 years at venesection. All HIV-seropositive participants were on HAART apart from one individual with active KS. CD4 counts of HIV-seropositive individuals ranged from 159 to 811. HIV viral loads ranged from undetectable (<50 copies per ml) to 12599. These characteristics are summarised in Table 5.10 and discussed in section 5.4.a. Individual characteristics of HIV-seropositive study participants are shown in Table 2.13.

Group	Participant ID	Age	CD4 Count	HIV Viral Load	Receiving HAART?	KSHV-related Disease History	KSHV-related Disease Status
2	B862	74	447	<50	Y	MCD	Remission
	C473	57	638	<50	Y	KS	Remission
	C991	58	406	<50	Y	KS	Remission
	D850	48	600	69	Y	MCD	Remission
	E043	43	188	<50	Y	KS	Remission
	F667	42	458	<50	Y	KS	Remission
	H501	43	707	<50	Y	KS and MCD	Remission
	J228	30	687	<50	Y	MCD	Remission
	K331	51	302	<50	Y	KS	Remission
	N047	56	810	<50	Y	MCD	Remission
	S343	42	413	<50	Y	KS	Remission
	S433	38	682	<50	Y	MCD	Remission
	S583	40	811	<50	Y	MCD	Remission
	V685	32	625	<50	Y	KS	Remission
3	B257	67	546	<50	Y	KS	Active
	C448	37	432	12599	N	KS	Active
	E881	40	575	102	Y	KS	Active
	N484	45	444	<50	Y	KS	Active
	N494	34	313	<50	Y	KS	Active
	R632	71	513	<50	Y	KS	Active
	V313	45	350	<50	Y	KS	Active
	W375	39	243	384	Y	KS	Active
	W481	40	159	110	Y	KS	Active

TABLE 2.13: Individual characteristics of HIV-positive study participants for whole-blood phenotyping. Y = yes; N = no.

2.8.b Whole Blood Staining

Peripheral blood samples were collected into EDTA tubes (BD Biosciences). One hundred microlitres whole blood was aliquoted into FACS tubes (Greiner Bio-one, Gloucestershire, UK) and 5µl of each antibody or isotype control (Table 2.14) were added and incubated for 30 mins at room temperature. Red blood cells were lysed

with 2ml FACS Lysing Solution (BD Biosciences) (10 mins, room temperature) then cells were washed three times in PBS and resuspended in 250µl CellFIX (BD Biosciences) before analysis on an LSR II flow cytometer (BD Biosciences). Twenty thousand CD4 events were collected for each sample and data were analysed using BD FACSDiva software (BD Biosciences). The live lymphocyte gate was set according to expected forward scatter and side scatter. Isotype controls were used to set gates for positive staining for each of the antibodies.

T-cell-surface molecule	Fluoro-chrome	Clone	Antibody Catalogue Number	Isotype	Isotype Control Clone	Isotype Catalogue Number
CD3	APCCy7	SK7	557832	IgG1κ	MOPC-21	557873
CD8	APC	RPA-T8	555369	IgG1κ	MOPC-21	555751
CD4	PerCPCy5.5	SK3	332772	IgG1κ	MOPC-21	550795
CD45RA	PeCy7	L48	337186	IgG1κ	MOPC-21	557872
CCR7	PE	150503	FAB197P	IgG_{2A}	20102	IC003P

TABLE 2.14: Antibodies used to distinguish T-cell memory subsets in whole blood. All antibodies and isotypes were purchased from BD Biosciences apart from CCR7 and its isotype, which were from R and D systems. All antibodies were mouse anti-human and all isotype controls were from mice.

2.9 Study Participant Characterisation Techniques

The following assays were used to characterise the phenotypes of participants from each of the studies described in this thesis (section 2.4.a; 2.6.a; and 2.8.a).

2.9.a Serology for KSHV Infection

An in-house developed MIX-MAP ELISA was used to detect KSHV seropositivity as previously described (Bourboulia et al., 2004) but with an improved, modified peptide containing two copies each of a lytic and a latent epitope (RSHLGFWQEGWSGQVYQDWLGRMNCSYENM derived from K8.1, and QPGPSREYRYVLRTSPPHRPGVRMRRV derived from ORF73, respectively). Briefly, 96-well plates (Nunc, Thermo Fisher Scientific, Rochester, New York, USA) were coated with 100µl/well peptide solution diluted to 2µg/ml in sterile PBS (Sigma) and incubated overnight at 4ºC. Plates were washed four times in a wash buffer of PBS/0.1%Tween-20 (Sigma) and then blocked with 200µl/well of blocking buffer [PBS/0.1%Tween-20/5% Milk (Premier International Foods Ltd., Spalding, Lincs., UK)] for 30 mins at 37ºC. Plates were washed as before and 95µl of serum samples or controls (diluted 1:101 in blocking buffer) were added to appropriate wells and incubated for 90 mins at room temperature. After another wash as above, 100µl/well secondary antibody solution (rabbit anti-human IgG, P0214; DAKO, Glostrup, Denmark; diluted 1:4000 in blocking buffer) was added and incubated for 1 hr at 37ºC. After a final wash as above, 200µl/well substrate solution (TMB Microwell Peroxidase Substrate System; KPL, Gaithersburg, Maryland, USA) was added and incubated for 20 mins at room temperature. Reactions were stopped by the addition of 50µl/well 2M H_2SO_4 (Sigma). Plates were read using a Varioskan plate reader (Thermo Electron, Thermo Fisher Scientific) at 450nm optical density. All experiments were performed in triplicate. A positive result was designated as a reading greater than the mean of four negative

control samples (each in triplicate) plus five times the standard deviation of the four negative controls.

2.9.b HLA Typing

i. From Cryogenically-preserved PBMC Samples

This method was used for HLA-typing the seven study participants who responded to the [ORF28/ORF36/ORF37] in the screen for immunogenic KSHV ORFs using lentiviral-transduced moDCs (section 2.4.a). Genomic DNA was extracted from a minimum of 5 $x10^6$ PBMCs using the standard Nucleon® BACC2 kit (Tepnel Life Sciences, Manchester, UK) protocol. HLA typing was performed by the Department of Histocompatibility and Immunogenetics, Clinical Immunology Laboratory, Hammersmith Hospital, London. Low resolution typing was performed using PCR-sequence specific primers (PCR-SSP). High resolution typing was achieved using reference strand conformational analysis (RSCA).

ii. From Whole Blood Samples

This method was used to identify HLA-A*0201 positive individuals with a history of KSHV-related neoplasia to test A*0201-restricted peptides and pentamers from ORF28 (section 2.5.c). Participants were recruited from the Chelsea and Westminster HIV-Oncology clinic and provided written, informed consent. Peripheral blood samples were collected into EDTA tubes and snap-frozen in liquid nitrogen before storage at -80ºC until use. Genomic DNA was extracted from 200µl

whole blood using a QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturers' instructions. At the end of the protocol, DNA was eluted in 200µl of the provided elution buffer. HLA typing was performed by the Histocompatibility Laboratories, Anthony Nolan Trust, Royal Free Hospital, London, UK. High and low resolution typing was performed by PCR-sequence specific oligonucleotides (PCR-SSO).

2.10 Statistics

Statistics were performed on Microsoft Office Excel 2003 software and GraphPad Prism 5.0 software. Significance was tested using a student T test or a Mann-Whitney test where appropriate.

CHAPTER 3: Preparation for and Design of a Lentiviral-based System for Investigating the CD8 and CD4 T-cell Responses Against KSHV

3.1 Cloning KSHV ORFs into the Lentiviral Vector

3.1.a Cloning Novel KSHV ORFs into the Lentiviral Vector

i. Cloning Novel KSHV ORFs into the pSIN Lentiviral Vector

Five putative immunogenic KSHV open reading frame (ORF) inserts (ORF8, ORF57, ORF59, ORF61 and ORF65; marked with a double asterisk on Table 1.1) were successfully cloned into the pSIN lentiviral vector. Plasmid DNA solutions were screened for the presence of the transgene insert. The insert was excised from the multiple cloning site of the plasmid vector by restriction digestion, and gel electrophoresis was used to check for the presence of the linearised pSIN vector (10kb) and a second DNA fragment of the expected length of the KSHV ORF insert (Figure 3.1).

ii. Sequencing the KSHV Transgene Inserts

For each of the five plasmid vector preparations that contained an insert of the expected size, the transgene was sequenced and compared to its published gene

sequence. This was to ensure that no point mutations had been introduced during the cloning process.

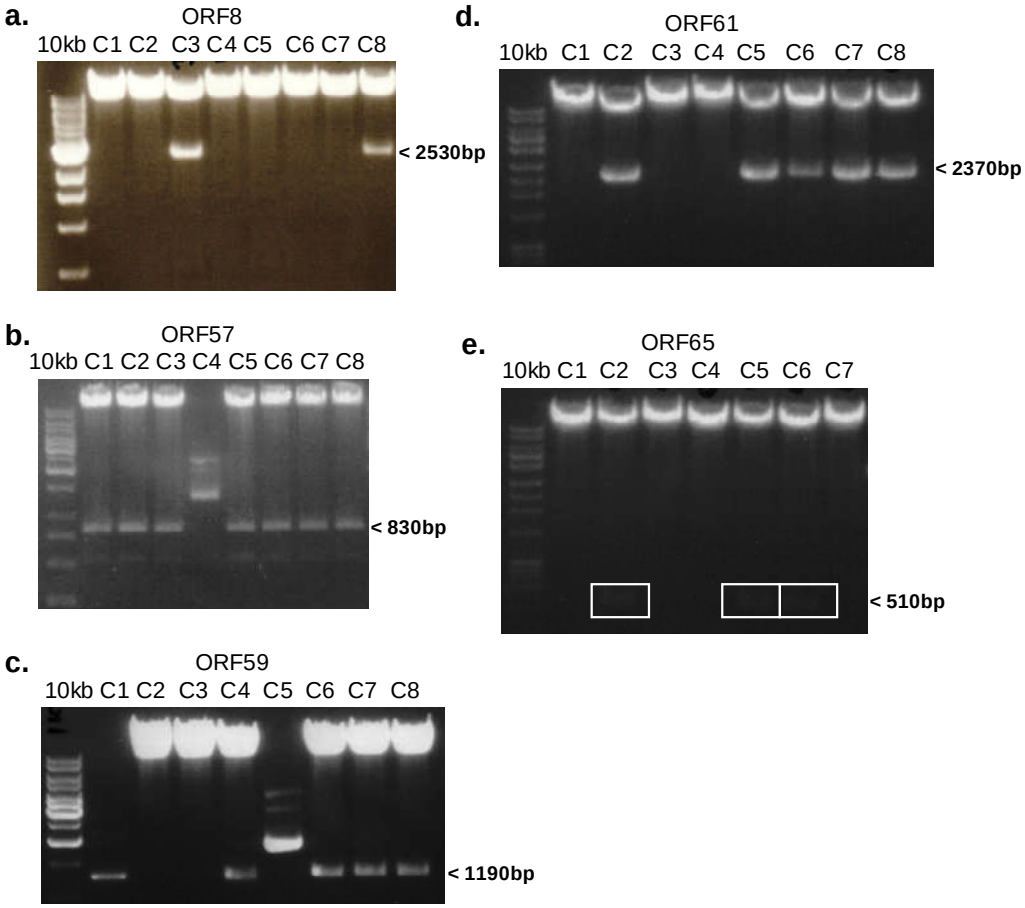


FIGURE 3.1: Electrophoresis gels showing lentiviral plasmid DNA after restriction digestion to check for presence of KSHV ORF insert. a. ORF8 (2530bp) insert is present in clones 3 and 8. b. ORF57 (830bp) insert is present in clones 1, 2, 3, 5, 6, 7 and 8. c. ORF59 (1190bp) insert is present in clones 4, 6, 7 and 8. d. ORF61 (2370bp) insert is present in clones 2, 5, 6, 7 and 8. e. ORF65 (510bp) insert is present in clones 2, 5 and 6. DNA bands are very faint and do not reproduce well here and so presence is indicated by white open boxes. In all gels, the bright band at 10kb is the linearised lentiviral vector with the insert removed.

The lentiviral vectors encoding ORF59 and ORF65 contained no point mutations in their respective KSHV gene sequences as compared to their published gene sequences.

The lentiviral vector encoding ORF57 contained a single point mutation in the ORF57 gene sequence at base pair 243, where a thymine base replaced a cytosine base in the published sequence. This is, however, a silent mutation that converts the codon CAC to the codon CAU, both of which code for the amino acid histidine.

The lentiviral vector encoding ORF61 contained three point mutations in the ORF61 gene sequence. The first point mutation was at base pair 1644, where a cytosine base replaced an adenine base in the published sequence. This is a silent mutation that converts the codon GGA to the codon GGC, both of which code for the amino acid glycine. The second point mutation was at base pair 1857, where a cytosine base replaced a thymine base in the published sequence. This is a silent mutation that converts the codon UCU to the codon UCC, both of which code for the amino acid serine. The third point mutation was at base pair 2070, where a thymine base replaced a cytosine base in the published sequence. This is also a silent mutation that converts the codon GAC to the codon GAU, both of which code for the amino acid aspartic acid.

The lentiviral vector encoding ORF8 contained two point mutations in the ORF8 gene sequence. One was at base pair 1422, where an adenine base was replaced by a guanine base in the published sequence. This is a silent mutation that converts the codon CAG to the codon CAA, both of which code for the amino acid glutamine. The other point mutation was at base pair 1199, where a cytosine base replaced a thymine base in the published sequence. This converts the codon GTC, which codes for the amino acid valine, to the codon GCC, which codes for the amino acid alanine. This mutation, however, lies outside of the coding sequence for the well-

documented optimal CD8 T-cell epitope within ORF 8, aa492-500 (LMWYELSKI) (Wang et al., 2002b). It is also not a particularly significant change as both valine and alanine are non-polar, neutral amino acids with similar molecular structures. The change was carefully noted, but it was considered acceptable to use this expression vector in a screen for immunogenic KSHV genes.

3.1.b Cloning the CMV Gene Phosphoprotein 65 into the Lentiviral Vector

A lentiviral vector encoding the immunodominant CMV gene phosphoprotein 65 (CMVpp65) was proposed for use as a positive control in a screen for immunogenic KSHV genes. Unfortunately, however, all attempts at cloning CMVpp65 into the pSIN lentiviral vector using the same strategy for cloning KSHV genes into pSIN (as outlined in Materials and Methods section 2.1.a) were unsuccessful. Numerous adjustments to the technique and alternative strategies were tried, but all to no avail.

Briefly, CMVpp65 was successfully amplified from both a pBluescriptII plasmid vector containing the gene (a kind gift from Dr John Zaia, National Medical Center and Beckman Research Institute, City of Hope, California, USA) and from total genomic DNA extracted from CMV (strain AD169)-infected neonatal dermal fibroblasts two days post-infection. However, all subsequent attempts at ligating either product into the pSIN vector were unsuccessful. The following techniques were tried to no avail: the use of different restriction enzyme sites from within the multiple cloning site for insertion; varying all possible parameters in the restriction digestion reaction; dephosphorylating the vector prior to ligation; varying all

possible parameters in the ligation reaction; the use of different ligation enzymes; the use of a ligation enzyme-free insertion system (In-Fusion™ PCR Cloning System; Clontech, Takara Bio Inc., Shiga, Japan); the use of a sub-cloning technique, successfully inserting CMVpp65 first into a TA vector (vector PCR 2.1, Invitrogen), then transferring into pSIN (which was again, unsuccessful). Finally, a biotechnology company, Biogenova Inc. (Ellicott City, Maryland, USA), was employed to perform the cloning reaction, but they were also unsuccessful. An adenovirus encoding CMVpp65 was thus used as a positive control to investigate the use of transduced moDCs to detect antigen-specific T-cell responses (see section 3.4).

3.2 Culture and Transduction of monocyte-derived Dendritic Cells (moDCs)

3.2.a moDC Isolation and Culture: Survival

CD14-positive monocytes were isolated from peripheral blood and cultured for 10 days in the presence of the cytokines interleukin 4 (IL4) and granulocyte/macrophage colony-stimulating factor (GMCSF), which promote differentiation of the monocytes into monocyte-derived dendritic cells (moDCs) (Sallusto and Lanzavecchia, 1994). moDCs were either left immature or matured by stimulation with either LPS or a ‘cytokine cocktail’. The differentiating monocytes were cultured in media supplemented with either 10% foetal calf serum (FCS), 5% human AB serum (AB) or 2% AB. Media supplemented with 10% FCS is

conventional for culturing moDCs, and the cells survive well in this serum (Jeras et al., 2005). However, moDCs cultured in FCS prime a high background in T-cell response assays when cultured with autologous T cells as they present antigens from bovine proteins present in the FCS (Jonuleit et al., 2001). This raised concern that subtle responses to KSHV genes could be masked in the proposed experiments. Therefore, survival of moDCs cultured in human AB serum was compared with survival of moDCs cultured in FCS, as moDCs grown in AB should prime a lower background T-cell response. Different batches of AB (Sigma-Aldrich, Poole, UK) were evaluated, and moDCs survived best in lot number 027K0432, so this batch was used for all future experiments.

Figure 3.2 shows the yield of different populations of moDCs after 10 days in culture in media supplemented with either 10% FCS or 5% or 2% AB (lot number 027K0432). Survival was measured as the number of live (trypan-blue excluding) moDCs recovered by aspiration of the culture plate on culture day 10, divided by the number of live monocytes plated on culture day 0. moDCs cultured in 2% AB showed very poor survival and consequently a low yield of cells (15.0% to 16.7%). However, there was no significant difference ($p>0.05$; student T test) between the yield of moDCs after culture in either 10% FCS (43.3% to 46.0%) or 5% AB (36.1 to 42.9%). Therefore, 10% FCS or 5% AB serum supplements were used in further experiments to compare the phenotype and function of moDCs cultured conventionally in FCS or in the selected batch of AB serum.

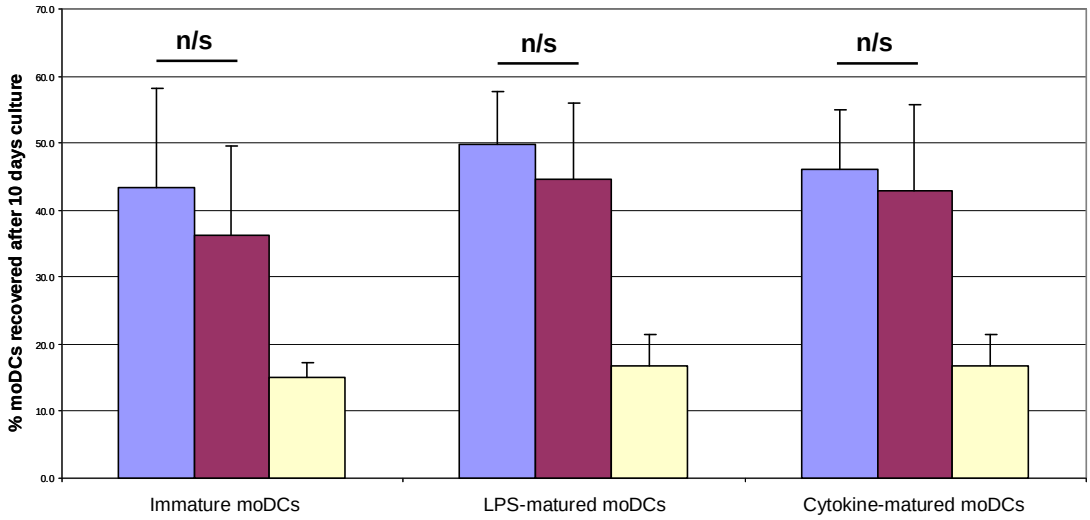


FIGURE 3.2: Yield of moDCs after 10 days in culture in media with different serum supplements. 10% FCS (blue bars), or 5% (purple bars) or 2% (yellow bars) human AB serum. moDCs were either left immature (left), or were matured by stimulation with either LPS (middle) or a cytokine cocktail (right). Survival of moDCs was assessed by yield of viable cells. Means and standard deviations of five (10% FCS and 5% AB) or two (2% AB) experiments are shown. moDCs did not survive well in 2% human AB serum, but there was no significant difference in survival of moDCs cultured in 10% FCS or 5% human AB serum (student T test).

3.2.b moDC Cell-surface Marker Expression and Maturation

After 10 days culture in the presence of IL4 and GMCSF, immature and mature moDCs were analysed for their expression of cell-surface markers by multi-parameter flow cytometry. The cell-surface markers examined were the protein complexes major histocompatibility complex I (MHC-I) and II (HLA-DR), which present antigens to CD8 and CD4 T cells respectively; the costimulatory molecules CD80 and CD86, which bind the T-cell molecules CD28 and CTLA-4 and provide the necessary stimuli to activate a T-cell response to antigens; CD40, a

costimulatory molecule which binds CD40L on CD4 helper T cells; and CD83, a cell surface molecule that is dramatically upregulated by DCs in response to maturation stimuli. Figure 3.3 shows an example of marker expression by immature and mature moDCs. Immature moDCs had high expression of MHC-I, mid-range expression of HLA-DR, CD86 and CD40 and low expression of CD80 and CD83. All markers were upregulated after exposure to a maturation stimulus. CD80 and CD83 showed the most dramatic upregulation, as these markers were expressed at the lowest levels prior to maturation.

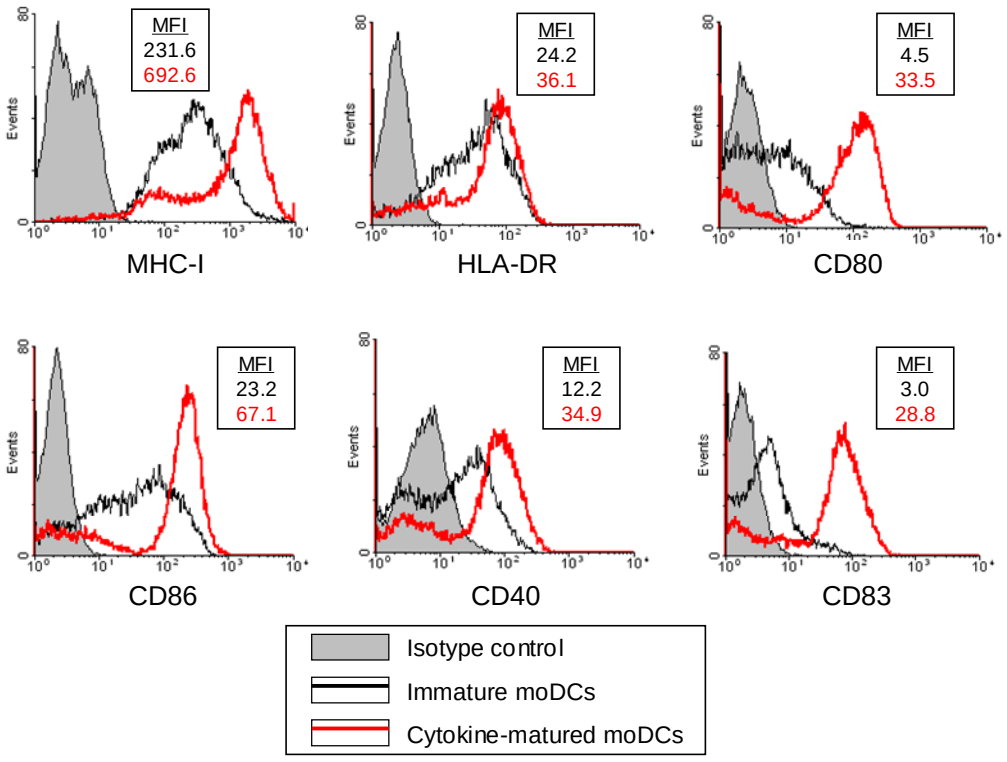


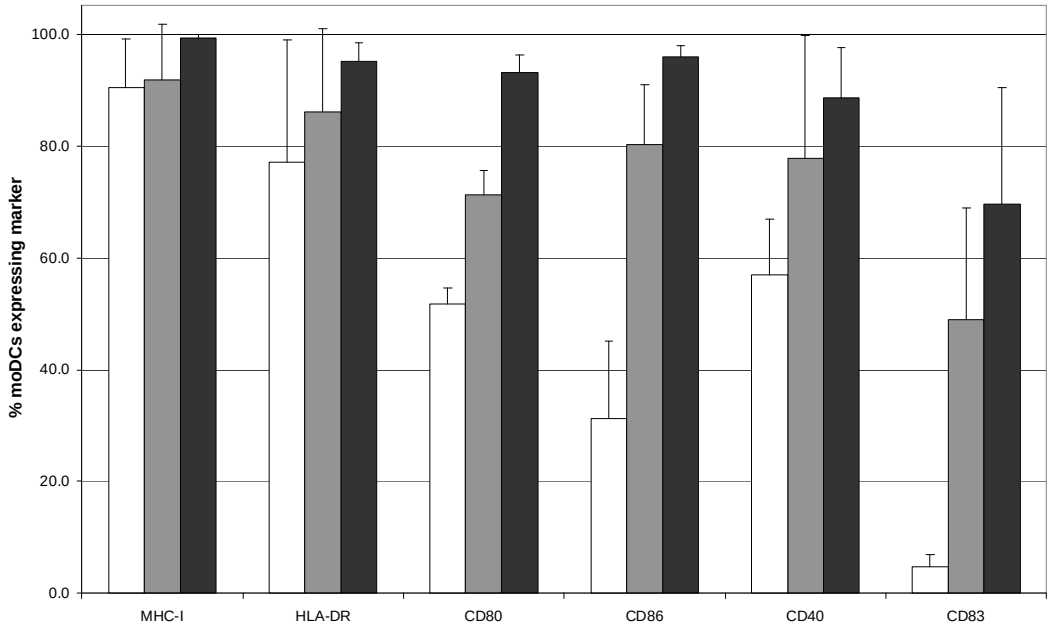
FIGURE 3.3: Cell-surface marker expression by moDCs. Flow cytometry histograms showing cell-surface marker expression by moDCs for one representative experiment out of three. moDCs were cultured in 5% human AB serum supplemented with IL4 and GMCSF for 10 days and either left in an immature state (black lines) or exposed to a 'cytokine cocktail' maturation stimulus (red lines). MFI indicates mean fluorescence intensity.

Figure 3.4 summarises three experiments investigating cell-surface marker expression by moDCs cultured in either 10% FCS (Figure 3.4.a) or 5% AB serum (Figure 3.4.b) and either left in an immature state or exposed to different maturation stimuli.

DC marker expression was comparable in moDCs cultured in either 10% FCS or 5% AB. The biggest variation in the immunophenotype between moDCs cultured in the two serum supplements was seen in immature moDCs, indicating that stimulating maturation strongly promotes moDC differentiation, giving rise to a more homogenous population of moDCs. For the most part, the 'cytokine cocktail' induced greater marker upregulation than LPS, and nearly all markers were expressed by 90% or more of moDCs after exposure to this maturation stimulus, regardless of which serum supplement the cells were cultured in.

In FCS, 90.5 ± 8.5% (mean ± s.d.) of immature moDCs expressed MHC-I, and in AB this rose slightly to 98.1 ± 2.7%. After exposure to either maturation stimuli MHC-I expression was maintained by between 91.7% and 99.2% of moDCs in either serum supplement. HLA-DR was expressed by 77.0 ± 21.8% of FCS-grown and 88.4 ± 9.6% of AB-grown immature moDCs, and was upregulated by both maturation stimuli so that 86.1% to 95.7% of moDCs cultured in either serum were positive for this marker. CD80 and CD40 were both expressed at mid-range levels by immature moDCs: 55.8% to 56.9% of moDCs cultured in either serum were CD40-positive and 30.7 ± 11.1% of AB-cultured moDCs or 51.8 ± 2.8% of FCS-cultured moDCs were CD80-positive.

a.



b.

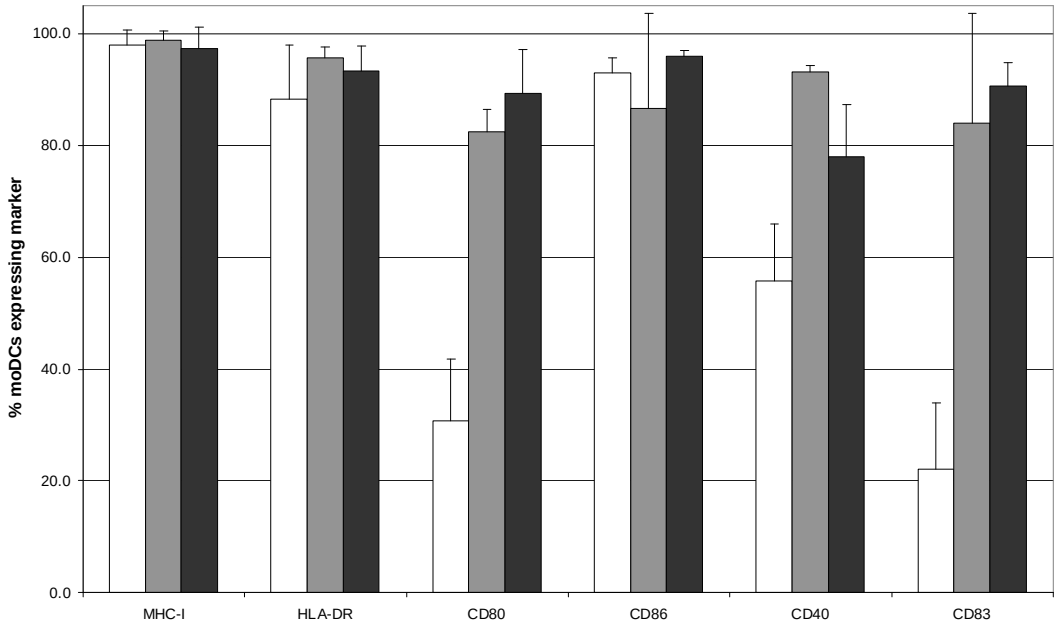


FIGURE 3.4: Antigen-presenting surface marker expression by moDCs cultured in either (a) 10% FCS or (b) 5% AB serum. moDCs were either left immature (white bars) or exposed to either LPS (pale grey bars) or a 'cytokine cocktail' (dark grey bars) maturation stimulus. Bars show the proportion of moDCs expressing each of the markers (MHC-I, HLA-DR, CD80, CD86, CD40 and CD83), as measured by flow cytometry. Means and standard deviations are shown (n = 3).

After exposure to either maturation stimuli, these costimulatory molecules were upregulated so that 77.8% to 93.0% of moDCs cultured in either serum expressed both CD40 and CD80. The only exception was FCS-cultured moDCs stimulated with LPS, which showed a slightly lower proportion of moDCs positive for CD80 (71.2 ± 4.4%). CD86 was expressed by 92.9 ± 2.9% of immature moDCs cultured in AB compared to only 31.2 ± 13.9% of FCS-cultured immature moDCs. Exposure to either maturation stimuli resulted in 80.2% to 96.7% of moDCs expressing this marker, regardless of culture serum. Only a small proportion of immature moDCs expressed CD83: 4.2 ± 2.2% of FCS-cultured moDCs and 22.0 ± 11.9% of AB-cultured moDCs. After exposure to either LPS or a 'cytokine cocktail', 84.0% or 90.7% of AB-cultured moDCs and 48.9% or 69.5% of FCS-cultured moDCs were CD83-positive, respectively.

Overall, the expression of these DC markers by the moDCs demonstrates that this method for deriving DCs worked in my hands; that the resulting cells display an antigen-presenting surface phenotype; and that the moDCs can be successfully cultured in either 10% FCS or 5% human AB serum. The 'cytokine cocktail' appears to be the most potent maturation stimulus, resulting in a heterogenous population of moDCs that expresses high levels of each the antigen-presenting cell-surface markers.

This is further summarised in Figure 3.5, which shows a direct comparison between the maturation profiles of moDCs cultured in either FCS or human AB serum before and after exposure to different maturation stimuli. The maturation profile was assessed as the percentage of cells that were both CD80-positive and HLA-DR-positive. Immature moDCs cultured in FCS showed a greater proportion of double-

positive mature cells than immature moDCs cultured in human AB serum (an average of 50% compared to 29% respectively; $p<0.005$, student T test). This is in correlation with the higher proportion of CD80-positive immature moDCs observed after culture in FCS as compared to AB, as shown in Figure 3.4. Also in agreement with the observations shown in Figure 3.4, Figure 3.5 reveals that the 'cytokine cocktail' was the most potent maturation stimulus for moDCs cultured in either serum. Although the cytokine-matured moDCs cultured in AB showed a slightly lower proportion of double-positive mature cells than those grown in FCS (82% compared to 94% respectively; $p<0.05$, student T test), there was still an average of over 80% of cells that were positive for both CD80 and HLA-DR. This was a higher proportion than that for moDCs cultured in either serum and matured by stimulation with LPS (73% for moDCs cultured in AB and 79% for moDCs cultured in FCS).

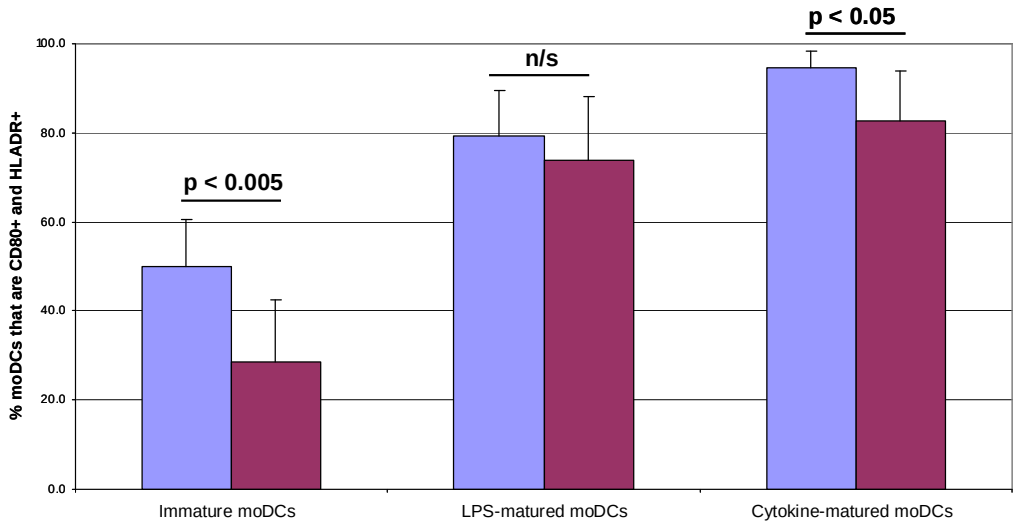


FIGURE 3.5: Maturation profiles of immature moDCs and moDCs exposed to different maturation stimuli. moDCs were cultured in 10% FCS (blue bars) or 5% human AB serum (purple bars). moDCs were left immature (left) or matured by stimulation with either LPS (middle) or a 'cytokine cocktail' (right). The maturation profile of each moDC population was assessed as the proportion of moDCs expressing both CD80 and HLA-DR. Means of nine (10% FCS) or eight (5% AB) experiments, and standard deviations are shown. P values are from a paired student T test for moDCs isolated from the same donor, cultured in parallel in different serum supplements.

3.2.c Transduction of moDCs with a GFP-encoding Lentivirus

i. Time-point Optimisation

To investigate the kinetics of lentiviral transduction, four wells of moDCs were transduced with 1ml of GFP-encoding lentivirus (pCSGW). On each day post-transduction, moDCs were examined for GFP-expression using a light fluorescence microscope. On day four GFP-expressing cells were observed, and these increased in number over each subsequent day. The percentage of moDCs expressing GFP was analysed by flow cytometry on days four, five, six and seven post-transduction. Figure 3.6 shows that the percentage of GFP-positive cells steadily increased over time post-transduction (4.78% GFP-positive cells on day four, rising to 41.96% on day seven). For the purpose of future experiments a five- or six-day period was

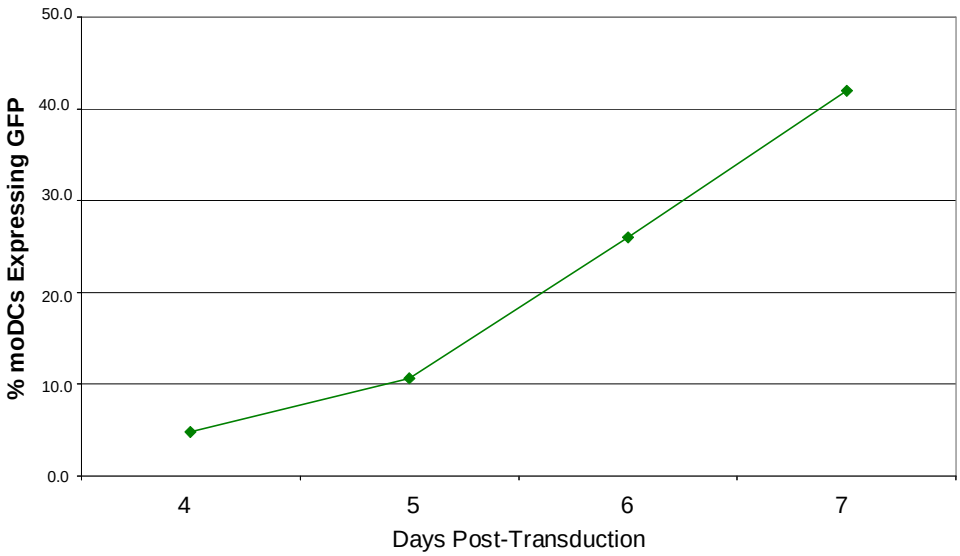


FIGURE 3.6: Percentage of moDCs expressing GFP at different time-points after transduction with a GFP-encoding lentivirus. The percentage of GFP-positive moDCs was measured by flow cytometry. It increased steadily over several days post-transduction. At the last time-point examined (seven days post-transduction) the highest proportion of GFP-positive moDCs was observed (41.96%). Results from one experiment are shown, and demonstrate the same trend of increasing transgene expression as reported in the literature (Schroers et al., 2000; Dyall et al., 2001).

selected as a suitable length of time for transduction of moDCs before using them in further assays, as this represented a balance between achieving good transgene expression (10.63% to 25.99% of GFP-positive cells) and optimal moDC viability. In three further experiments, a mean transduction efficiency of 12.5 ± 2.5% GFP-positive immature moDCs was observed at six-day's post-transduction.

ii. Multiplicity of Infection

In order to determine the optimal multiplicity of infection (MOI) for lentiviral transduction of moDCs, a titration experiment was performed. moDCs were transduced with 0.05, 0.1, 0.3, 1.0 or 1.5 ml of a GFP-encoding lentivirus. On day five post-transduction, the lentiviral copy number per cell for each transduction was determined by qPCR and the percentage of GFP-expressing moDCs was assessed by flow cytometry (Figure 3.7). Lentiviral copy number per moDC increased with increasing volume of lentivirus used in transduction. At low MOIs, the percentage of moDCs that were GFP-positive increased slightly from 10.6% GFP-positive moDCs at 3.0 lentiviral copies per cell to 17.2% at 5.6 lentiviral copies per cell. However, interestingly, at higher MOIs the proportion of GFP-positive moDCs appeared to reach a plateau, and then declined slightly to 16.6% GFP-positive moDCs at 16.9 lentiviral copies per cells and 13.2% GFP-positive cells at 30.1 lentiviral copies per cells. This may be due to a toxic effect of high MOIs on the moDCs, as reported by other groups (Gruber et al., 2000; Dyall et al., 2001). Therefore an optimal MOI of between three and eight lentiviral copies per moDC was used for future experiments, as MOIs greater than this did not appear to significantly increase that proportion of GFP–positive moDCs.

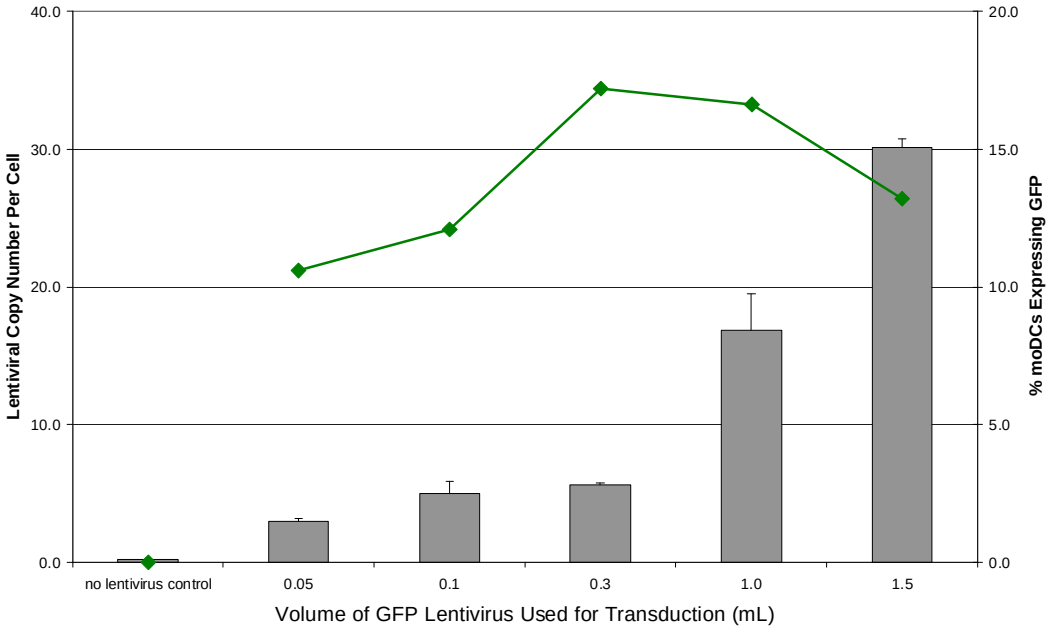


FIGURE 3.7: Titration to determine optimal multiplicity of infection (MOI) for transgene expression using the pSIN lentiviral vector. qPCR was used to determine the lentiviral copy number per cell (grey bars) for moDCs transduced with different volumes of GFP-encoding lentivirus. Means and standard deviations from two experiments are shown. Flow cytometric analysis was used to determine the percentage of moDCs that were GFP-positive (green diamonds) after transduction with different volumes of lentivirus. Results from one experiment are shown.

iii. Characterisation of moDCs Transduced with a GFP-encoding Lentivirus

To investigate whether lentiviral transduction of moDCs affected their antigen-presenting surface phenotype, MHC-I expression by GFP-transduced (MOI = 5.6) and non-transduced moDCs was examined by flow cytometry. moDCs were stained with an MHC-I antibody, and GFP-positive and GFP-negative moDCs from three donors were compared for MHC-I expression (Figure 3.8.a and 3.8.c). LPS stimulation resulted in a 1.7- or 1.9-fold increase in MHC-I mean fluorescence intensity (MFI) in non-transduced and GFP-transduced moDCs respectively.

Stimulation with a ‘cytokine cocktail’ resulted in a 2.9- or 3.2- fold increase in MHC-I MFI in non-transduced and GFP-transduced moDCs respectively. Importantly, there was no significant difference ($p>0.05$; student T test) between the MHC-I MFI of non-transduced and GFP-transduced moDCs from each individual, and the fold increase in MHC-I MFI after exposure to either maturation stimuli was equivalent for both non-transduced and GFP-transduced moDCs.

To confirm that lentiviral transduction did not affect maturation of moDCs, CD80 expression by GFP-transduced and non-transduced moDCs was examined by flow cytometry. moDCs were stained with a CD80 antibody conjugated to PE and GFP-positive and GFP-negative moDCs from three donors were compared for CD80 expression (Figure 3.8.b and 3.8.c). The CD80 MFIs of both non-transduced and GFP-transduced moDCs were equivalent, and increased by 1.7- or 1.8-fold after stimulation with LPS and 3.6- or 3.4-fold after stimulation with a ‘cytokine cocktail’.

Interestingly, the proportion of moDCs that were GFP-positive decreased after moDCs were exposed to maturation stimuli (Figure 3.9). GFP expression appeared to be downregulated in correlation with the potency of the stimulus used to induce moDC maturation. Stimulation with a ‘cytokine cocktail’ induced strong, uniform maturation of immature moDCs (82% to 94% of moDCs double-positive for CD80 and HLA-DR, Figure 3.5) and resulted in a 37.1% (FCS-grown moDCs) to 38.5% (AB-grown moDCs) decrease in the proportion of GFP-positive moDCs that was statistically significant (student T test; see Figure 3.9). LPS, which at 20ng/ml gave rise to around 73% to 79% of double-positive moDCs, caused a smaller decrease in

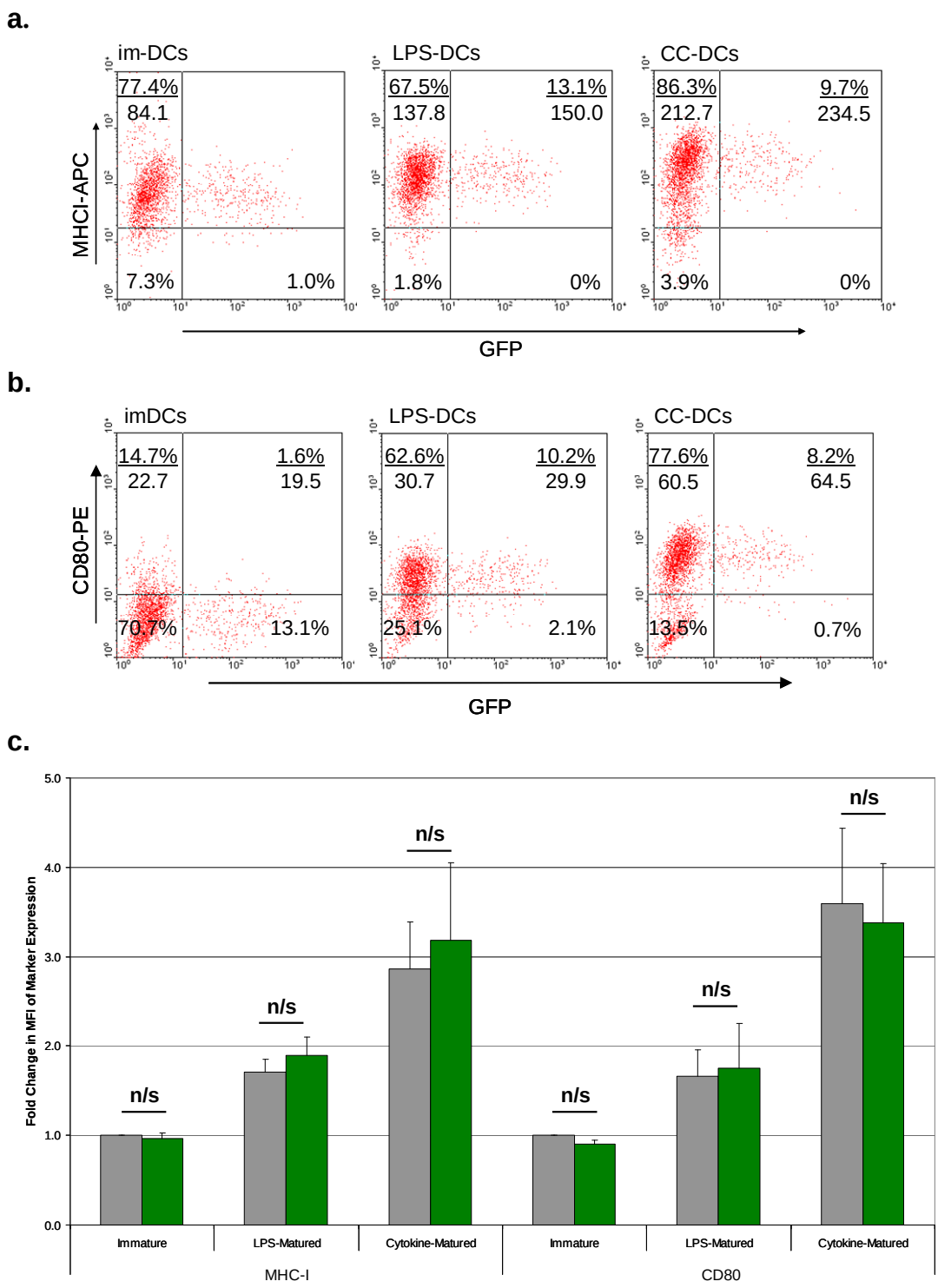


FIGURE 3.8: Expression of MHC-I and CD80 by non-transduced and GFP-transduced moDCs. a. and b. show examples of flow cytometry plots showing MHC-I (a) and CD80 (b) surface expression by non-transduced (left-hand quadrants) and GFP-transduced (right-hand quadrants) moDCs before and after maturation. Numbers in each quadrant indicate the percentage of cells in the quadrant (top) and the MHC-I or CD80 MFI of the cells in that quadrant (bottom). c. shows fold change in MHC-I and CD80 surface expression by non-transduced (grey bars) and GFP-transduced (green bars) before and after maturation by different stimuli. Mean and standard deviations from three experiments are shown. In each experiment, MFIs were normalised to non-transduced immature moDCs.

the proportion of GFP-expressing cells that was not significantly different from the proportion of GFP-positive immature moDCs.

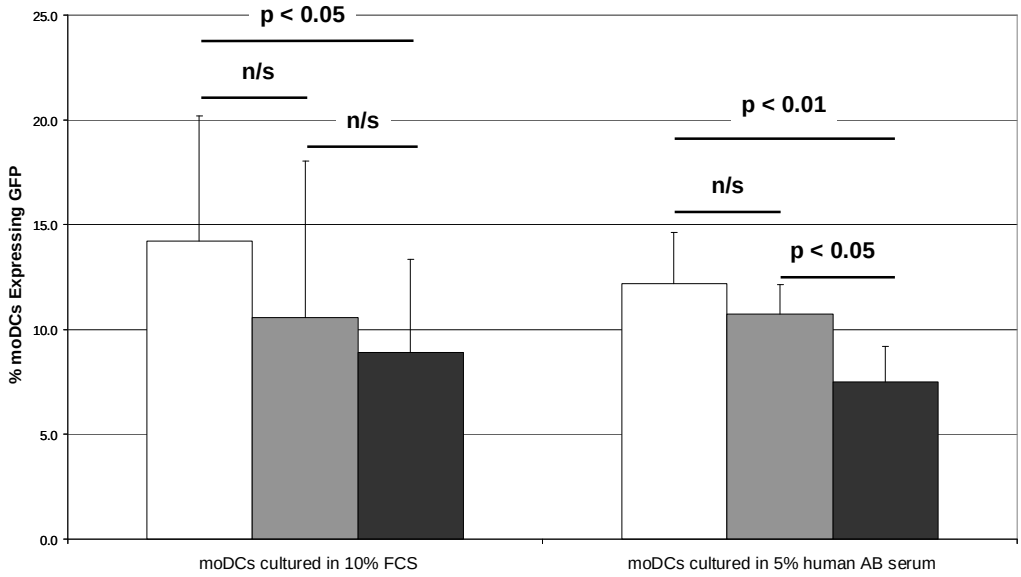


FIGURE 3.9: Percentage of GFP-positive moDCs before and after exposure to different maturation stimuli: immature (white bars); LPS-matured (pale grey bars); cytokine matured (dark grey bars). Maturation induced downregulation of GFP. Stimulating moDCs with a 'cytokine cocktail' resulted in a 37.1% – 38.5% decrease in the proportion of GFP-positive cells compared to immature moDCs that was statistically significant (paired student T test). LPS stimulation resulted in a lesser decrease in the proportion of GFP-positive cells that was not statistically significant. Means and standard deviations from three experiments are shown.

3.2.d Transduction of moDCs with the KSHV Lentiviral Library

i. Confirmation of Viral Gene Expression by RT-PCR

To confirm that all the 31 different KSHV-ORF-encoding lentiviral vectors in the KSHV lentiviral library (26 previously existing constructs and five newly constructed as part of the work described in this thesis, section 3.3.1) successfully transduced moDCs resulting in expression of the appropriate transgene insert, moDCs were transduced with individual KSHV ORFs. On day six post-

transduction, moDCs were harvested, RNA was extracted and reverse-transcription (RT)-PCR was used to confirm KSHV transgene expression (Figure 3.10).

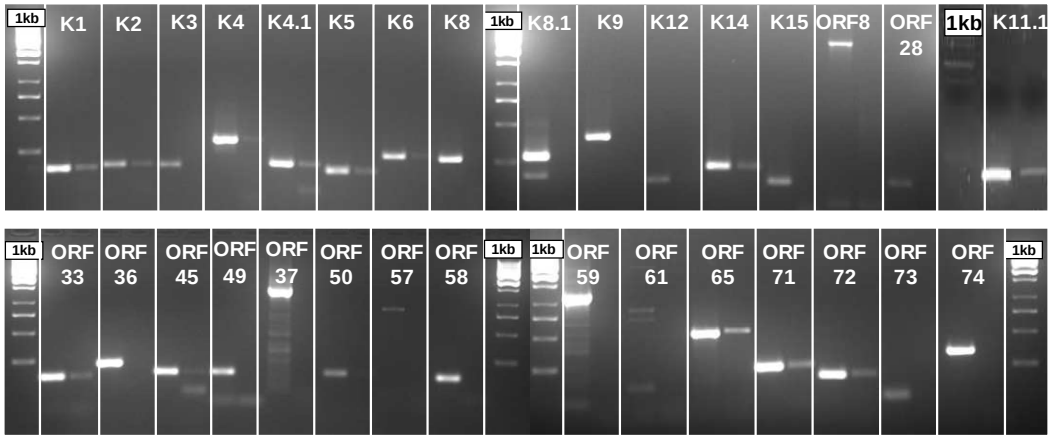


FIGURE 3.10: KSHV transgene expression by moDCs after transduction with individual KSHV-ORF-encoding lentiviral vectors. For each ORF, left-hand lane shows PCR product and right-hand lane shows no reverse transcriptase control. moDCs were transduced with 300µl of lentivirus encoding individual KSHV ORFs. On day six post-transduction, moDCs were harvested, RNA was extracted, cDNA was synthesised with and without reverse transcriptase, and the resulting product was used as a template for RT-PCR to confirm viral gene expression. All viral genes were expressed. Faint bands were sometimes seen in the no reverse-transcriptase controls, likely due to some sample contamination, however the true RT-PCR product was always brightest, indicating true transgene expression.

ii. Titre of Lentivirus by qPCR

qPCR was used to determine the MOI of each KSHV-ORF-encoding lentiviral vector. Three hundred thousand moDCs were transduced with 300µl of each lentivirus preparation. On day six post-transduction, moDCs were harvested, DNA was extracted and qPCR was used to determine the lentiviral copy number per cell. Results are shown in Figure 3.11. Virus preparations had an MOI of between 1.9 and 8.9. In order to achieve a uniform MOI for all lentivirus preparations in future

experiments (particularly the screen for immunogenic ORFs) the volume of lentivirus used for transduction was increased to 600µl for preparations with an MOI of less than 3.4, and decreased to 150µl for preparations with an MOI greater than 7.2. This achieves an MOI range of 3.4 to 7.2 for all preparations (median = 4.5; interquartile range = 3.9 to 5.4; mean = 4.75). With the GFP–encoding lentivirus, an MOI in this range achieved good transgene expression (between 10.6% and 17.2% GFP-positive immature moDCs; see Figure 3.7) with no notable improvement if the MOI was increased further. Furthermore, whilst there is a consensus that lentiviral transduction of moDCs at MOIs of less than ten does not affect moDC viability, immunophenotype or antigen-presenting function (Gruber et al., 2000; Dyall et al., 2001; Koya et al., 2003; Dullaers et al., 2004), the evidence regarding higher transduction with higher MOIs is less clear (Chen et al., 2004).

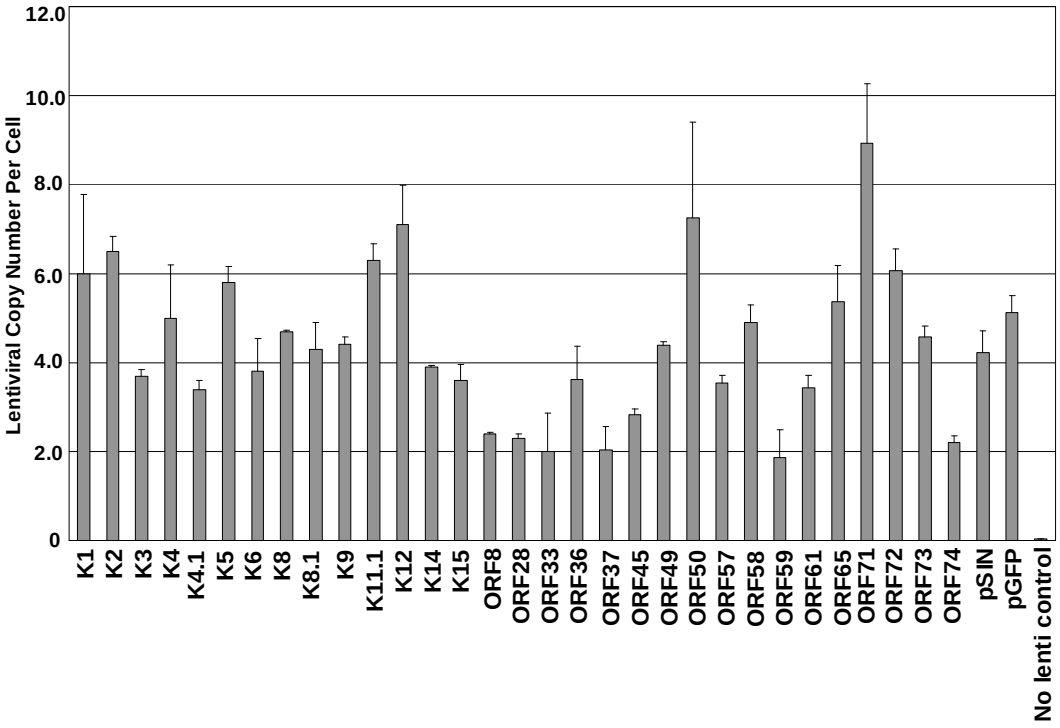


FIGURE 3.11: Lentiviral copy number per cell for moDCs after transduction with 300µl of different KSHV-ORF-encoding lentiviral vector preparations. qPCR was used to determine the multiplicity of infection (MOI) by measuring the lentiviral copy number and normalising to cell number by measuring the GAPDH copy number. Means and standard deviations from two experiments are shown.

iii. Multiple Transductions

As the KSHV lentiviral library consisted of 31 KSHV ORFs, it was decided to perform the immunogenic screen with moDCs transduced with up to three KSHV ORFs simultaneously, in order to make the experiment more manageable and to make the best use of clinical samples. KSHV ORFs were grouped according to expression profile to determine whether latent or immediate-early, early or late lytic gene products elicit the strongest T-cell responses. To ensure moDCs could be transduced with up to three ORFs, resulting in the co-expression of all three genes, a multiple transduction experiment was performed (Figure 3.12). Briefly, moDCs were transduced with one, two or three different KSHV-ORF-encoding lentiviral vectors. Transduction was normalised to an MOI of approximately 6 for each construct. On day six post-transduction, cells were harvested, RNA was extracted, cDNA was synthesised and RT-PCR was used to confirm KSHV transgene expression. All single, double and triple transductions showed the expected transgene expression. No transgene expression was seen in RT-PCR products from reactions performed on 'no reverse transcriptase' control preparations.

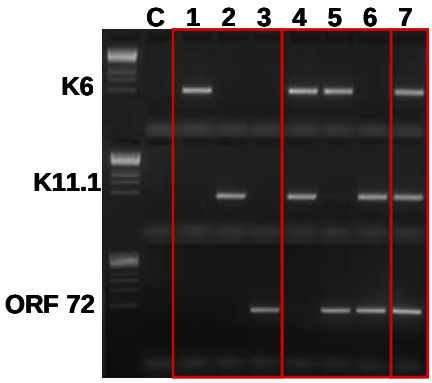


FIGURE 3.12: Viral gene expression by moDCs transduced with one, two or three KSHV-ORF-encoding lentiviral vectors. Electrophoresis gel showing KSHV gene expression (K6, top panel; K11.1, middle panel; ORF72, bottom panel) by non-transduced moDCs (lane c) and moDCs transduced with one (lanes 1, 2 and 3), two (lanes 4, 5 and 6) or three (lane 7) different KSHV-gene-encoding lentiviral vectors.

3.3 T-cell Response Assays

3.3.a T-cell Proliferation Responses Measured by CFSE Fluorescence

Allogenic mixed lymphocyte cultures were used to refine methods for measuring T-cell responses. moDCs were cultured for 10 days (as this is the length of time the moDCs transduced to express KSHV genes were cultured for) and then harvested and cocultured with allogenic CFSE-stained T cells.

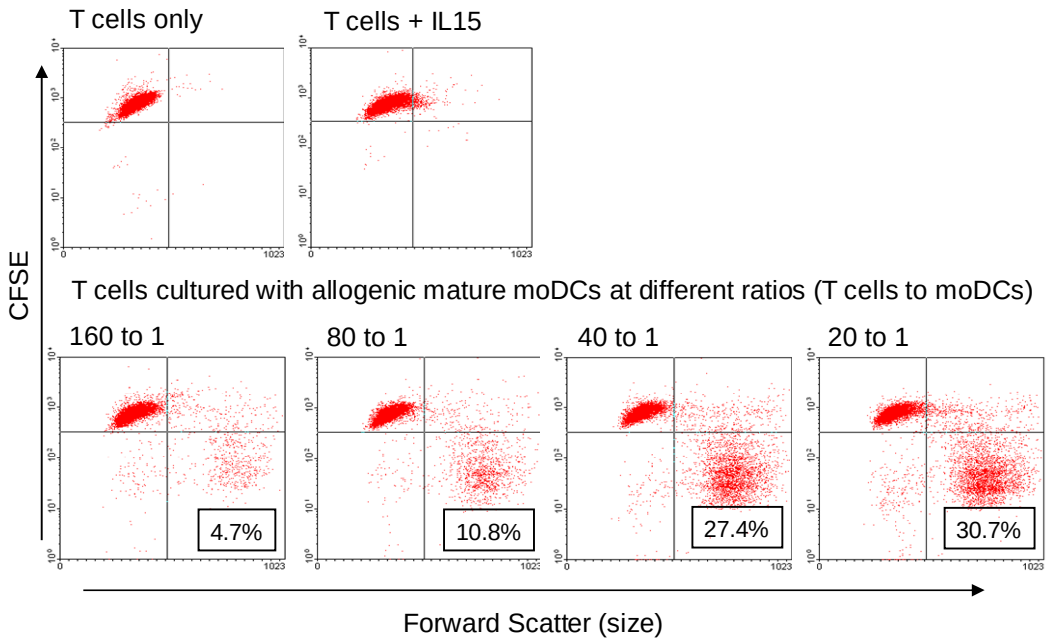


FIGURE 3.13: Flow cytometry dot plots showing progressive loss of CFSE fluorescence by proliferating T cells in allogenic mixed lymphocyte reactions. Non-proliferating T cells that are small in size and have high CFSE fluorescence are represented in the top left quadrant. In response to stimulation by allogenic mature moDCs, T cells proliferate, growing in size and losing CFSE fluorescence. Proliferating T cells are represented in the bottom right quadrant. The barred pattern that can be seen indicates the different generations of daughter cells – each bar is representative of each new generation of cells. The strength of the response can be assessed by the proportion of proliferating cells in the bottom right quadrant, as indicated by the percentage values shown on each plot. Decreasing the ratio of T cells to moDCs increases the strength of the response.

CFSE is a fluorescent vital dye that is partitioned with high fidelity between daughter cells, leading to serial halving of fluorescence down each new generation of cells (Lyons and Parish, 1994; Lyons, 2000). T-cell proliferative responses can therefore be measured by flow cytometry as the proportion of T cells that are CFSE-low (Figure 3.13 shows representative results from one experiment). The number of distinct populations with different levels of CFSE fluorescence indicates the number of divisions.

In the experimental design for the screen for immunogenic KSHV ORFs, CD14-negative PBMCs were cryopreserved at -80ºC whilst the autologous moDCs are cultured from CD14-positive monocytes and transduced with KSHV-ORF-encoding lentiviral vectors. T cells were then isolated from the frozen cells and cultured with the transduced moDCs. Therefore, T cells isolated from cryogenically preserved CD14-negative PBMCs were compared to T cells isolated from fresh blood for their ability to mount a proliferation response in allogenic mixed lymphocyte reactions. Results are shown in Figure 3.14.

In two experiments using different moDC donors, T cells isolated from frozen CD14-negative PBMCs mounted slightly, though not significantly ($p > 0.05$; student T test), stronger responses than T cells isolated from fresh blood. Different donors provided the fresh and frozen T cells, and therefore the difference in the strength of the response may be attributable to the degree of HLA-type mismatch between the moDC donor and the T-cell donor. Nevertheless, T cells clearly remain capable of mounting a proliferative response after cryopreservation.

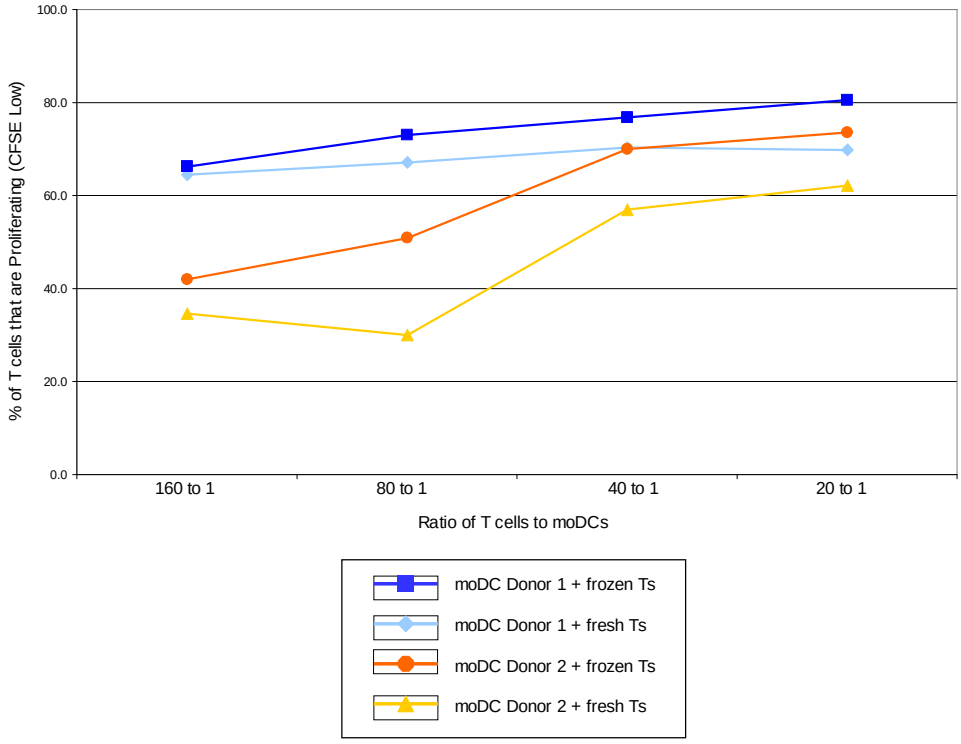


FIGURE 3.14: Proliferation response by fresh or frozen T cells in allogenic mixed lymphocyte reactions. Mature moDCs were cultured with CFSE-stained allogenic T cells isolated from either fresh blood or frozen CD14-negative PBMCs at four different ratios of T cells to moDCs. The proliferation response was measured by flow cytometry as the proportion of T cells that were CFSE-low. Decreasing the ratio of T cells to moDCs increased the strength of the T cell proliferation response. For each moDC donor, T cells from frozen PBMCs showed a slightly stronger proliferative response than T cells from fresh blood.

To test the stimulatory capacity of moDCs cultured in different serum supplements and matured by exposure to different stimuli, four allogenic mixed lymphocyte reactions were performed using moDCs from four different donors, each cultured and matured under all of the different conditions. moDCs were cultured with allogenic CFSE-stained T cells at four different ratios. After six days, the T-cell proliferation response was measured as the proportion of cells that were CFSE low using flow cytometry. Results are shown in Figure 3.15. Decreasing the ratio of T cells to moDCs increased the strength of the proliferation response. There was considerable variation in the size of responses between the different donor-matched

pairs as indicated by the error bars, but this is likely just a reflection of variation between individuals as well as the degree of HLA-type mismatch between pairs.

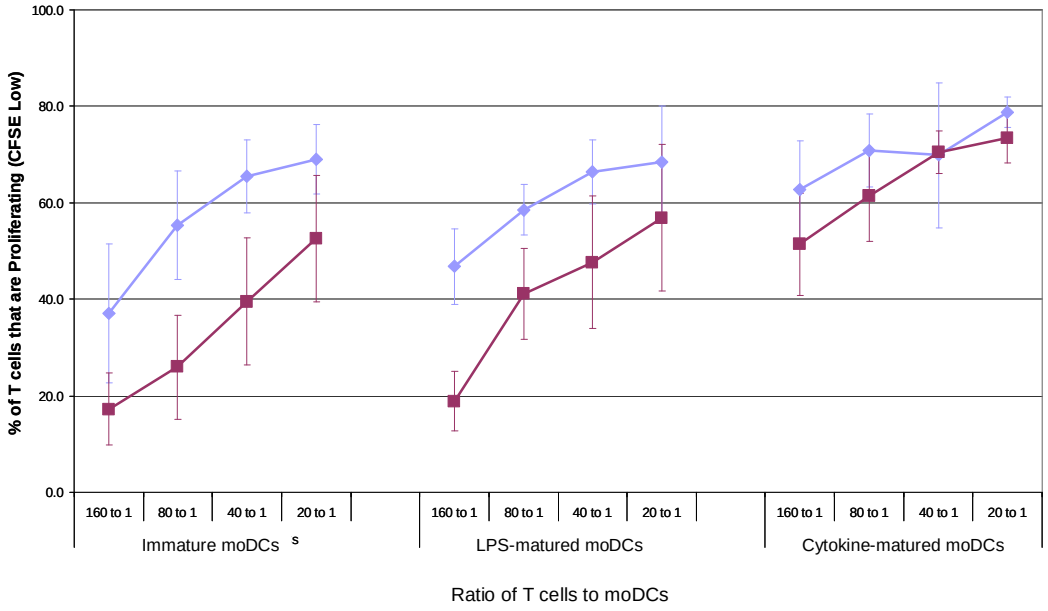


FIGURE 3.15: Allogenic stimulatory capacity of moDCs cultured in different serum supplements and matured by exposure to different stimuli. moDCs from four donors were cultured in either FCS (blue) or human AB serum (purple) and left either immature (left) or matured by stimulation with either LPS (centre) or a 'cytokine cocktail' (right). moDCs were then cultured with allogenic CFSE-stained T cells at four different ratios of T cells to moDCs for six days. Mature moDCs were more stimulatory than immature; and cytokine-matured moDCs were more stimulatory than LPS-matured moDCs. moDCs cultured in FCS were more stimulatory than moDCs cultured in AB. Means and standard deviations from four experiments are shown.

As would be expected, mature moDCs were more stimulatory than immature moDCs, as assessed by the strength of the T-cell proliferative response primed by these cells. moDCs matured by stimulation with a 'cytokine cocktail' were more stimulatory than LPS-matured moDCs ($p<0.001$; paired student T test). This was in keeping with Figure 3.4, which showed that the 'cytokine cocktail' is a more potent maturation stimulus than LPS at these working concentrations, resulting in a higher proportion of mature (CD80-positive and HLA-DR-positive) moDCs, and thus presumably a more stimulatory population of moDCs.

moDCs cultured in FCS were slightly more stimulatory than moDCs cultured in human AB serum ($p<0.01$; student T test), although the difference in the strength of the response they elicit was greater between immature moDCs than for mature moDCs. On average, immature moDCs cultured in FCS primed a 22.9% stronger response than immature moDCs cultured in AB. However, there was little difference in the strength of response primed by cytokine-matured moDCs cultured in the two serum supplements – on average 6.3% more of the total T-cell population proliferated in response to FCS-grown moDCs as compared to AB-grown moDCs – although this was still statistically significant ($p<0.05$; student T test). Again, this fitted with the maturation profile of the different populations of moDCs shown in Figure 3.4. Immature moDCs cultured in different serum supplements had quite different maturation profiles – 50% of immature moDCs cultured in FCS were HLA-DR-positive and CD80-positive, compared to 28% of immature moDCs cultured in AB serum. This was reflected in the stimulatory capacity of these moDC populations. There was less difference in the maturation profile of the cytokine-matured moDCs cultured in different serum supplements – 95% of cytokine-matured moDCs cultured in FCS were double-positive, compared to 83% of cytokine-matured moDCs cultured in AB serum. Again, this was reflected in the stimulatory capacity of these moDC populations. When T cells were cocultured with mature moDC populations (that showed more than 80% of moDCs displaying a mature profile) at low ratios of T cells to moDCs (40 to 1 or 20 to 1), more than 70% of the T cells were stimulated into a proliferative response and appeared to reach a threshold in the strength of the T-cell response.

3.3.b Cell-surface Marker Expression and Intracellular Cytokine Production

One of the advantages of using CFSE staining to measure T-cell responses is that it can be used alongside immunostaining in order to characterise the proliferating cells. Cell-surface marker staining for the expression of CD8 or CD4 can determine whether the proliferating T cells are CD8-positive CTLs or CD4-positive helper T cells or both. Staining for intracellular cytokine production can determine the functional profile of proliferating CTLs, or whether proliferating helper T cells are Th1 or Th2 cells. To refine techniques for cell-surface and intracellular immunostaining in conjunction with CFSE-staining, allogenic mixed lymphocyte assays were used. In the first instance, staining for the production of the intracellular cytokines IFNγ and IL4 was used as an indication of whether proliferating helper T cells were Th1 or Th2, respectively.

In order to stain for intracellular cytokine production, T cells were stimulated with TPA and ionomycin. This stimulation results in downregulation of the CD4 cell-surface molecule. Therefore, experiments were performed to determine whether staining for CD8 expression alone was sufficient to distinguish between CD4 helper T cells and CD8 CTLs. The proportions of CD4-positive or CD8-positive cells in stimulated and non-stimulated T-cell populations were measured by flow cytometry. In non-stimulated T cells, the mean proportion of CD4-positive cells was 51% and the mean proportion of CD8-positive cells was 45% (n = 12). In a student T Test there was no significant difference between the proportions of CD8-negative T cells and the proportions of CD4-positive T cells. This indicates that the proportion of CD8-negative T cells is approximately the same as the proportion of

CD4-positive T cells, and thus staining for the CD8 cell surface marker alone, and gating on either CD8-positive or CD8-negative T cells is sufficient to distinguish helper T cells and CTLs. In stimulated T cells the mean proportion of CD4-positive cells was 22% and the mean proportion of CD8-positive cells was 48% (n = 12). There was no significant difference between the mean proportion of CD8-positive cells in non-stimulated and stimulated T-cell populations. However, there was a significant difference ($p < 0.00001$) between the mean proportion of CD4-positive cells in non-stimulated and stimulated T-cell populations, illustrating that CD4 is indeed downregulated by cells after stimulation with TPA and ionomycin.

CD8 cell-surface marker staining was therefore used in conjunction with CFSE staining and staining for intracellular production of the cytokines IFNγ and IL4 to characterise the responding T cells in allogenic mixed lymphocyte reactions. Different populations of moDCs were used to stimulate allogenic T cells in order to investigate whether moDCs cultured in different serum supplements or matured by exposure to different maturation stimuli primed polarised T-cell responses. This was to determine the most appropriate conditions for the culture and maturation of moDCs for use in an investigation into KSHV-specific T-cell responses, in order to avoid masking the true polarisation of these memory responses. moDCs from four different donors were cultured in media supplemented with either 10% FCS or 5% human AB serum and were matured by stimulation with either LPS or a ‘cytokine cocktail’ and then cocultured with allogenic T cells. Flow cytometry was used to assess intracellular cytokine production by CD8-positive and CD8-negative (CD4) T-cell fractions as illustrated in Figure 3.16. The results from the four experiments are summarised in Figure 3.17.

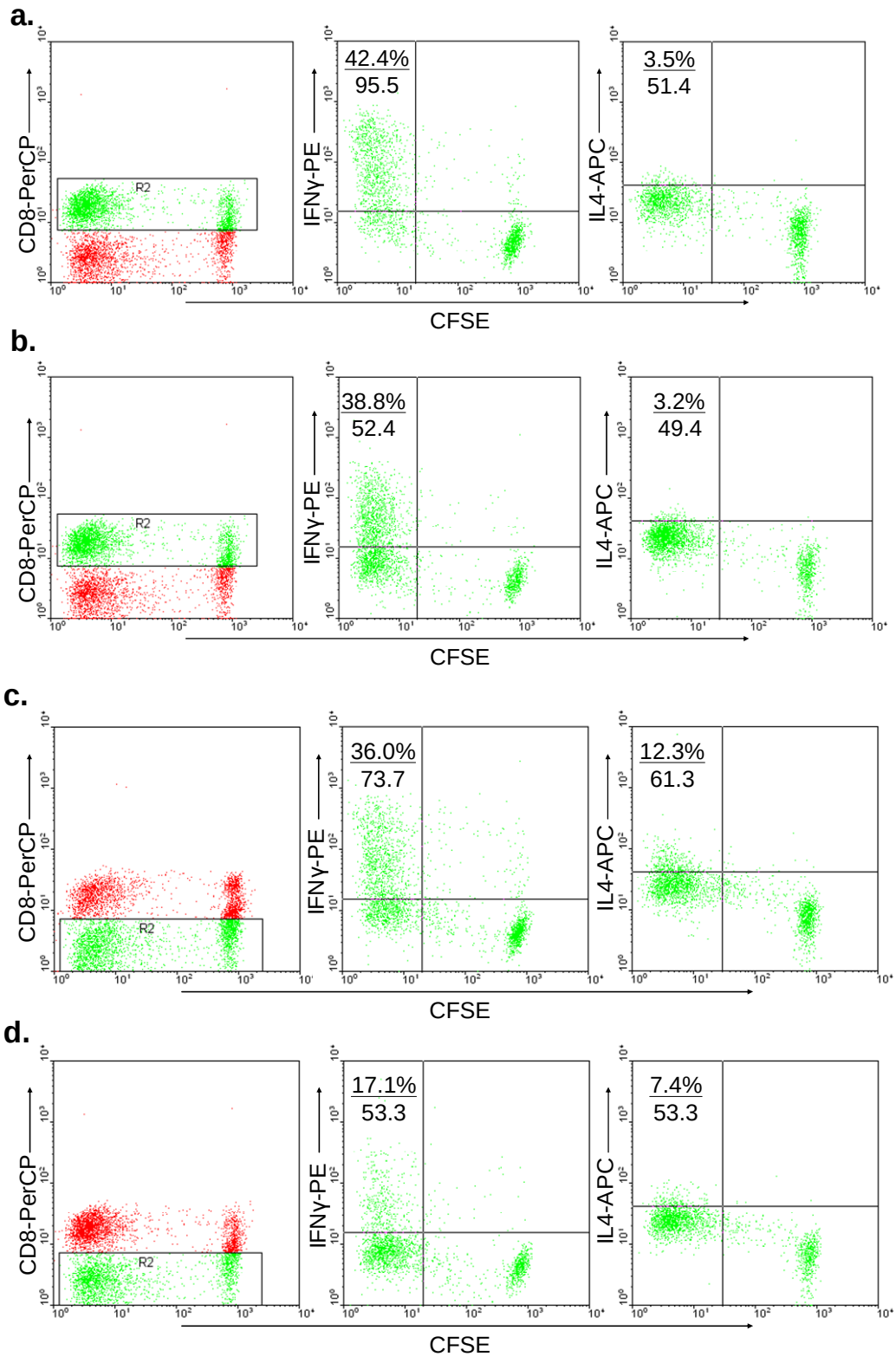
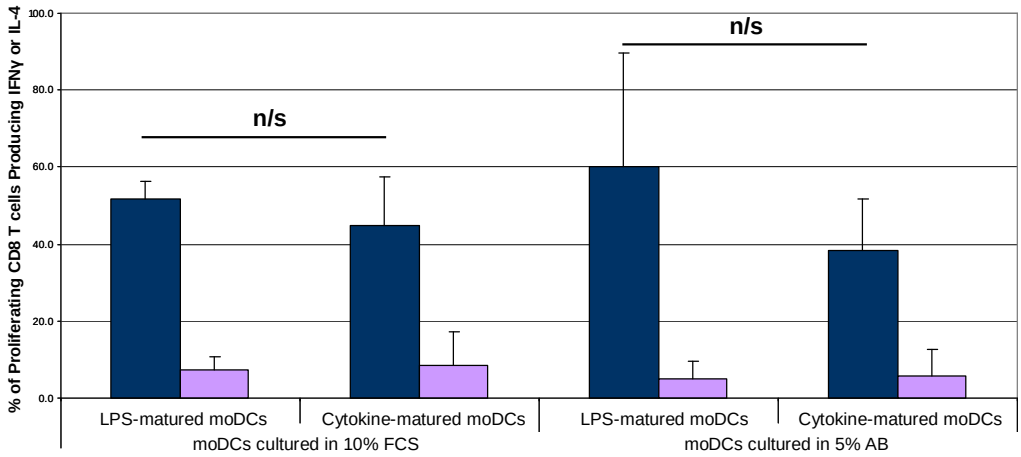


FIGURE 3.16: Flow cytometry dot plots showing intracellular cytokine production by T cells in an allogenic mixed lymphocyte reaction with moDCs cultured in FCS. Numbers indicate % of total cells in that quartile (top) and MFI of those cells (bottom). a. and b. Cytokine production by CD8 T cells in response to stimulation by LPS-matured (a) or cytokine-matured (b) moDCs. c. and d. Cytokine production by CD8-negative (CD4) T cells in response to stimulation by LPS-matured (c) or cytokine-matured (d) moDCs.

a.



b.

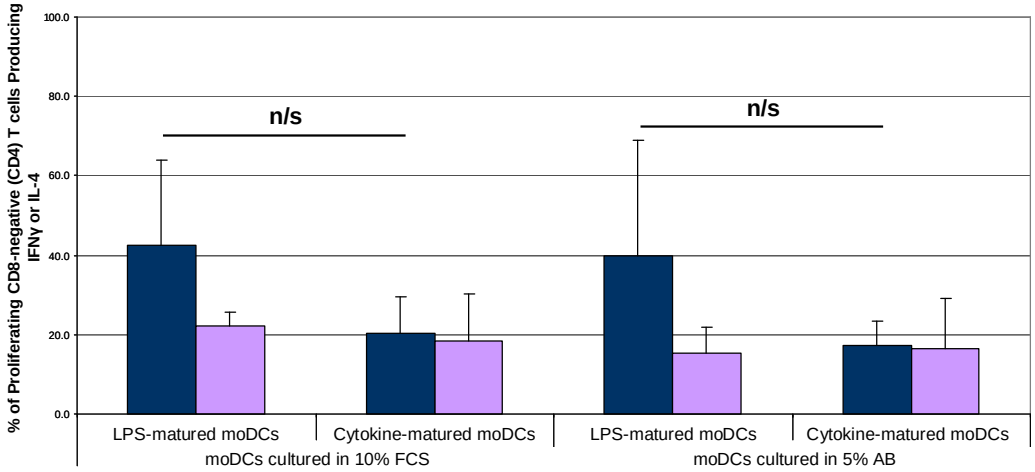


FIGURE 3.17: IFNγ (navy bars) and IL4 (lilac bars) production by proliferating T cells in allogenic mixed lymphocyte reactions with different populations of moDCs. Means and standard deviations from four experiments are shown. a. Cytokine production by CD8-positive T cells. Between 39% and 60% of proliferating CD8 cells produced IFNγ and 5% to 9% produced IL4. There was no difference in cytokine production by CD8 cells stimulated with moDCs cultured in different serum supplements or matured by exposure to different stimuli. b. Cytokine production by CD8-negative (CD4) T cells. Between 15% and 22% of proliferating CD8-negative cells produced IL4 after stimulation with all four different moDC populations. Between 40% and 42% of proliferating CD8- cells produced IFNγ after stimulation with LPS-matured moDCs compared to 17% to 20% after stimulation with cytokine-matured moDCs, although this did not reach statistical significance.

Cytokine production by CD8-positive T cells was similar after stimulation with all four different populations of moDCs (Figure 3.17.a). Between 39% and 60% of proliferating CD8-positive T cells produced IFNγ, whereas only 5% to 9% of proliferating CD8-positive T cells produced IL4. There was considerable variation in levels of cytokine production between donors, as indicated by the error bars. However, the serum supplement and maturation stimuli used in moDC culture did not appear to affect moDC priming of CD8-positive T-cell cytokine production.

Cytokine production by CD8-negative T cells appeared slightly different depending on the method used to mature the stimulating moDCs (Figure 3.17.b). Between 15% and 22% of CD8-negative T cells responding to all four different populations of moDCs produced IL4. However, between 40% and 42% of proliferating CD8-negative T cells stimulated by LPS-matured moDCs produced IFNγ, compared to between 17% and 20% of proliferating CD8-negative T cells stimulated by cytokine-matured moDCs. This did not, however, reach statistical significance in these four experiments using a paired student T test ($p=0.169$ and $p=0.0127$ for moDCs cultured in FCS or AB respectively). Again, there was considerable variation in levels of cytokine production between donors, as indicated by the error bars, but the serum supplement used in moDC culture did not appear to affect moDC priming of cytokine production by CD8-negative T cells.

3.4 Using Transduced moDCs to Investigate Antigen-specific Memory T-cell Responses

Due to the lack of success cloning the immunodominant CMV gene phosphoprotein 65 (CMVpp65) into the pSIN lentiviral vector (section 3.1.b), moDCs transduced with an adenovirus encoding CMVpp65 (Adpp65) were used to investigate the use of transduced moDCs to prime an antigen-specific memory T-cell response. Adpp65 was kindly provided by Dr Magnus Essand of Uppsala University, Sweden (Carlsson et al., 2003).

3.4.a Transduction of moDCs with an Adenovirus Encoding the CMV Gene Phosphoprotein 65

Expression of CMVpp65 by moDCs transduced with Adpp65 was confirmed by reverse-transcription PCR (Figure 3.18).

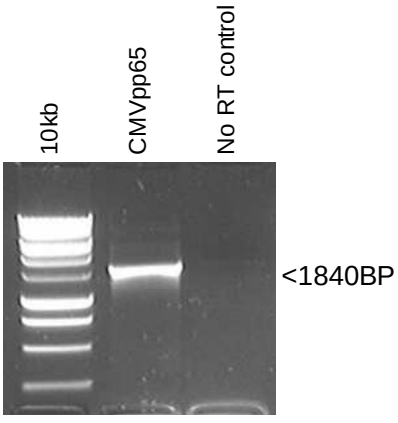


FIGURE 3.18: Electrophoresis gel of RT-PCR product showing expression of CMVpp65 by moDCs transduced with Adpp65. No RT control = no reverse transcriptase control.

i. Immunophenotype of moDCs Transduced with Adpp65

To ensure that transduction with Adpp65 did not affect the immunophenotype of moDCs, cell-surface marker expression by moDCs transduced with Adpp65 was compared to that of non-transduced moDCs at 48 hours post-transduction. The experiment was repeated twice for both moDCs cultured in FCS and moDCs cultured in human AB serum, and the immunophenotypes of both immature moDCs and moDCs matured by stimulation with a 'cytokine cocktail' were examined. Representative results from one experiment are shown in Figure 3.19.

In summary, there was no difference observed in the expression of moDC markers CD80 and HLA-DR between transduced and non-transduced moDCs, as assessed by the proportion of marker-positive cells and the MFI of each marker. Transduction with Adpp65 did not affect moDCs' antigen-presenting phenotype, and did not affect their maturation after exposure to an appropriate stimulus. In this particular experiment, MFI values for moDCs cultured in AB serum were lower than for moDCs cultured in FCS. However, the fold change in MFI after maturation was comparable for moDCs cultured in the two different serum supplements.

ii. Allogenic Stimulatory Capacity of moDCs Transduced with Adpp65

To ensure that moDCs transduced with Adpp65 retained their allogenic stimulatory capacity as well as their immunophenotype, allogenic mixed lymphocyte reactions comparing non-transduced moDCs and Adpp65-transduced moDCs were carried out. Stimulatory capacity was assessed by the strength of the T-cell proliferation response to these cells, measured by loss of CFSE fluorescence (see section 3.3.a).

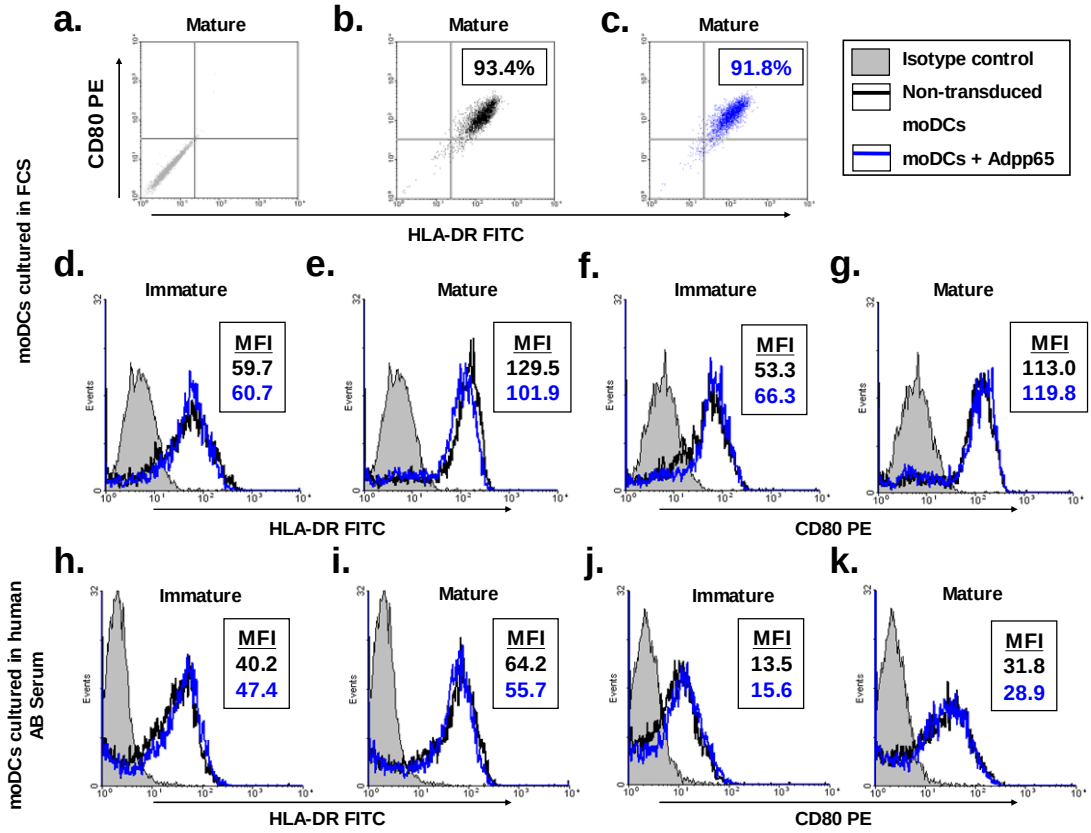


FIGURE 3.19: Marker expression profile of moDCs transduced with Adpp65. Results from one representative experiment are shown. a. – c. Co-expression of CD80 and HLA-DR by mature moDCs cultured in FCS. There was no difference between the immunogenic profiles of non-transduced moDCs (b; black dots) and moDCs transduced with Adpp65 (c; blue dots). Both were highly homogenous populations of double-positive cells. d. – g. Flow cytometry histograms showing mean fluorescence intensity (MFI) of HLA-DR (d and e) and CD80 (f and g) in immature (d and f) and mature (e and g) moDCs cultured in FCS. Marker expression is shown in black for non-transduced moDCs and in blue for transduced moDCs. There is no difference in the expression of either HLA-DR or CD80 between transduced and non-transduced immature moDCs. Both transduced and non-transduced moDCs upregulated HLA-DR and CD80 in a comparable manner after exposure to a maturation stimulus. h. – k. Flow cytometry histograms showing MFI of HLA-DR (h and i) and CD80 (j and k) in immature (h and j) and mature (i and k) moDCs cultured in human AB serum. Marker expression is shown in black for non-transduced moDCs and in blue for transduced moDCs. As for moDCs cultured in FCS, there is no difference in the expression of either HLA-DR or CD80 between transduced and non-transduced, immature or mature moDCs.

The experiment was repeated twice with two different donors. Representative results from one experiment are shown in Figure 3.20. Briefly, there was no difference between the allogenic T-cell response to transduced and non-transduced moDCs cultured in FCS. In moDCs cultured in AB, the response to transduced moDCs was slightly stronger than that to non-transduced moDCs (on average 7.65% more proliferation was seen to transduced moDCs; $p < 0.05$, student T test). This could be due to the additional activation of memory T cells specific for CMV antigens presented by the transduced moDCs. The transduced moDCs clearly retain their capacity to simulate T cells.

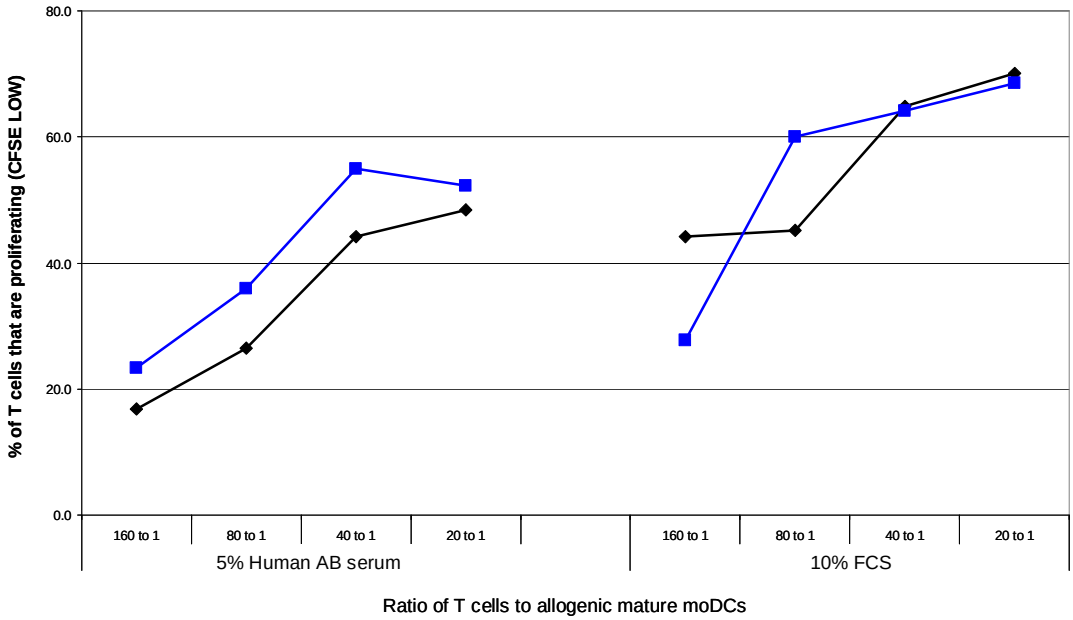


FIGURE 3.20: Allogenic stimulatory capacity of non-transduced (black lines) and Adpp65-transduced moDCs (blue lines) cultured in two different serum supplements. Allogenic stimulatory capacity of non-transduced and transduced moDCs was assessed by the strength of the T-cell response to these cells, as measured by the loss of CFSE fluorescence. moDCs capacity to stimulate T cells is not impaired by transduction with Adpp65. Results from one representative experiment are shown.

3.4.b Antigen-specific Memory Responses to moDCs Transduced with Adpp65

i. T-cell Proliferation Measured by CFSE Fluorescence

CFSE-stained T cells were cocultured with autologous moDCs that were either non-transduced or transduced with Adpp65. After six days, the T-cell proliferation response was assessed using CFSE fluorescence as outlined in section 3.3.a. Both the proportion of CFSE-low T cells and the CFSE MFI of the total T-cell population were used to determine the strength of the T-cell response. The experiment was repeated with two different donors, and for each donor the experiment was performed with moDCs that had been cultured in either human AB serum or FCS, and left either immature or matured using a 'cytokine cocktail'. Representative results from one experiment are shown in Figures 3.21 and 3.22.

Figure 3.21 illustrates the clear detection of an antigen-specific memory T-cell response by T cells cocultured with moDCs transduced with Adpp65 as compared to T cells cocultured with non-transduced moDCs. There is a dramatic increase in the proportion of CFSE-low T cells and a decrease in the CFSE MFI of the total T-cell population in T cells exposed to the CMV antigen. Both these parameters indicate a strong proliferation response. The strength of the response increased with decreasing T cell to moDC ratio.

Figure 3.22 shows the different responses to moDCs cultured in different media serum-supplements and left either immature or matured by stimulation with a 'cytokine cocktail'. Mature moDCs cultured in FCS were the most potent

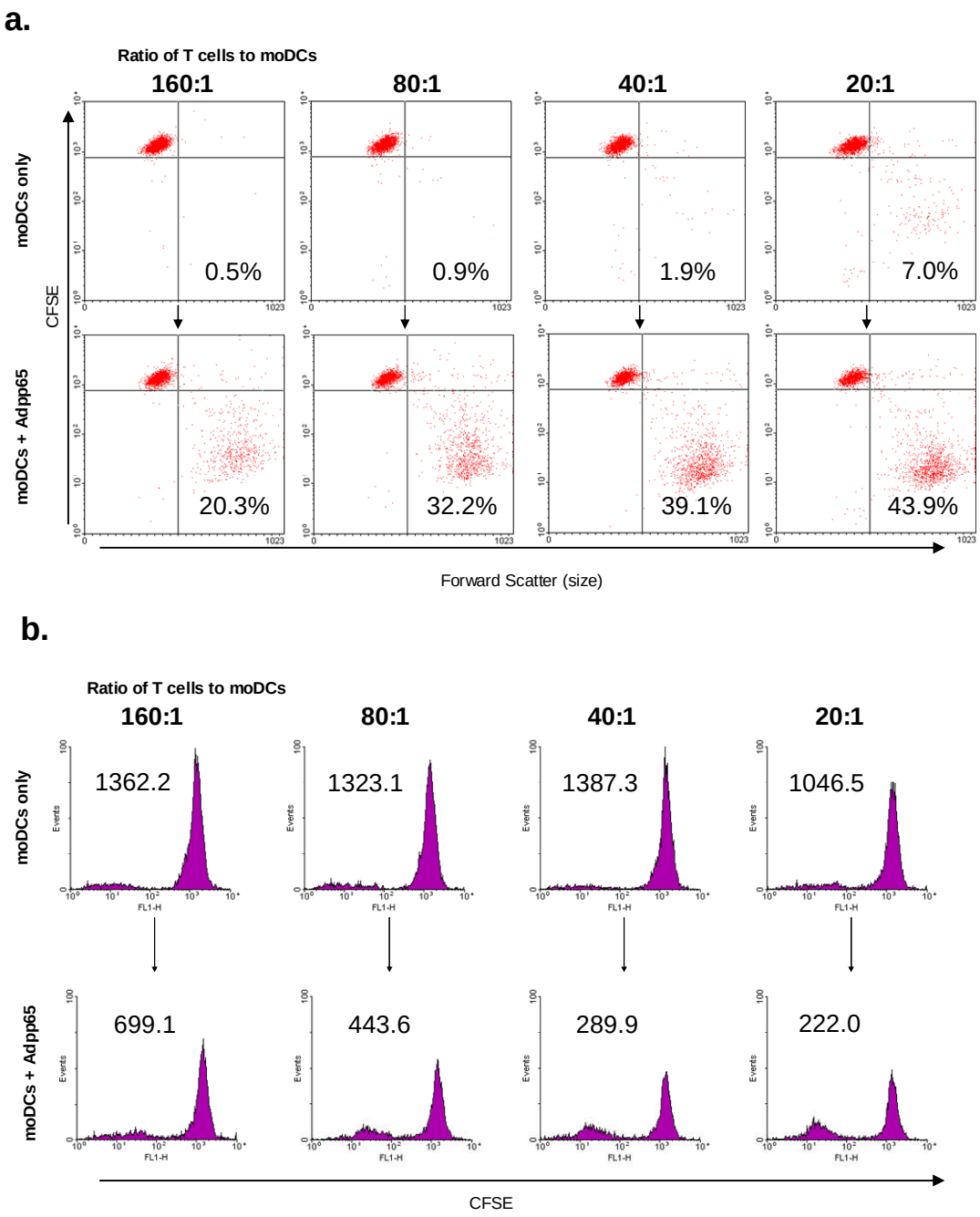


FIGURE 3.21: T-cell responses to non-transduced and Adpp65-transduced autologous moDCs measured by CFSE fluorescence. Results from one representative experiment are shown. a. Flow cytometry dot plots showing T-cell proliferation response to autologous non-transduced (top) and transduced (bottom) moDCs measured by the proportion of T cells that are proliferating (CFSE-low; indicated by the percentages in the bottom right quadrants). b. Flow cytometry histograms showing T-cell proliferation response to autologous non-transduced (top) and transduced (bottom) moDCs measured by the CFSE MFI of the total T-cell population (indicated by the numbers on each histogram). There is a distinct, strong response to the CMV antigen that increases as the ratio of T cells to moDCs decreases.

stimulators of an antigen-specific response to CMV pp65 (priming proliferation by 29.5% to 58.0% of total T cells, dependent on T cell to moDC ratio in culture). However, these cells also primed a comparatively high background response to non-transduced moDCs (6.5% to 33.7% proliferating T cells). Mature moDCs cultured in human AB serum elicited very little background response when they were non-transduced (0.5% to 7.0% proliferating T cells), but were potent stimulators of an antigen-specific response when they were transduced with Adpp65 (20.3% to 43.9% proliferating T cells).

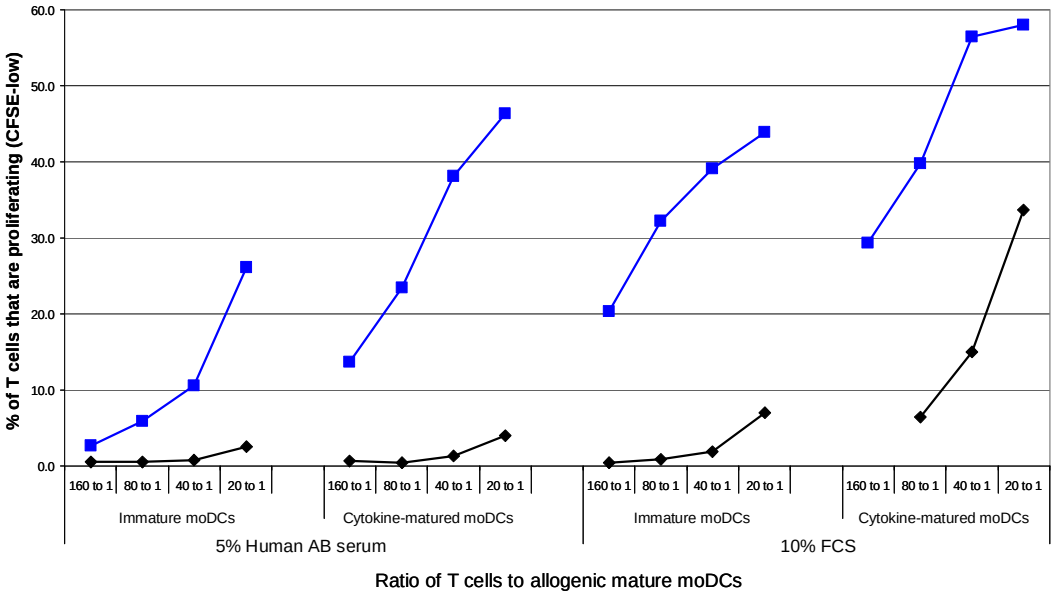


FIGURE 3.22: T-cell responses to non-transduced (black lines) and Adpp65-transduced (blue lines), immature and mature autologous moDCs cultured in different serum supplements. For all populations of moDCs, a strong response to the CMV antigen was observed above the background response to non-transduced moDCs. Mature moDCs were more potent stimulators than immature moDCs. moDCs cultured n FCS were more potent stimulators than moDCs cultured in AB serum, but also primed a higher background response to non-transduced moDCs. Results from one representative experiment are shown.

Mature moDCs cultured in human AB serum were thus selected as the most appropriate cells to use in the screen for immunogenic KSHV genes, although there

was the caveat that this was demonstrated using a different viral construct to the one used in this screen.

Results in Figure 3.22 also indicated that the most appropriate ratio of T cells to moDCs to use to investigate antigen-specific responses was 40 to 1. At this ratio, when using mature moDCs cultured in AB serum, there was very little background response to non-transduced moDCs (1.9% proliferating T cells), but a strong response to Adpp65-transduced moDCs (39.1% proliferating T cells). The same trend was seen when CFSE MFI values were used to analyse data instead of the proportion of CFSE-low T cells.

ii. Cell-surface Marker Expression and Intracellular Cytokine Production

To investigate the type of helper T-cell response primed by moDCs transduced with Adpp65, T cells that had been cocultured with these moDCs were stained for the cell-surface molecule CD8, and for intracellular production of IFNγ and IL4 (see section 3.3.b). The Adpp65-transduced moDCs used in this experiment were cultured in AB serum and matured by exposure to a 'cytokine cocktail'. The experiment was repeated for each of the two donors with a similar outcome, and representative results from one experiment are shown in Figure 3.23.

Seventy-seven percent of the CFSE-low, proliferating CD8-negative T cells produced IFNγ in response to stimulation with the Adpp65-transduced moDCs, whereas only 14% produced IL4. This strongly indicates that moDCs presenting antigens derived from Adpp65 prime a Th1-type helper T response. This is particularly evident when the cytokine production by these T cells is compared to

cytokine production by T cells primed with non-transduced allogenic moDCs cultured in the same way (in AB serum-supplemented media and matured by stimulation with a 'cytokine cocktail'). On average, only 17% of these proliferating CD8-negative T cells produced IFNγ and 16% produced IL4, as shown in Figure 3.17.

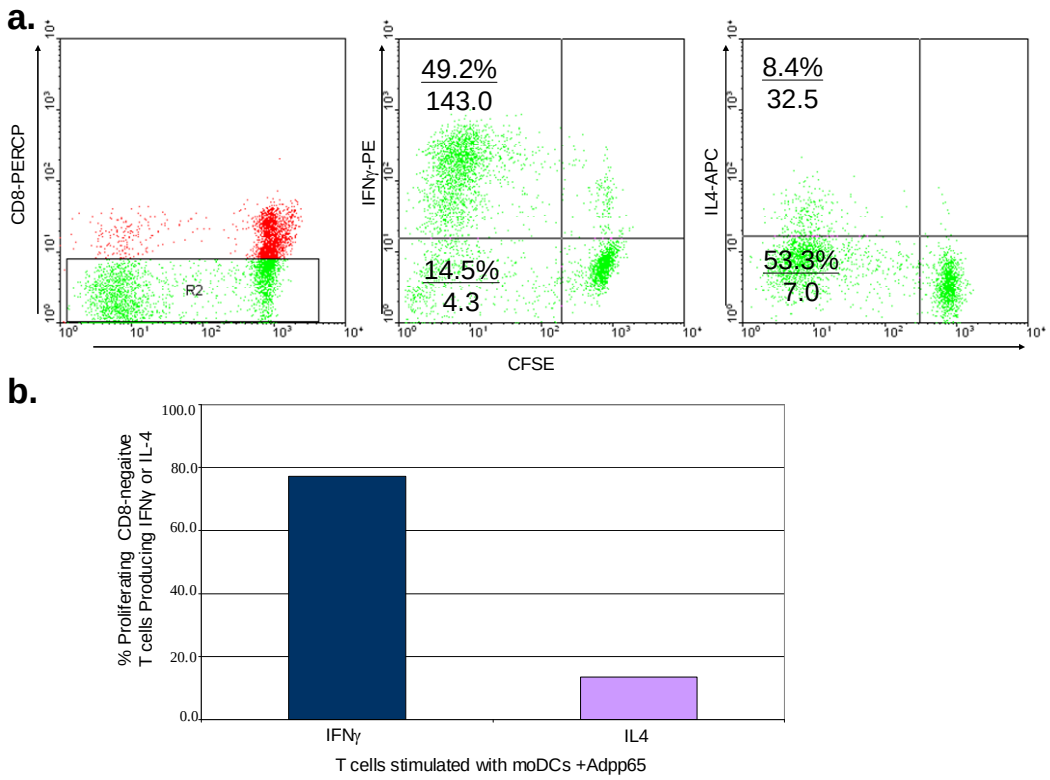


FIGURE 3.23: Intracellular cytokine production by proliferating CD8-negative T cells stimulated with moDCs transduced with Adpp65. a. Sample flow cytometry dot plots. T cells were gated on the CD8-negative fraction (left panel) and the % cells producing IFNγ (middle panel) and IL4 (right panel) was assessed. In the middle and right panels, the proliferating fraction of CD8-negative cells is represented by the CFSE-low cells in the two left-hand quadrants, and the proportion of these that are producing the cytokine of interest are represented by the top left quadrants. Numbers indicate the % of total T cells in that quartile (top) and the IFNγ or IL4 MFI of the cells in that quartile (bottom)**.** b. Summarises the proportion of proliferating CD8-negative cells producing either IFNγ or IL4, based on the results of the flow cytometry shown in panel a. Nearly 80% of the CD8-negative T cells proliferating in response to stimulation with moDCs transduced with Adpp65 produced IFNγ, which is strongly indicative of a Th1-type helper T response.

3.5 Summary

In this chapter, experiments that were performed in order to prepare for and design a protocol for a lentiviral-based system to investigate the KSHV-specific T-cell response have been described.

Five putative or known immunogenic KSHV ORFs (ORF8, ORF57, ORF59, ORF61, and ORF65) were cloned into the lentiviral vector for addition into a previously existing library of lentiviral expression vectors encoding individual KSHV ORFs. The resulting cumulative library contained 31 KSHV ORFs. The proposed experimental design to screen these ORFs for immunogenicity was to isolate moDCs from KSHV-seropositive individuals and transduce these moDCs with each KSHV ORF. The moDCs would naturally process the KSHV gene products and present the resulting optimal epitopes for that individual in the context of MHC-I and MHC-II. The transduced moDCs would then be cocultured with autologous T cells and a CFSE-dye based assay would be used to determine which of the KSHV ORFs yielded epitopes that elicited a T-cell response. To this end, protocols for the isolation, culture and transduction of moDCs were first investigated and refined. T-cell response assays were then investigated and refined in both an allogenic system and an autologous system that used moDCs transduced with an adenovirus encoding CMVpp65 to stimulate antigen-specific memory responses.

moDCs were derived from CD14+ monocytes, and different culture conditions were investigated to identify the most suitable for use in the immunogenic screen. A

culture media supplemented with 5% human AB serum and a ‘cytokine cocktail’ maturation stimulus were selected for use in the immunogenic screen, as moDCs cultured using these conditions showed good viability and differentiated into a homogeneous population of mature moDCs with strong stimulatory capacity. AB serum was deemed preferable to FCS as moDCs cultured in a selected AB serum primed a lower background response in autologous T-cell response assays. The ‘cytokine cocktail’ was selected in preference to LPS as it provided the most potent moDC maturation stimulus. Furthermore, cytokine-matured moDCs did not prime polarised Th responses so it seemed a more neutral maturation stimulus for use in investigation of polarisation of Th responses by KSHV antigens.

A GFP-encoding lentivirus (pCSGW) was used to demonstrate that moDCs could be successfully transduced using this lentiviral vector, and to determine the optimal time-point and MOI for achieving good transgene expression with this vector. pCSGW was additionally used to show that lentiviral transduction did not affect the moDCs’ antigen-presenting surface phenotype or their maturation. Next, each of the KSHV-ORF-encoding lentiviruses was demonstrated to transduce moDCs, resulting in expression of the desired transgene and the MOI for each of the lentiviral preparations was determined.

As the library of lentiviral expression vectors available for the immunogenic screen consisted of 31 KSHV ORFs, it was decided to perform the screen with moDCs transduced with up to three KSHV ORFs (grouped according to each ORF’s expression profile during the viral life cycle). This was in order to make the experiment more manageable; to make the best use of clinical samples; and to

determine whether latent, immediate-early lytic, early lytic or late lytic ORFs are the major targets of KSHV-specific T-cell response. Thus, it was demonstrated that moDCs could be transduced with up to three KSHV ORFs simultaneously, resulting in expression of all three transgenes.

Finally, a CFSE-dye based assay in conjunction with immunostaining for cell surface markers and intracellular cytokine production was used to assess T-cell responses to both allogenic moDCs and autologous moDCs transduced to express a viral antigen. For future experiments, a ratio of T cells to moDCs of 40 to 1 was selected, as this achieved a balance between a strong antigen-specific response to transduced moDCs and a low background response to non-transduced moDCs in autologous assays.

Based on these findings, a protocol for a screen for immunogenic KSHV ORFs was established, and this screen was carried out as described in the following chapter.

CHAPTER 4: Investigating the Targets of the CD8 and CD4 T-cell Responses against KSHV

4.1 Lentiviral-based Screen for Immunogenic KSHV ORFs

In Chapter 3 of this thesis, a pre-existing library of lentiviral expression vectors encoding individual KSHV ORFs was extended to include a total of 31 KSHV ORFs. Preparations of each of these lentiviruses were synthesised; they were each shown to express their transgene in monocyte-derived dendritic cells (moDCs); each MOI was determined; and simultaneous transduction of moDCs with up to three KSHV genes was demonstrated. Lentiviral transduction of moDCs was shown not to affect their immunophenotype, and the best serum supplement and maturation stimulus for preparing moDCs to use in a screen for immunogenic KSHV ORFs were decided. T-cell response assays for measuring antigen-specific memory responses were refined. Based on this work, an experimental design for a screen for immunogenic KSHV genes was decided, and is shown in Figure 4.1.

As the KSHV lentiviral library consisted of 31 KSHV ORFs, it was decided to perform the immunogenic screen with moDCs transduced with up to three different KSHV ORFs simultaneously, in order to make the experiment more manageable and to make the best use of clinical samples. KSHV ORFs were grouped according to expression profile to determine whether latent, immediate-early lytic, early lytic or late lytic KSHV genes elicit the strongest T-cell responses. KSHV ORFs known

to affect the expression of MHC-I [K3, K5, K9 and ORF 71; (Coscoy and Ganem, 2000; Lagos et al., 2007)] were used to singly transduce moDCs, since these genes' functions may affect priming of T cells by moDCs thus skewing the results (see Materials and Methods 2.4.b).

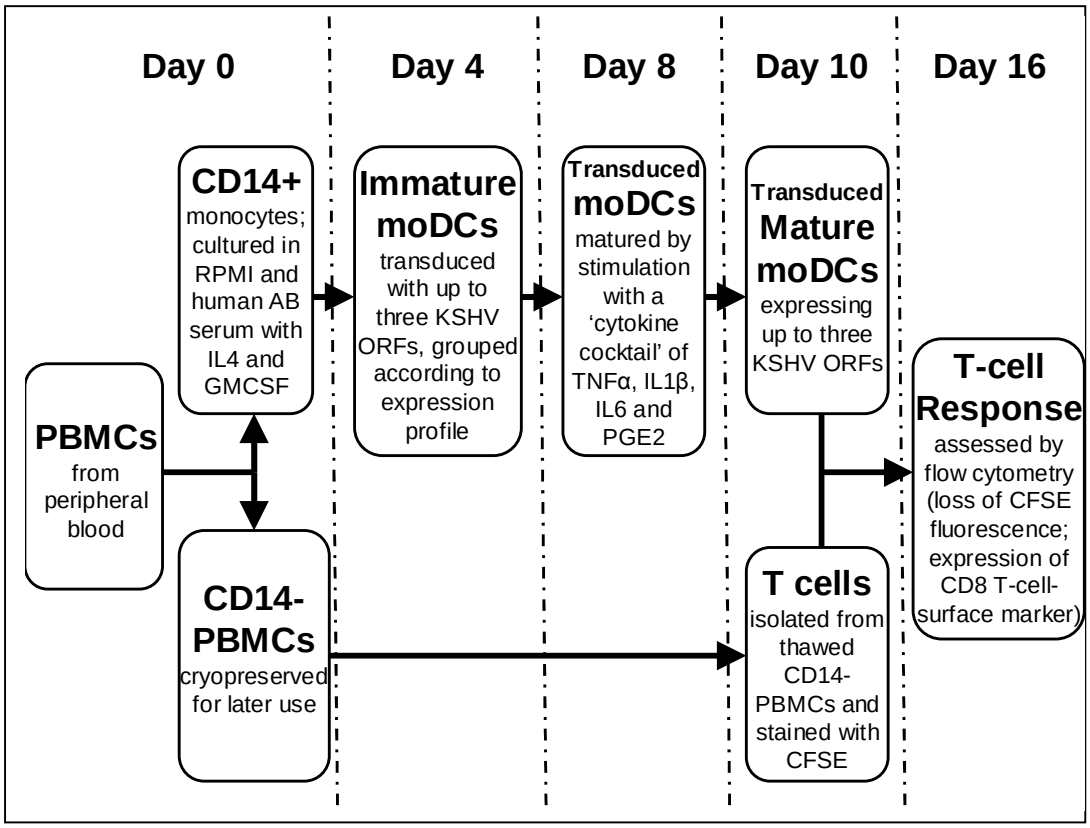


FIGURE 4.1: Schematic illustration of the experimental design for a screen for immunogenic KSHV ORFs using lentiviral-transduced moDCs.

4.1.a Study Participants

For the screen, 14 KSHV-seropositive and seven KSHV-seronegative study participants were recruited from the Chelsea and Westminster Hospital, London, UK. All participants provided written, informed consent. Study protocols were

approved by the Riverside Research Ethics Committee. Study participants were classified into five groups based on their KSHV-serostatus, HIV-serostatus and history of KSHV-related disease. These included two KSHV-seronegative control groups and three KSHV-seropositive groups and were as follows:

1) HIV-negative, KSHV-seronegative individuals (n = 3).

2) HIV-positive, KSHV-seronegative individuals (n = 4).

3) HIV-positive, KSHV-seropositive individual with no history of KSHV-related disease (asymptomatic carrier; n = 1).

This individual was originally intended for inclusion in the study as an HIV-positive, KSHV-negative control participant, as they had no history of KSHV-related disease and were considered to be at low risk of KSHV infection, and so was assumed to be KSHV-negative. However, when the KSHV immunogenic screen was performed in this individual, strong T-cell responses to several pools of KSHV ORFs were observed. The individual's KSHV-serostatus was thus retrospectively tested using plasma frozen at the time of venesection, which proved to be positive (see section 4.1.b).

4) HIV-positive, KSHV-seropositive individuals with a history of KSHV-related disease (KS; KS and MCD; or PEL) but in remission at the time of venesection and on HAART (n = 11).

Individuals in this group were the original target cohort of KSHV-positive individuals, as this cohort have been shown to have comparatively high

levels of KSHV-specific T cells (Bihl et al., 2007a; Bourboulia et al., 2004), and have the additional advantages that they are easily identifiable and continue to attend clinics regularly after entering remission.

5) HIV-negative, KSHV-seropositive individuals with active KSHV-related disease (KS), in regression at the time of venesection (n = 2).

I was eager to take any opportunities that arose to perform the screen in HIV-negative, KSHV-seropositive individuals, to see what results were obtained in individuals who didn't have the complication of a background of immunosuppression. Unfortunately, classic KS is rare in the UK and blood samples from individuals with no active neoplasm are difficult to obtain as these individuals do not regularly attend clinics after entering remission. I was able to collect two samples from individuals with classic KS, although these were collected at the time of their final hospital visit, when their KS was regressing but minor lesions were still present.

All study participants were male apart from one HIV-positive, KSHV-seronegative female. Study participants' age at venesection ranged from 30 to 74 years. All HIV-positive individuals were on HAART and had an HIV viral load of <50 copies per ml. CD4 counts ranged from 171 to 785 cells/mm^3. Individual characteristics of HIV-positive participants are shown in Table 2.6. Summarised characteristics of each group of study participants are shown in Table 4.1. The different groups of study participants were well matched for age and HIV+ groups were well matched for CD4 count.

Group	Group Description	Number	Male/ Female	Mean Age (Range)	KSHV status	HIV status	Mean CD4 Count (Range)
1	HIV-/KSHV- Healthy Controls	3	3/0	37 (30 to 45)	Seronegative	Negative	ND
2	HIV+/KSHV- Controls	4	3/1	48 (32 to 70)	Seronegative	Positive On HAART HIV viral load suppressed	345 (174 to 652)
3	HIV+/KSHV+; asymptomatic carriers of KSHV	1	1/0	56	Seropositive Asymptomatic	Positive On HAART HIV viral load suppressed	373
4	HIV+/KSHV+; history of KSHV-related neoplasia	11	11/0	47 (36 to 74)	Seropositive* KSHV-related disease in remission	Positive On HAART HIV viral load suppressed	389 (171 to 785)
5	HIV-/KSHV+; history of KSHV-related neoplasia	2	2/0	37, 41	Seropositive Active KS, regressing	Negative	550, ND

TABLE 4.1: Summary of characteristics of study participants in the screen for immunogenic KSHV ORFs. * indicates except for two individuals (K058 and B625) for whom plasma were unavailable for serological testing; ND indicates not determined. Detailed individual characteristics of HIV-positive participants can be seen in Table 2.6.

4.1.b KSHV Serostatus of Study Participants

All study participants had their KSHV serostatus at the time of venesection assessed by multi-antigenic ELISA, except for two individuals who had a history KSHV-related disease (K058 and B625) for whom plasma samples were unavailable for serological testing. Results are shown in Table 4.2.

	Participant ID	KSHV Serostatus		Comments
		Positive	Negative	
HIV-negative, no history of KSHV-related disease	HC1		N	
	HC2		N	
	HC3		N	
HIV-positive, no history of KSHV-related disease	D919		N	
	C122		N	
	S810		N	
	D403		(N)	Borderline negative
	H419	P		Asymptomatic Carrier
HIV-positive, KSHV-related disease in remission	D208	P		
	S079	P		
	K058	Not Tested		
	P940	P		
	B625	Not Tested		
	M620	P		
	V689	P		
	A556	P		
	B196	P		
	T541	P		
	T328	P		
HIV-negative, active KS	S346	P		
	W946	P		

TABLE 4.2: KSHV serostatus of study participants at the time of venesection for the screen for immunogenic KSHV ORFs. P = positive; N = negative; (N) = borderline negative.

All participants had very clear serostatus, with the exception of D403, an HIV-positive individual with no history of KSHV-related neoplasm who was KSHV seronegative but had a fairly high antibody titre (0.129 ± 0.02) only just below the cut-off for positivity (0.145). It is possible, therefore, that D403 had either a very low level of latent KSHV infection or had been exposed to KSHV in the past but infection had been abortive.

4.1.c Antigen-presenting Surface Phenotype of Transduced moDCs Expressing KSHV ORFs

moDCs from each study participant and transduced with each KSHV ORF or group of ORFs were checked for their expression of HLA-DR and CD80 by flow cytometric analysis. This was to ensure that no KSHV ORF (or group) significantly affected the antigen-presenting surface phenotype of the moDCs or their maturation in such a way that would skew the results of T-cell response assays using the transduced moDCs. Figure 4.2 shows the mean proportion of HLA-DR-positive and CD80-positive moDCs after transduction with each KSHV ORF or group of ORFs across all 21 study participants included in the screen for immunogenic KSHV ORFs. On average, 92.8% of non-transduced control moDCs had a mature phenotype (i.e. were double-positive for these markers). Control moDCs transduced with the empty vector alone (pSIN) were on average 89.2% double-positive. This slight reduction in the proportion of double-positive moDCs reached statistical significance ($p<0.001$; paired student T test) across this large number of samples ($n = 21$). This was in contrast to what we observed when comparing the phenotypes of non-transduced moDCs with moDCs transduced with a GFP-encoding lentivirus in

Chapter 3 (section 3.2.c). It may be that lentiviral transduction has some subtle affect on moDCs – either mild toxicity or triggering or blocking of moDC differentiation pathways – that only becomes apparent when examining a large number of samples. Nonetheless, the effect was very slight and did not interfere with the capacity of transduced moDCs to stimulate T-cell responses (see below, sections 4.1.d – f).

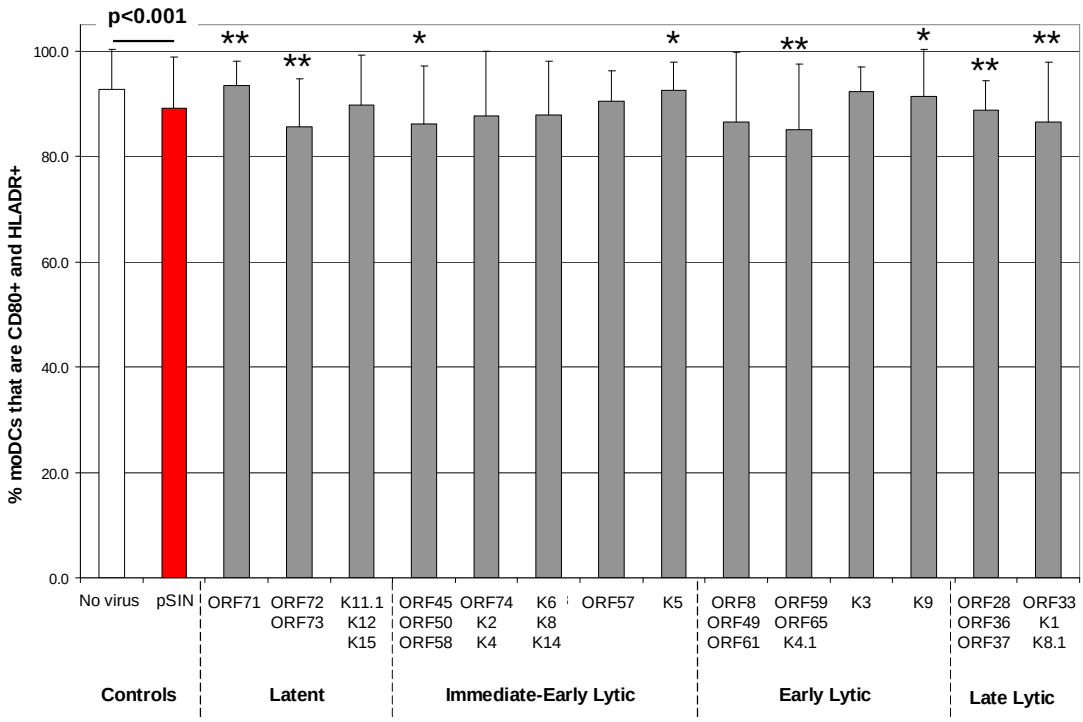


FIGURE 4.2: Maturation profile of moDCs transduced with different KSHV ORFs or groups of ORFs. Maturation profile measured as the proportion of moDCs [non-transduced (white bar); transduced with the pSIN empty lentiviral vector (red bar); or transduced with KSHV ORFs or groups of ORFs (grey bars)] that were both HLA-DR-positive and CD80-positive using flow cytometry. Means and standard deviations across all study participants (n = 21) are shown. Stars indicate proportion of double-positive moDCs significantly different from that in moDCs transduced with empty pSIN. ** indicates $p<0.01$; * indicates $p<0.05$ (paired student T test).

Eight different populations of moDCs transduced with KSHV ORFs showed a maturation profile that was statistically significantly different from moDCs

transduced with the empty pSIN vector (paired student T test; Figure 4.2). However, none of these differences were numerically large, and all were within 5% of the proportion of double-positive moDCs transduced with pSIN alone (89.2%). The range across all transduction conditions was 85.1% to 93.5%; the inter-quartile range was 86.6% to 91.7%; the median was 89.0%; and the mean was 89.2%. Populations of moDCs with 85.1% to 93.5% HLA-DR/CD80 double-positive cells can be considered to represent effectively homogenous populations of mature, highly stimulatory moDCs. This level of variation would not be expected to affect the stimulatory capacity of moDCs in T-cell response assays (and, indeed, was demonstrated not to; sections 4.1.d – 4.1.f).

Interestingly, moDCs transduced with ORF71, K5 or K9 (ORFs known to affect MHC-I expression) showed a modest but statistically significant increase in the proportion of double-positive cells as compared to moDCs transduced with pSIN alone (93.5%, 92.5% and 91.5%, respectively compared to 89.2%). ORF71 (which codes for the latency protein vFLIP) upregulates MHC-I expression at the translational level by activating the NFκB pathway (Lagos et al., 2007) and has recently been shown to promote the upregulation of CD80 and other costimulatory molecules and to drive moDC maturation through the same pathway (Rowe et al., 2009). Paradoxically, both K5 (MIR2) and K9 (vIRF1) downregulate MHC-I expression. K9/vIRF1 downregulates MHC-I by binding to the transcriptional co-activator p300 in competition with IRF1 and IRF3 and thereby blocking type I and II IFN-mediated activation of MHC-I transcription (Lagos et al., 2007). K5/MIR2 downregulates MHC-I by enhancing its endocytosis from the cell surface. The internalised MHC-I molecules are delivered to endolysosomal vesicles where they

are degraded (Coscoy and Ganem, 2000). It is thus surprising that moDCs transduced with these ORFs showed an increase (albeit very slight) in the proportion of HLA-DR/CD80 double-positive cells.

moDCs transduced with [ORF72/ORF73], [ORF45/ORF50/ORF58], [ORF59/ORF61/K4.1], [ORF28/ORF36/ORF37] and [ORF33,K1/K8.1] all showed a modest but statistically significant decrease in the proportion of double-positive cells compared to moDCs transduced with pSIN alone (85.6%, 86.5%, 85.1%, 88.9% and 86.7% respectively compared to 89.2%). Infection of CD14-positive monocytes with KSHV has been shown to inhibit their differentiation into moDCs, and to reduce the moDCs allogenic stimulatory capacity both before and after maturation. In particular, a strong reduction in the expression of CD80 on the cell surface of KSHV-infected mature moDCs was reported (Cirone et al., 2007). Further investigation of the effect of the ORFs above on moDC maturation and CD80 expression may therefore be warranted, in particular those groups of ORFs in which the greatest reduction in double-positive cells was seen ([ORF72/ORF73] and [ORF59/ORF61/K4.1]).

4.1.d T-cell Proliferation Responses to Lentiviral-transduced moDCs Expressing KSHV ORFs

CFSE-stained T cells were cultured with autologous moDCs transduced to express up to three KSHV ORFs grouped according to their expression profile. After six days, T cells were harvested and flow cytometry was used to assess the CD8-positive cytotoxic lymphocyte (CTL) response and the CD8-negative (CD4) helper

T-cell response to each KSHV ORF or pool, as measured by the proportion of CFSE-low proliferating cells. Gating strategy is shown in Figure 4.3. See section 3.3.b for the rationale behind staining for CD8 only.

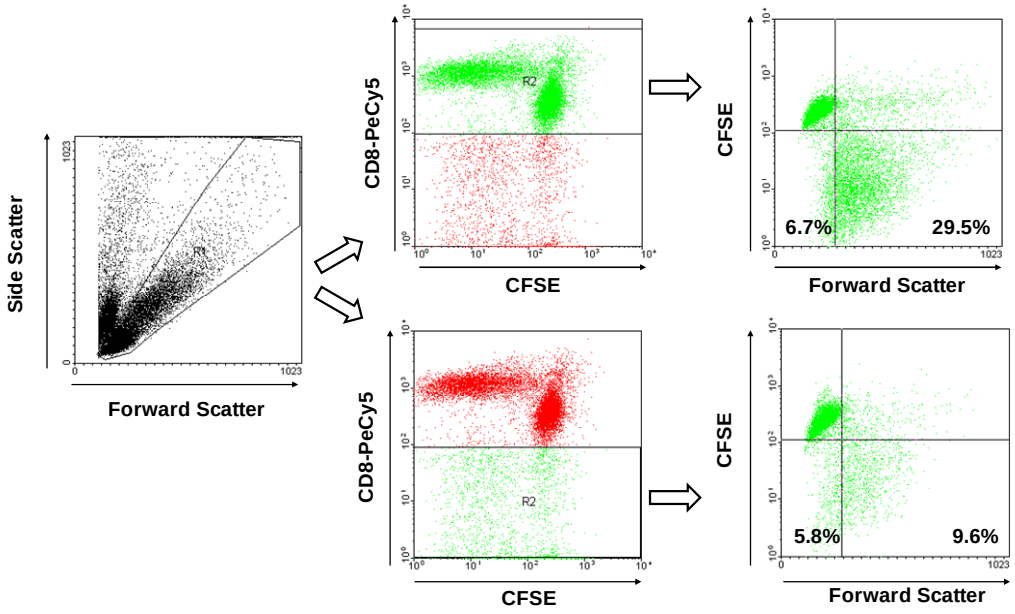


FIGURE 4.3: Gating strategy for T-cell proliferation responses to moDCs expressing KSHV ORFs. Live T lymphocyte gate was set according to forward scatter and side scatter. Gates were then set on either the CD8-high or CD8-low (CD4) T-cell population. The proportion of proliferating CD8 or CD4 T cells was measured by the proportion of CFSE-low cells within each population.

An example of CD8 and CD4 responses by one HIV-positive, KSHV-seropositive individual (V689; group 4) are shown in Figures 4.4.a and 4.4.b, respectively. Strict criteria were used to designate positive and borderline positive responses (see section 2.4.d) to ensure that no false positives were recorded as a result of the slight variation observed in individuals' background response to moDCs transduced with the empty vector alone. This may mean that some weak T-cell responses were not identified in some or all KSHV-seropositive individuals. However, it allows us to observe patterns of immunodominant T-cell responses against KSHV.

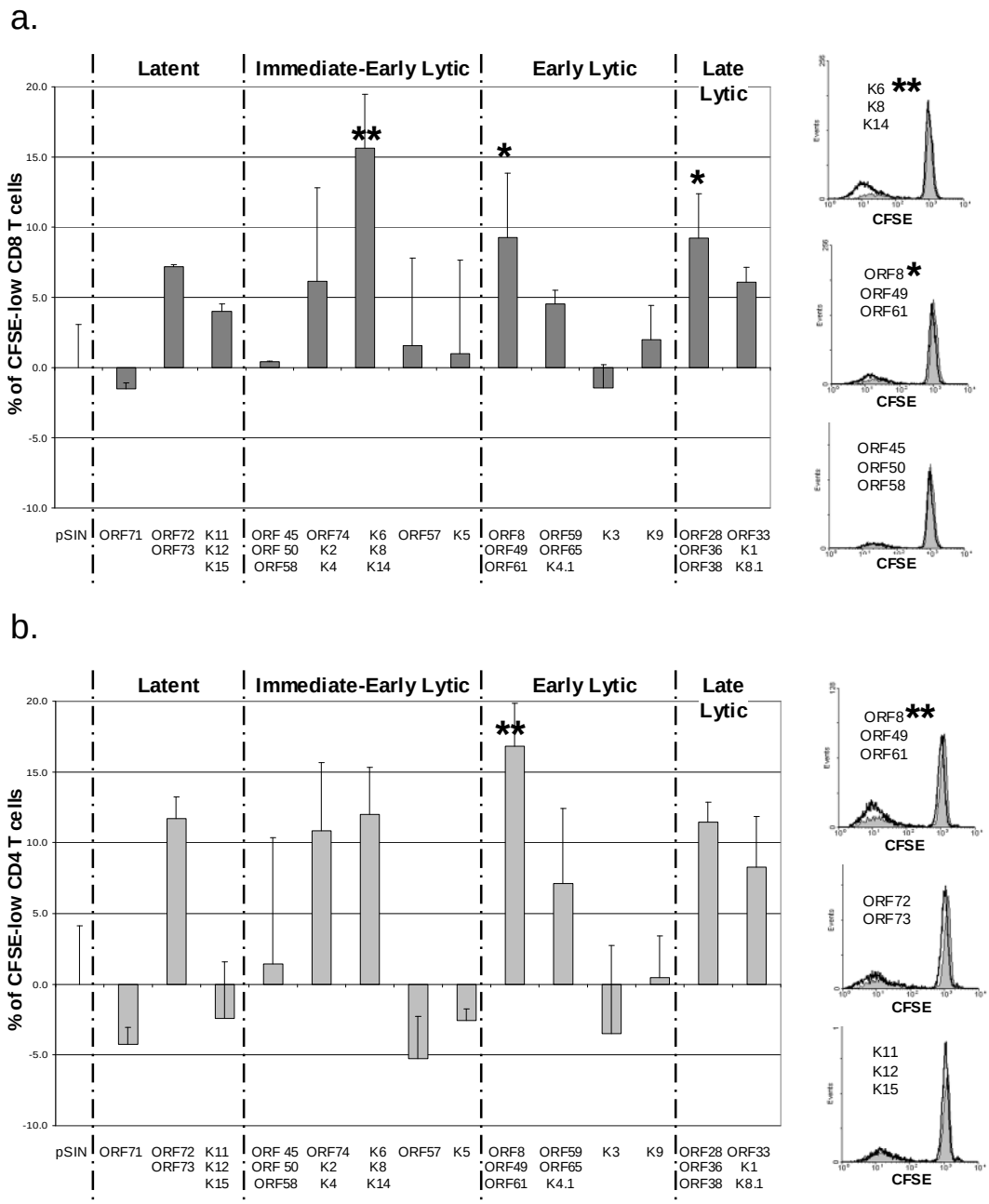


FIGURE 4.4: Example of CD8 (a) and CD4 (b) responses to moDCs transduced with each KSHV ORF or pool of ORFs by T cells from one study participant (V689; HIV+, KSHV+, history of KSHV-related neoplasm, in remission). Bar graphs show the proportion of CFSE-low cells in response to each ORF or pool of ORFs (with the background response to moDCs transduced with the empty pSIN vector subtracted). Mean of triplicates and standard deviations are shown. Two stars indicate a response that was considered to be positive; one star indicates a response that was considered to be borderline positive according to the criteria described in section 2.4.d. Flow cytometry histograms show examples of strong (top), medium (middle) and negative (bottom) CD8 (a) or CD4 (b) responses to different KSHV antigens from the same study participant (V689). In all histograms, grey shading represents CD8 or CD4 T cells cultured with moDCs transduced with the empty pSIN vector and black open lines represent CD8 or CD4 T cells cultured with moDCs transduced with different KSHV antigens.

CD8 and CD4 responses by all participants to all KSHV ORFs or pools are summarised in Figure 4.5.a and Figure 4.5.b, respectively. A single borderline CD8 response (participant C122 to [ORF74/K2/K4]) and two borderline CD4 responses (participant HC3 to [ORF59/ORF65/K4.1]; and participant D403 to [ORF28/ORF36/ORF37]) were observed in KSHV seronegative individuals compared to 32 CD8 responses and 21 CD4 responses by KSHV seropositive individuals. This gives us confidence that the responses observed to KSHV ORFs are indeed KSHV-specific. Interestingly, one of the borderline CD4 responses observed in a KSHV seronegative individual was in D403, who had a KSHV antibody titre only just below the cut-off value for seropositivity (0.129 ± 0.02 compared to 0.145; see section 4.1.b). It is possible that this weak T-cell response against KSHV and relatively high KSHV antibody titre both result from previous abortive exposure to KSHV.

One of the two participants with active classic KS (W962) did not respond to any of the KSHV ORFs (CD8 or CD4) and the other (S346) gave only one borderline CD4 response (against [ORF8/ORF49/ORF61]). The asymptomatic carrier of KSHV (H419) gave six strong positive CD8 responses and one borderline and four strong CD4 responses to different KSHV ORFs. Of the 11 HIV+ individuals with a history of KSHV-related disease, six (D208, S079, K058, B625, T514 and T328) gave one or no responses (total of CD8 and CD4), and were classified as poor responders. There were no notable differences in the ages, CD4 counts or years in remission from KSHV-related disease between the poor responders and good responders (i.e. those that gave one or more total CD8- and CD4-mediated responses). However, interestingly, one poor responder (D208) suffered a relapse of KS within a year of

			Latent			Immediate-Early Lytic					Early Lytic				Late Lytic		
CD8 RESPONSES		ID	ORF71	ORF72 ORF73	K11.1 K12 K15	ORF45 ORF50 ORF58	ORF74 K2 K4	K6 K8 K14	ORF57	K5	ORF8 ORF49 ORF61	ORF59 ORF65 K4.1	K3	K9	ORF28 ORF36 ORF37	ORF33 K1 K8.1	Total
	HIV-, KSHV- Controls	HC1				nd											0
		HC2															0
		HC3															0
	HIV+, KSHV- Controls	D919															0
		C122															0 (1)
		S810															0
		D403															0
	HIV+, KSHV+ Asymptomatic	H419															6
	HIV+, KSHV+ KSHV-related disease in remission	D208															0
		S079															0
		K058								nd			nd				0
		P940															3
		B625															0
		M620															4(5)
		V689															1(3)
		A556															8
		B196															5(6)
		T541															0(1)
		T328															0
	HIV-, KSHV+, Active KS	S346															0
		W962	nd						nd	nd			nd	nd			0
	Total responses in KSHV+ individuals		1	2	1	0	2	4	0	0	5(6)	4(5)	0	0	5(7)	3(4)	27(32)

FIGURE 4.5.a: Summary of CD8 T-cell responses to KSHV ORFs by all study participants. Dark blue boxes represent positive responses; light blue boxes represent borderline responses; unfilled boxes represent no response. Nd indicates experiment not done due to insufficient numbers of PBMCs.

			Latent			Immediate-Early Lytic					Early Lytic				Late Lytic		
CD4 RESPONSES		ID	ORF71	ORF72 ORF73	K11.1 K12 K15	ORF45 ORF50 ORF58	ORF74 K2 K4	K6 K8 K14	ORF57	K5	ORF8 ORF49 ORF61	ORF59 ORF65 K4.1	K3	K9	ORF28 ORF36 ORF37	ORF33 K1 K8.1	Total
	HIV-, KSHV- Controls	HC1				nd											0
		HC2															0
		HC3															0 (1)
	HIV+, KSHV- Controls	D919															0
		C122															0
		S810															0
		D403															0 (1)
	HIV+, KSHV+ Asymptomatic	H419															4(5)
	HIV+, KSHV+ KSHV-related disease in remission	D208															0
		S079															1
		K058								nd			nd				0(1)
		P940															1(2)
		B625															0
		M620															3(5)
		V689															1
		A556															5
		B196															0
		T541															0
		T328															0
	HIV-, KSHV+, Active KS	S346															0(1)
		W962	nd						nd	nd			nd	nd			0
	Total responses in KSHV+ individuals		0	1(2)	0(1)	0	1	1	0	0	4(6)	3(4)	0	0	2	3(4)	15(21)

FIGURE 4.5.b: Summary of CD4 T-cell responses to KSHV ORFs by all study participants. Dark green boxes represent positive responses; light green boxes represent borderline responses; unfilled boxes represent no response. Nd indicates experiment not done due to insufficient numbers of PBMCs.

venesection for this experiment. Three other poor responders (S079, B625 and T328) had received no cancer-specific chemotherapy treatment in addition to their antiretroviral therapy, whereas all good responders received HAART in combination with cancer chemotherapy. All poor responders had a history of KS alone, except for K058, who had been treated for PEL. Of the good responders, three (M620, A556 and B196) had a history of KS alone and two (P940 and V689) had a history of KS and MCD. These study participant characteristics are summarised in Table 4.3.

moDCs transduced with an adenovirus encoding the immunodominant CMV gene phosphoprotein 65 were used as a positive control to demonstrate that T cells from KSHV seronegative individuals were capable of responding to transduced autologous moDCs. All seven KSHV seronegative individuals mounted strong positive CD8 responses and five out of seven gave positive CD4 responses (and a further one gave a borderline CD4 response) to Adpp65-transduced moDCs (Figure 4.6). The T-cell responses, in particular CD8 responses, to Adpp65-transduced moDCs were much stronger (an average of 52.6 ± 16.0 % proliferating CD8 T cells) than responses to moDCs transduced with KSHV ORFs (an average of 15.7 ± 4.9% proliferating CD8 T cells). This may be due to a number of factors. The T-cell responses against KSHV recorded to date have been consistently lower in both frequency and magnitude than responses to other viruses (Bihl et al., 2007b; Brander et al., 2001). Thus, circulating CMV-specific T cells may be of higher frequency than KSHV-specific T cells and may hence mount a stronger proliferation response to stimulation. However, it is likely that the different expression vectors used (adenoviral versus lentiviral) result in different transduction efficiencies as well as differences in levels of antigen processing and presentation and so differences between CMV-specific and

	Participant ID	Age	CD4 Count	KSHV-related disease history	Treatment for KSHV-related disease	Approximate years between active disease and venesection
Poor Responders	D208	46	785	KS	HAART + chemotherapy + radiotherapy	<1, since relapsed
	S079	74	183	KS	HAART only	2
	K058	46	565	PEL	HAART + chemotherapy	3.5
	B625	39	406	KS	HAART only	<1
	T541	51	171	KS	HAART + chemotherapy	5
	T328	40	308	KS	HAART only	<1
	Mean	**49**	**403**			**2**
Good responders	P940	36	645	KS and MCD	HAART + chemotherapy	<1
	M620	38	496	KS	HAART + chemotherapy + radiotherapy	8.5
	V689	53	281	KS and MCD	HAART + chemotherapy	1.5
	A556	49	226	KS	HAART + chemotherapy	3.5
	B196	49	209	KS	HAART + chemotherapy	<1
	Mean	**45**	**371**			**3**

Table 4.3: Characteristics of HIV-positive study participants in remission from KSHV-related disease.

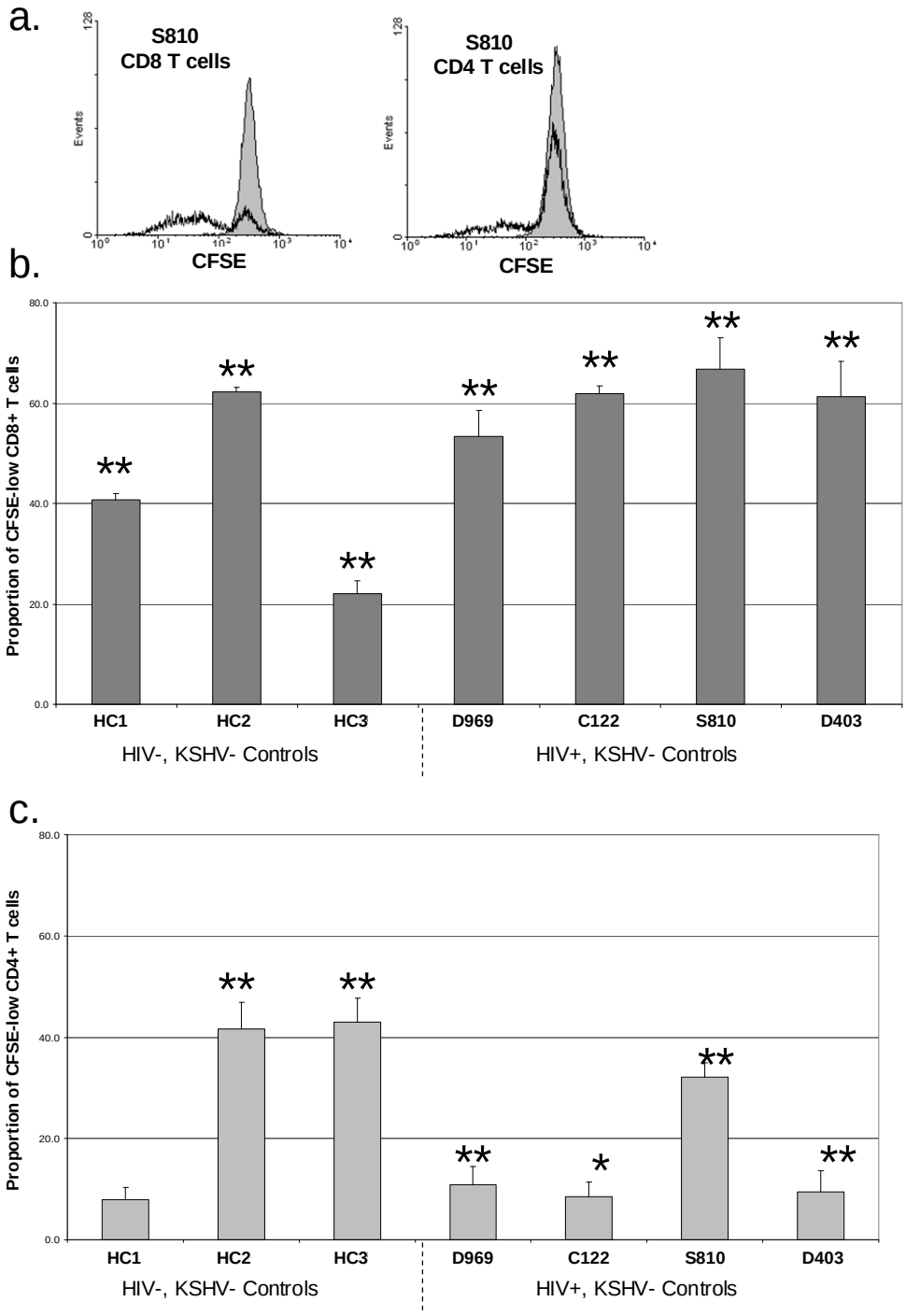


FIGURE 4.6: T-cell responses by KSHV-seronegative controls to autologous moDCs transduced with Adpp65. a. Example flow cytometry histograms showing CD8 (left) and CD4 (right) responses to Adpp65-transduced moDCs by one individual (S810; HIV+, KSHV-seronegative). Grey shading represents T cells cultured with non-transduced autologous moDCs; black open lines represent T cells cultured with Adpp65-transduced autologous moDCs. b. and c. Summary of CD8 (b) and CD4 (c) responses to Adpp65 by all KSHV-seronegative controls (with the background response to non-transduced moDCs subtracted). Means and standard deviations of triplicate experiments are shown. ** indicates positive response above background; * indicates borderline positive response above background.

KSHV-specific responses could not be quantitatively compared using the results from this experiment. Furthermore, an empty adenoviral vector was not available as a control, and thus the measured T-cell responses to Adpp65 represent the combined responses to the CMV antigen as well as any antigens derived from the adenoviral vector. With responses to moDCs transduced with KSHV ORFs, vector-specific response was controlled for by subtracting the background response to moDCs transduced with the empty lentiviral vector alone. Vector-specific responses are reportedly stronger to adenoviral vectors than to lentiviral vectors, partly due to inherent differences in the host immune response to the viruses from which they are derived, and partly due to differences in the construction of the vectors [(Collins and Cerundolo, 2004; Palmowski et al., 2004); see section 1.4.b *Antigen Delivery to Dendritic Cells*].

4.1.e Targets of the KSHV-specific CD8 CTL T-cell Response

CD8 responses to KSHV ORFs by all responsive KSHV-seropositive individuals showed a bias towards early lytic and late lytic gene products (Figure 4.5.a.). Out of a total of 32 CD8 responses observed, four were directed against latent ORFs; six against immediate-early lytic ORFs; 11 against early lytic ORFs; and 11 against late lytic ORFs. Two pools of early lytic ORFs were frequently recognised CD8 targets: [ORF8/ORF49/ORF61] was recognised by six individuals and [ORF59/ORF65/K4.1] was recognised by five individuals. ORF8 codes for glycoprotein B, which contains a well-described CD8 epitope, aa492-500 [LMWYELSKI; (Wang et al., 2002b)]. ORF61 codes for a large ribonucleotide reductase essential for DNA synthesis and also contains a previously documented

CD8 epitope, aa505-513 [GLADVFAEL; (Lambert et al., 2006)]. ORF65 codes for the minor capsid protein which has also been identified as a target of the KSHV-specific CD8 T-cell response, and contains one identified CD8 epitope [aa35-43; NMSQAEYLV; (Lambert et al., 2006)]. One pool of late lytic ORFs – [ORF28/ORF36/ORF37] – was recognised by seven individuals, and thus was the most frequently recognised target of CD8 T cells by this study cohort. This was of particular interest to us as none of the gene products in this pool have been previously investigated for immunogenicity. For the same reason, a pool of immediate-early ORFs – [K6/K8/K14] – that was recognised by four individuals was also of interest.

4.1.f Targets of the KSHV-specific CD4 T-cell Response

CD4 responses to KSHV ORFs by all KSHV-seropositive individuals also showed a bias towards early lytic and late lytic gene products (Figure 4.5.b). Out of a total of 21 CD4 responses observed, three were directed against latent ORFs; two against immediate-early lytic ORFs; 10 against early lytic ORFs; and six against late lytic ORFs. The most frequently recognised CD4 targets were the pool [ORF8/ORF49/ORF61], comprised of early lytic antigens, which was recognised by six individuals; and the pools [ORF59/ORF65/K4.1] (early lytic antigens) and [ORF33/K1/K8.1] (late lytic antigens), which were each recognised by four individuals. Out of these pools, the K8.1 gene product has previously been identified as a target of the KSHV-specific CD4 T-cell response, although no specific epitope was identified (Barozzi et al., 2008), but none of the remaining

ORFs have been previously investigated as potential CD4 antigens. Interestingly, the only pool which contained ORFs in which CD4 epitopes have been previously identified ([K11.1/K12/K15]) was only recognised by CD4 T cells from one individual (H419; HIV-positive, KSHV-seropositive asymptomatic carrier of KSHV). This pool contains K12 and K15, both ORFs in which CD4 epitopes were previously identified that elicited a response in one HIV-positive, asymptomatic carrier of KSHV out of 52 KSHV-positive individuals (35 of which HIV-positive) tested (Guihot et al., 2006).

4.2 Asymptomatic Carriers of KSHV

The strong CD8 and CD4 responses mounted by the asymptomatic carrier of KSHV (H419) and the often weak or lack of responses seen in individuals with a history of KSHV-related disease prompted me to look for further asymptomatic carriers of KSHV in which to perform the immunogenic screen.

4.2.a KSHV Seroprevalence in HIV-positive Individuals

Regular attendees to an HIV-clinic at Chelsea and Westminster Hospital were tested for KSHV seropositivity by multi-antigenic ELISA. Results are shown in Table 4.4.

Out of 27 individuals tested, eight were KSHV seropositive giving a 30% seroprevalence of KSHV in this cohort of HIV-positive individuals. Seroprevalence was higher in men (seven out of 22; 32%) than in women (one out of five; 20%).

No particular association was observed between an individual's KSHV serostatus and whether or not they originated from a country where KSHV is endemic (Ghana, Italy, Nigeria, Uganda, Zimbabwe). Two men from Italy were seronegative; one man and one woman from Nigeria were both seronegative; two women from Sub-Saharan Africa (one from Uganda and one from Zimbabwe) were also seronegative. Only one of the eight KSHV-seropositive individuals was born in a country where

Participant ID	KSHV Serostatus		Gender	Ethnicity	Country of Birth
	Negative	Positive			
V245	N		M	White – British	UK
I418	N		M	Black – African	Nigeria
H850		P	M	White – British	UK
F692	N		M	White – Other	Italy
C971		P	M	White – Other	Uruguay
W864	N		M	White – British	UK
K305	N		M	Black – Caribbean	Jamaica
E176		P	M	White – Other	France
G020	N		M	White – Other	Portugal
W688		P	M	White British	UK
T378		P	M	White – Other	Germany
M582	N		M	White – Irish	Ireland
C329	N		M	White – Irish	Ireland
D786	N		M	Mixed	Ethiopia
B661	N		M	White – British	UK
J145		P	M	White – British	UK
T407		P	M	White – British	UK
P722	N		M	White – British	UK
V340	N		M	White – Other	Italy
B084	N		M	White – British	UK
S577	N		M	White – Other	Portugal
L451	N		M	Chinese	China
S562	N		F	Mixed White/Black	UK
G781		P	F	Black African	Ghana
O763	N		F	Mixed White/Black	Uganda
A266	N		F	Black African	Nigeria
K081	N		F	Black African	Zimbabwe

TABLE 4.4: Prevalence of asymptomatic KSHV infection in an HIV-positive cohort.
P = positive; N = negative; M = male; F = female.

KSHV is endemic (G781; a woman born in Ghana). Seven of the eight KSHV-seropositive individuals were 'White – British' or 'White – Other' males, and six of these were born in Western Europe in countries where KSHV seroprevalence in the general population is low (UK, France or Germany). It thus seems likely that the majority of KSHV prevalence observed in this cohort is associated with homosexual practices, although this information was not available.

4.2.b Screen for Immunogenic KSHV ORFs in Three Asymptomatic Carriers of KSHV

Of the eight HIV-positive individuals with no history of KSHV-related disease who were identified as KSHV seropositive, I was able to collect further peripheral blood samples from three individuals (E176, G781, H850) for use in the screen for immunogenic KSHV ORFs. However, disappointingly, no CD8 or CD4 responses were observed to any of the KSHV ORFs or pools of ORFs in any of these individuals. Furthermore, none of the three individuals mounted CD4 responses to moDCs transduced with Adpp65 (used as a positive control), and CD8 responses to Adpp65-transduced moDCs were absent in individual H850 and greatly reduced in individual G781 (16.6% proliferating T cells) compared to CMV-specific CD8 responses observed in the seven KSHV-seronegative controls (mean 52.6% proliferating T cells; see section 4.1.d and Figure 4.6). Only CD8 T cells isolated from E176 responded significantly to Adpp65-transduced moDCs, with 63.9% of CD8 T cells proliferating in response to stimulation.

On further consultation with the physicians treating these individuals, I discovered that each of these individuals were receiving chemotherapy for other types of cancer (E176 had anal cancer; G781 had ovarian cancer; and H850 had Hodgkin's lymphoma) as well as drugs to suppress opportunistic infections at the time of venesection. It seems likely that the chemotherapy drugs taken by these individuals suppressed either the ability of their moDCs to process and present antigens or the ability of their T cells to proliferate in response to stimulation in this culture system, or had substantially depleted the individuals' memory T cells, thus explaining these negative results. Although CD8 T cells from individual E176 responded to Adpp65 transduced moDCs, CD8 responses to KSHV ORFs by this individual were still not observed. This may yet be a suppressive effect of the individual's chemotherapy regime, damping down the weaker responses to KSHV ORFs but still allowing stronger responses to Adpp65-transduced moDCs to be observed (see section 4.1.d).

The cell-surface expression of HLA-DR and CD80 by moDCs from these individuals was not notably reduced as compared to the other participants in this study (see section 4.1.c). Across all three individuals and all different transduction conditions the mean proportion of HLA-DR and CD80 double-positive cells was 90.0 ± 6.3% compared to a mean proportion of 89.2 ± 9.5% double-positive cells seen across all transduction conditions in all 21 participants included in the previously described immunogenic screen (Figure 4.2).

It thus seemed that although moDCs from individuals with non-KSHV-related cancers and undergoing chemotherapy were not notably lacking in the expression of antigen-presenting cell-surface markers, these individuals were still not appropriate

for use in this model system for investigating KSHV-specific T-cell responses. It seems likely that disease- or therapy-related damage to the cells of their immune system reduced their ability to prime, recognise or respond to antigens, or had caused substantial loss of the individuals' memory T-cell compartment. Unfortunately, I found that comparatively healthy asymptomatic carriers of KSHV attended clinics too infrequently for follow-up collection of peripheral blood samples to be practical. Thus, the original target cohort of individuals with a history of KSHV-related neoplasia attending follow-up clinics after treatment does indeed seem to be the most appropriate for further immunological studies.

4.3 Summary

The experimental method of using lentiviral-transduced moDCs expressing KSHV ORFs to investigate the targets of the KSHV-specific T-cell response had several advantages as a system for performing a broad screen for immunogenic KSHV ORFs. In particular, no prior knowledge of the optimal epitope or HLA-restriction was required, and far more ORFs could be investigated for immunogenicity than would be possible using artificial peptides. The use of overlapping peptides to test the immunogenicity of the 31 KSHV gene products included in this screen would be impractical, and even the use of epitope prediction software (Immune Epitope Database; www.immuneepitope.org.uk) yielded over 1000 potential nine-mer epitopes (IC50 value less than 5000 nM; see sections 2.5.c and 5.1.e) from these 31 genes for HLA-A*0201 alone (data not shown). The lentiviral library system offered a good overview of the immunogenic profile of KSHV, and enabled the identification of antigenic hotspots within the KSHV genome. Unexpectedly, the

results indicated that early lytic and late lytic gene products are the major targets of the KSHV-specific immune response.

However, the lentiviral system also had limitations. The experiments were very long and time-consuming. The variable background response to moDCs transduced with the empty pSIN vector meant that strict criteria were applied to designate positive and borderline positive responses (see Materials and Methods section 2.4.d) in order to ensure that no false positives were recorded. Thus the system may not have been very sensitive, and some weak T-cell responses may not have been identified in some or all KSHV-seropositive individuals. Moreover, the long duration of each experiment, coupled with the background response to the empty vector, made the system unsuitable for further functional characterisation of the KSHV-specific T-cell response, for example by using intracellular staining techniques such as those described in section 3.3.b. It was thus decided to use the information that had been gathered using the lentiviral system to embark on an epitope discovery project to identify new late lytic KSHV epitope(s) and to investigate alternative protocols for the exploration of the functional properties of KSHV-specific T cells. This work is described in Chapter 5.

CHAPTER 5: Identification of an Immunodominant Late Lytic KSHV Epitope; Pentamers as Tools to Explore Phenotypes and Functions of Virus-Specific T cells; T-cell Memory Phenotypes in KSHV Infection

Although several HLA-restricted, KSHV-specific CD8 epitopes have been identified, the frequency of recognition of these epitopes is low and the responses they elicit appear weak compared with responses to known epitopes from other viruses such as HIV-1 and EBV (Bihl et al., 2007b; Brander et al., 2001). For example, Table 5.1 shows all published HLA-A*0201-restricted KSHV epitopes with an accumulated tally of the number of responses observed to each of these epitopes by all individuals (from all publications) in which the epitope has been tested. Strikingly, only three of these 21 epitopes have been confirmed by more than one study (LLNGWRWRL from K12; LMWYELSKI from ORF8; and FLNWQNLLNV from ORF22), and less than half (10 out of 21 epitopes) have been tested in more than 10 individuals. Of these 10 epitopes, only six elicited responses in more than five percent of individuals tested (LLNGWRWRL from K12; VLGDEVLSL from ORF6; LMWYELSKI from ORF8; GLADVFAEL from ORF61; NMSQAEYLV from ORF65; and FLNWQNLLNV from ORF22). For these six epitopes, the percentage of responders to each epitope ranged from 16% to 46% of all individuals (HIV-negative and HIV-positive) tested, and 13% to 32% of all HIV-positive individuals tested. The two most frequently recognised epitopes (of those tested in more than 10 individuals) appear to be LLNGWRWRL from K12

Viral Gene Expression	KSHV ORF	Peptide	Total HIV+ and HIV- Responders (Percentage)		Total HIV+ Responders (Percentage)		References
Latent	ORF73	FTSGLAPAFV	1 of 52	(2)	1 of 35	(3)	Guihot et al., 2006
		WATESPIYV	2 of 52	(4)	2 of 35	(6)	Guihot et al., 2006
		QMARLAWEA	2 of 52	(4)	2 of 35	(6)	Guihot et al., 2006
	K12	*LLNGWRWRL	23 of 61	(38)	12 of 37	(32)	Brander et al., 2001 Bourboulia et al., 2004 Lambert et al., 2006
		VLLNGWRWRL	2 of 4	(50)	1 of 3	(30)	Micheletti et al., 2002
	K15	ILFTSTFAV	1 of 52	(2)	1 of 35	(3)	Guihot et al., 2006
Immediate-Early Lytic	K5	*ALYAANNTRV	4 of 7	(57)	Nt		Ribechini et al., 2006
	ORF6	*VLGDEVLSL	18 of 46	(39)	4 of 23	(17)	Lambert et al., 2006
Early Lytic	K3	GLAAATWVWL	4 of 8	(50)	Nt		Ribechini et al., 2006
		FVFYQLFVV	1 of 8	(13)	Nt		Ribechini et al., 2006
	ORF8	**LMWYELSKI	33 of 131	(25)	16 of 85	(19)	Wang et al., 2002b Bourboulia et al., 2004 Guihot et al., 2006 Lambert et al., 2006 Guihot et al., 2008
	ORF61	*GLADVFAEL	13 of 46	(28)	3 of 23	(13)	Lambert et al., 2006
	ORF65	*NMSQAEYLV	22 of 48	(46)	6 of 24	(25)	Lambert et al., 2006
	ORF70	VVQELLWFL	2 of 5	(40)	Nt		Ribechini et al., 2006
		SLLTYMLAHV	1 of 5	(20)	Nt		Ribechini et al., 2006
		*YMLAHVTGL	5 of 5	(100)	Nt		Ribechini et al., 2006
Late Lytic	K8.1	*LVLILYLCV	4 of 8	(50)	4 of 8	(50)	Bourboulia et al., 2004
	ORF22	*FLNWQNLLNV	14 of 85	(16)	13 of 67	(19)	Micheletti et al., 2002 Bourboulia et al., 2004 Guihot et al., 2006 Guihot et al., 2008
	ORF26	FQWDSNTQL	3 of 6	(50)	Nt		Ribechini et al., 2006
		IVLESNGFDL	3 of 6	(50)	Nt		Ribechini et al., 2006
		VLDDLSMYL	4 of 6	(67)	Nt		Ribechini et al., 2006

TABLE 5.1: Frequency of recognition of all published A*0201-restricted KSHV CD8 epitopes. * indicates epitopes tested for recognition by *ex vivo* T cells from A*0201-positive, KSHV-positive individuals (section 5.2); ** indicates epitope included in HLA-binding and off-rate assays and *in silico* analysis (section 5.1) as well as in later *ex vivo* assays.

(recognised by 38% of all individuals tested and 32% of all HIV+ individuals tested) and NMSQAEYLV from ORF65 (recognised by 46% of all individuals tested and 25% of all HIV+ individuals tested). However, it seems reasonable to conclude that there is no immunodominant HLA-A*0201 epitope evident from the compiled data from these studies (see Table 5.1).

From the work described in Chapter 4 of this thesis, a search for new epitopes derived from within the late lytic KSHV antigens ORF28, ORF36 and ORF37 (the three ORFs in the pool that elicited the most responses in the lentivirus-based screen) seemed warranted. MHC binding assays and computer algorithms were used to identify potential HLA-A*0201 epitopes from within ORF28, ORF36 and ORF37. These potential epitopes were then tested in clinical samples alongside nine of the most frequently recognised known HLA-A*0201-restricted epitopes (indicated in Table 5.1) in order to investigate further the patterns of recognition of these epitopes, and in an attempt to identify an immunodominant epitope(s).

Identification of such an epitope would be a useful tool for further exploration of the KSHV-specific T-cell response. In particular the functions and memory phenotypes of KSHV-specific CD8 T cells remain largely unexplored, but novel technology may allow better investigation and understanding of this field. Since their introduction 13 years ago (Altman et al., 1996), tetrameric moieties (‘tetramers’) have been useful tools for investigating the memory phenotypes and functions of T cells specific for viruses such as CMV and HIV-1 (Appay et al., 2000; Chen et al., 2001). Tetramers are synthetic T-cell receptor (TCR) ligands comprised of four MHC-peptide complexes conjugated to a fluorescent tag via a

streptavidin moiety, thus allowing fluorescent labelling of T cells specific for a known epitope. More recently, pentamers have been developed with the goal of improving TCR labelling. Such pentamers are pentavalent (and so have five MHC-peptide complexes available for TCR binding) and also have a higher number of fluorescent tags, designed to increase the brightness of fluorescence. However, with KSHV, a limitation to such investigations has been the paucity of known immunodominant epitopes to target.

Such an epitope would also be a useful tool for tracking changes in the KSHV-specific T-cell response during chronic infection and disease. Furthermore, it could have a potential clinical application, for example, in vaccine design.

5.1 Identification of Potential CD8 Epitopes from Late Lytic KSHV Gene Products

5.1.a Target HLA-restriction

Before embarking on a search for potential new late lytic epitopes, a target HLA-restriction needed to be selected. The seven individuals who mounted CD8 responses against the late lytic KSHV gene pool [ORF28/ORF36/ORF37] were HLA typed. Results are shown in Table 5.2. The most frequently arising HLA alleles amongst these seven individuals were A*0201 (four out of six typed); B*0702 (three out of six typed); Cw*0702 (three out of seven); and DRB1*1501 (four out of seven).

Participant HIV/KSHV Status	Participant ID	High Resolution HLA Types			
		A	B	Cw	DRB1
HIV+, KSHV-related neoplasia in remission	**P940**	***0201** *2902	*0702 *4403	*0702 *1601	*0701 *1501
	M620	*2402 *2902	*5101 *1402	*0802 *1502	*0402 *1302
	V689	***0201** *0301	*0702 *3501	*0702 *0401	*0101 *1501
	A556	***0201** *2402	*1302 *4402	*0602 *0501	*0401 *1501
	B196	nd	nd	*0602 *1601	*0701 *1102
	T541	***0201** *2601	*0801 *1801	*0701 *1203	*0301 *1103
HIV+, Asymptomatic	**H419**	*0301	*0702 *1501	*0702 *0304	*0401 *1501

Table 5.2: High resolution HLA types of all individuals who showed CD8 T-cell responses to the [ORF 28/ORF 36/ORF37] KSHV gene pool.

It was decided to focus on HLA-A*0201-restricted epitopes, as this was the most common MHC-I allele in the seven responders to the late lytic ORF pool. Moreover, HLA-A*0201 is recognised to be one of the most common alleles in the general population. For example, in the population of North America, HLA-A*0201 is the most common HLA allele carried by Caucasians and Hispanics, found in 45.6% and 37.1% of individuals respectively. HLA-A*0201 is also the fourth most-common HLA allele amongst African-Americans, carried by 22.3%; and the eighth most-common HLA allele in those of South-East Asian origin, carried by 18.1% (source: HLA matchmaker; http:tpis.upmc.edu/tpis/HLAMatchmaker/). Thus HLA-

A*0201-restricted epitopes are likely to be useful for future immunological investigations or the development of vaccines or immunotherapies.

5.1.b Target Immunogenic KSHV Open Reading Frame

As discussed in section 4.1.e, the gene pool most frequently recognised by CD8 T cells from KSHV+ individuals was the late lytic pool [ORF28/ORF36/ORF37] (recognised by seven out of 14 individuals) and none of the gene products from this pool have been previously investigated for immunogenicity. Thus a search for novel KSHV CD8 epitopes from within this pool seemed warranted.

To narrow down this search, the likelihood of each of the gene products in the [ORF28/ORF36/ORF37] pool being immunogenic was considered. Firstly, the function of each of these proteins was examined. ORF28 has been classified as an envelope glycoprotein based on positional and structural similarities to EBV BDLF3 (Zhu et al., 2005), which encodes the EBV glycoprotein gp150. Viral glycoproteins are often targets of anti-viral T-cell responses (Torseth et al., 1987; Wallace et al., 1999). Three other KSHV glycoproteins (glycoprotein B, encoded by ORF8; glycoprotein H, encoded by ORF22; and glycoprotein 35/37 encoded by K8.1) are documented targets of the KSHV-specific CD8 T-cell response (Micheletti et al., 2002; Wang et al., 2002b; Wilkinson et al., 2002). KSHV ORF36 encodes a serine protein kinase (Hamza et al., 2004) and ORF37 a modified DNA exonuclease involved in host mRNA shut off (Glaunsinger and Ganem, 2004). There is no evidence to indicate that either of these gene products is likely to be immunogenic.

Secondly, the amino acid (aa) sequence of each of the three ORFs was examined. In the search for immunodominant virus-specific epitopes it seems logical to focus on viral gene products with aa sequences that share the least similarity with proteins from other pathogens, as epitopes derived from such proteins are the most likely to be biologically relevant in terms of effective host control of viral infection. This is supported by observations of the EBV-specific T-cell response (personal communication with Professor Alan Rickinson). The NCBI BLAST 'blastp' tool was used to search for proteins that shared regions of aa sequence with each of the three KSHV ORFs of interest. Results are shown in Figure 5.1.

The results for ORF28 returned a match to two identical published aa sequences for the KSHV ORF28 gene product. Other than this, there was no sequence similarity between ORF28 and any other known proteins from other viruses. The only matches returned were occasional loose similarity between the ORF28 aa sequence and proteins from thermal bacteria, yeast and rice. This confirmed reports that although KSHV ORF28 is the positional equivalent of EBV BDLF3, its gene product shares no aa sequence similarity to EBV gp150 (Zhu et al., 2005).

The results for ORF36 and ORF37 showed considerable sequence similarity between these gene products and their equivalent proteins in a host of other herpesviruses that infect a range of animal species. Of particular significance, KSHV ORF36 shares several aa residues with its EBV homologue BGLF4 and KSHV ORF37 shares several aa residues with its EBV homologue BGLF5. It was thus decided to focus initially on KSHV ORF28 in a search for new epitopes.

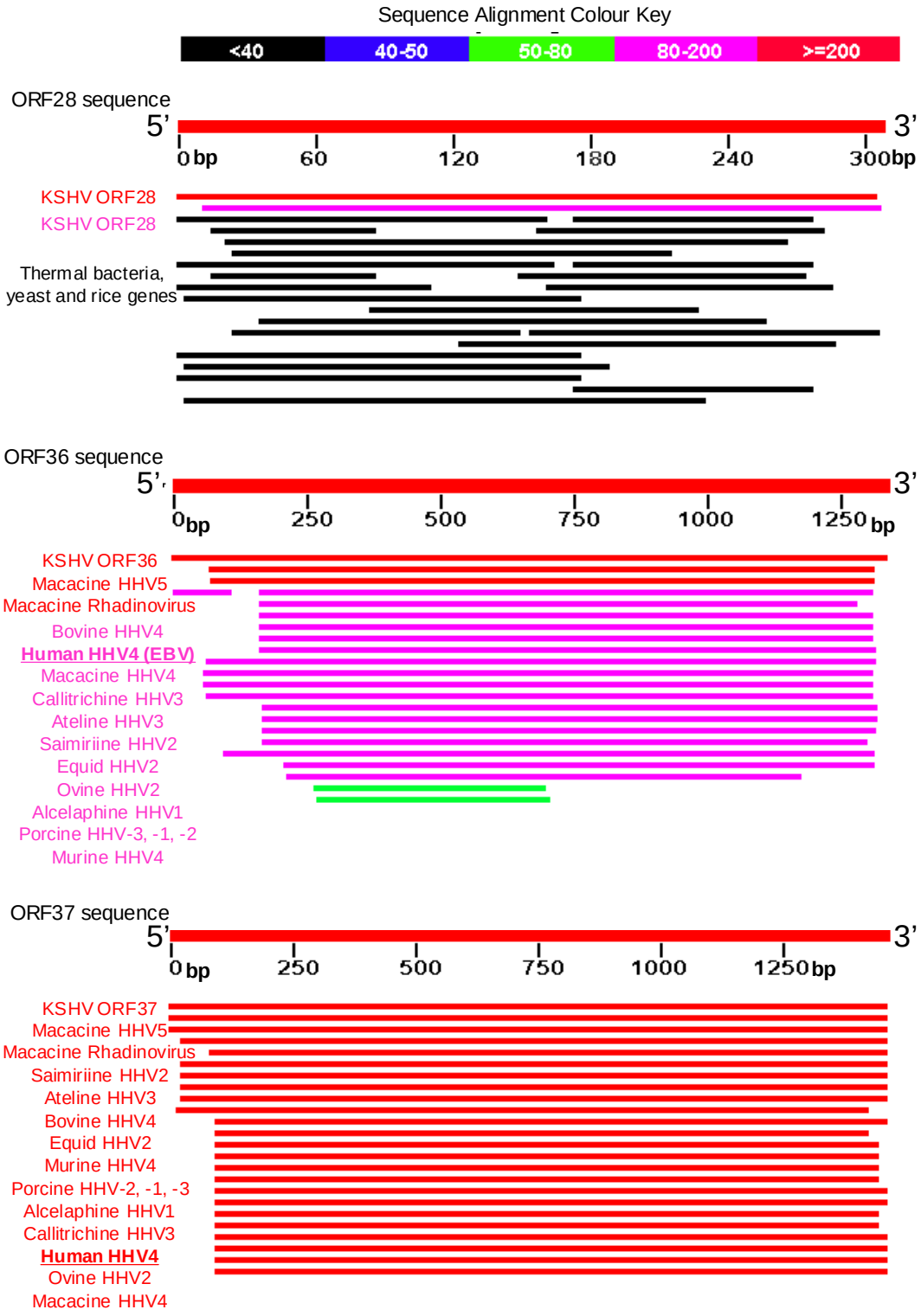


FIGURE 5.1: Sequence similarity between KSHV ORF28, ORF36 and ORF37 and genes from other viruses. Results from an NCBI 'BLAST' search for sequences that align to ORF28 (top panel), ORF36 (middle panel) and ORF37 (bottom panel). Each ORF's sequence is indicated by the red line with base pair graduations at the top of each panel. The lines below represent genes with sequence similarity. The degree of sequence alignment is indicated by the colour of the line (colour key at top). Colour-coordinated labels at the side show the origins of the genes represented by each line.

5.1.c Binding Affinities of KSHV ORF28-derived Peptides to HLA-A*0201

REVEAL™ assays (ProImmune Ltd., Oxford, UK), which measure MHC-peptide binding affinity and denaturation rates (section 5.1.d), were used to identify putative candidate HLA-A*0201-restricted epitopes from within ORF28.

Ninety-four overlapping nine-mer peptides, off-set by one amino-acid residue, were synthesised spanning the entire amino acid sequence of ORF28 and numbered P1 to P94 sequentially (see Materials and Methods Table 2.8 for peptide sequences). One of the best documented KSHV HLA-A*0201-restricted epitopes, LMWYELSKI (aa492-500 from glycoprotein B/ORF8 [$Gb_{492-500}$]; indicated on Table 5.1), was also included in the screen for comparison, and was designated P95. The REVEAL™ binding assay measured the ability of each peptide to bind to HLA-A*0201, and compared this to the binding affinity of a known high-affinity A*0201-restricted T-cell epitope (the influenza matrix protein epitope, GILGFVFTL) in order to identify putative immunogenic peptides. An intermediate-affinity control (whose identity was undisclosed by the manufacturer, but was described as: ‘a known T-cell epitope of marginal binding’) was also included in the assay for comparison. Results are shown in Figure 5.2 and Table 5.3.

Each peptide was given a binding score as a percentage relative to the binding of the high-affinity positive control, GILGFVFTL. A ‘pass’ rate was set at 45%. This was a fairly arbitrary cut-off score, designed to identify the best peptide binders to prioritise in future experiments. Twelve peptides qualified as ‘passed’ using this scoring system. Significantly, the ‘pass’ score was higher than the binding score of

the known intermediate-affinity control (17.54 ± 2.7%). Five further peptides (P31, P47, P42, P79 and P39) had binding scores above that of the intermediate control (38.34%, 28.84%, 27.42%, 26.72%, and 20.29% respectively), and so may also be weaker-binding T-cell epitopes and may warrant further investigation in the future.

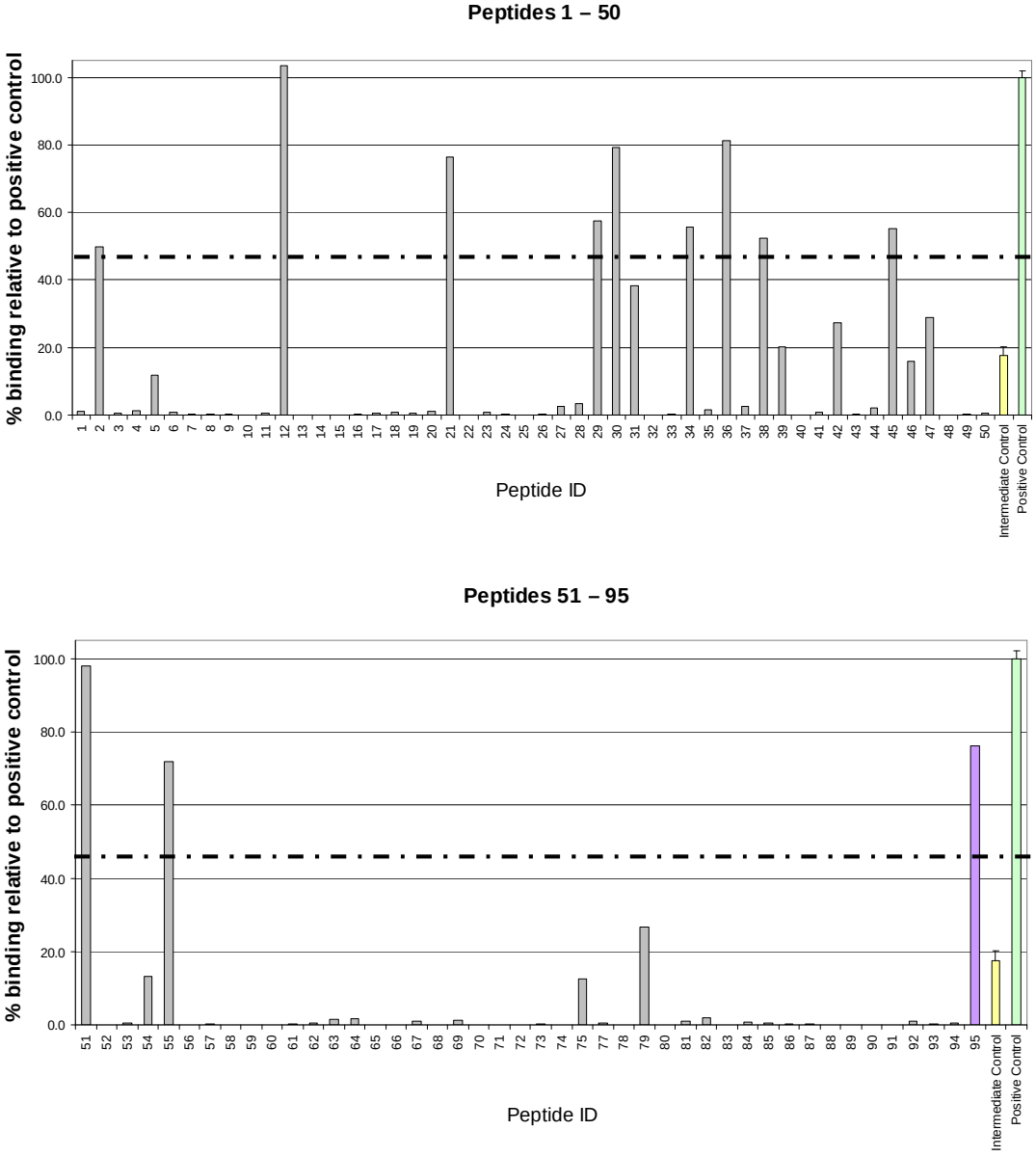


FIGURE 5.2: Binding affinity of ORF28-derived peptides to HLA-A*0201. Binding shown as a percentage relative to the binding of the known high-affinity HLA-A*0201-restricted epitope, GILGFVFTL (from the influenza matrix protein; 'positive control'; green bars). Dashed line indicates the 'pass' rate of 45%, designated to identify the best binders for use in future assays. Yellow bars indicate the intermediate-affinity control epitope and the lilac bar indicates the KSHV control epitope ($Gb_{492-500}$).

Peptide ID	Binding Affinity	Peptide ID	Binding Affinity
12*	103.49*	53	0.47
Positive Control*	100 ± 2.0*	19	0.42
51*	97.95*	17	0.42
36*	81.20*	11	0.41
30*	79.09*	3	0.41
21*	76.27*	87	0.30
95*	76.25*	8	0.30
55*	71.89*	7	0.30
29*	57.52*	33	0.27
34*	55.69*	73	0.25
45*	55.20*	61	0.25
38*	52.35*	49	0.23
2*	49.80*	43	0.23
31	38.34	9	0.20
47	28.84	93	0.15
42	27.42	86	0.15
79	26.72	57	0.13
39	20.29	26	0.13
Intermediate Control	17.54 ± 2.7	24	0.13
46	15.90	16	0.13
54	13.29	48	0.12
75	12.61	40	0.12
5	11.71	14	0.10
28	3.23	13	0.09
27	2.66	10	0.09
37	2.60	91	0.00
44	2.01	90	0.00
82	1.95	89	0.00
64	1.64	88	0.00
35	1.53	83	0.00
63	1.51	80	0.00
4	1.35	78	0.00
69	1.13	74	0.00
92	1.05	72	0.00
81	1.05	71	0.00
67	1.01	70	0.00
20	0.97	68	0.00
1	0.93	66	0.00
6	0.83	65	0.00
84	0.75	60	0.00
41	0.70	59	0.00
18	0.70	58	0.00
23	0.69	56	0.00
94	0.60	52	0.00
85	0.60	32	0.00
77	0.60	25	0.00
50	0.58	22	0.00
62	0.50	15	0.00

TABLE 5.3: HLA-A*0201 binding affinity of ORF28-derived peptides as a percentage relative to positive control (highlighted green). KSHV control epitope ($Gb_{492-500}$) highlighted lilac; intermediate-affinity epitope highlighted yellow. * indicates peptides that scored above the 'pass' rate of 45%.

Of the 12 ‘passed’ peptides, one peptide (P12) had a higher binding score than the positive control, showing 103.49% binding relative to the positive control. Importantly, the KSHV control epitope, P95, qualified as a passed peptide with a binding score of 76.25%, indicating that the results of the assay concur with the results from previous *ex vivo* studies used to identify KSHV epitopes. However, five of the peptides from within ORF28 (P12, P51, P36, P30 and P21) were better binders than the P95 KSHV control peptide epitope, with binding scores of 103.49%, 97.95%, 81.20%, 79.09% and 76.27% respectively.

5.1.d Stability of KSHV ORF28 Peptides in the Context of HLA-A*0201

For each of the 12 highest-scoring (‘passed’) peptides in the binding assay, the off-rates for peptide binding to HLA-A*0201 were measured to assess how long each individual peptide could be presented to T cells to help identify the most useful epitope. The rate of dissociation for each A*0201-peptide complex was measured at 0 hours (hr), 2 hr and 24 hr at 37ºC (Figure 5.3), and A*0201-peptide complex half-life values, $t_{1/2}$ (h), were calculated for each peptide (Figure 5.4 and Table 5.4).

One of the peptides, P30, had an estimated A*0201-binding half-life (40.49h) that was greater than the positive control (40.37 ± 3.1 hr). Two further peptides (P21 and P51) had estimated A*0201-binding half-lives (24.98hr and 17.89hr respectively) that were greater than that of the intermediate control (13.54 ± 1.0 hr). Interestingly, the five peptides (P12, P51, P36, P30, P21; marked with a * on Table 5.4) that scored highest in the REVEAL™ binding assay (section 5.2.a), were also

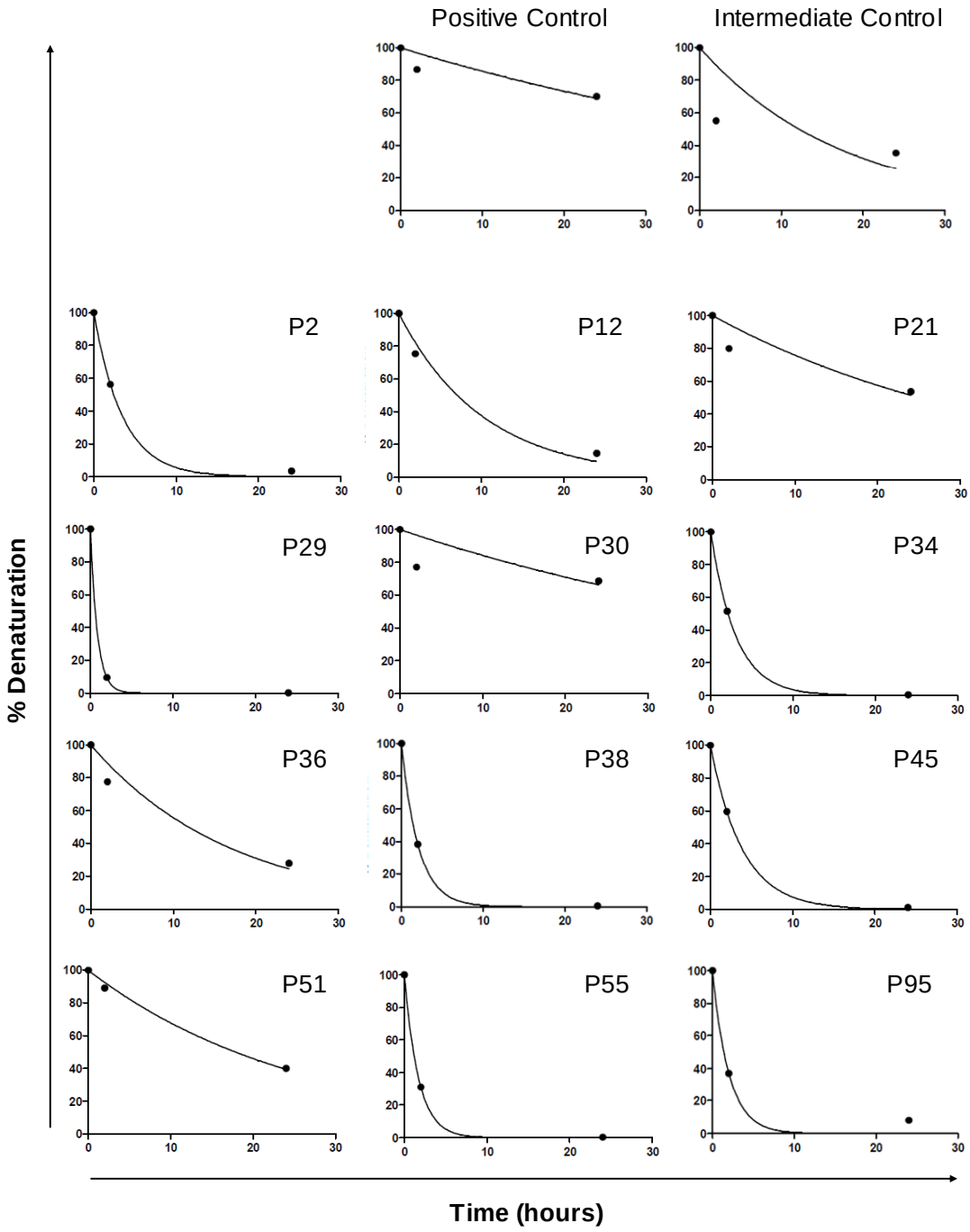


FIGURE 5.3: Rate of dissociation of ORF28-derived peptides from HLA-A*0201. Denaturation of peptide-HLA-A*0201 complexes were measured at 0 hours (hr), 2 hr and 24 hr at 37ºC and compared to the denaturation rates of positive and intermediate controls (top two panels) and the KSHV control peptide (P95; bottom right panel).

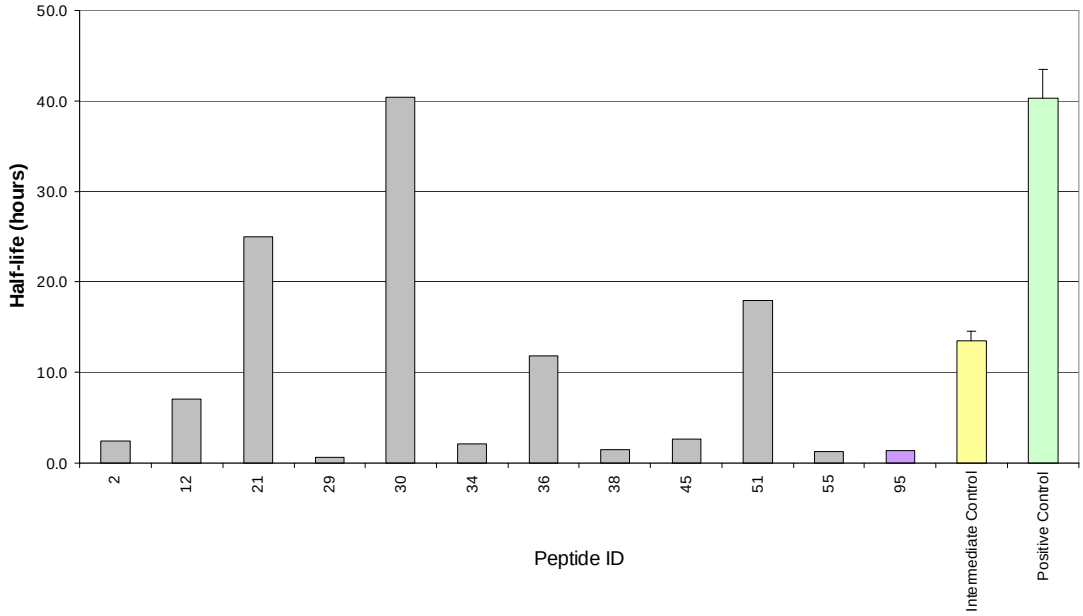


FIGURE 5.4: Estimated half-lives of peptide-HLA-A*0201 complexes for ORF28-derived peptides, KSHV control peptide (P95; lilac bar) and positive (green bar) and intermediate (yellow bar) controls. Half-lives were calculated based on the rate of dissociation of each peptide from HLA-A*0201 at 37ºC.

Peptide ID	Peptide Sequence	Half-life (hr)	Binding Affinity
2	SMTSPSPVT	2.42	49.80
12*	GMVDGSVLV	7.10	103.49
21*	RMATKPPVI	24.98‡	76.27
29	IGLITVLFL	0.59	57.52
30*	GLITVLFLL	40.49‡	79.09
34	VLFLLVIGA	2.10	55.69
36*	FLLVIGACV	11.81	81.20
38	LVIGACVYC	1.45	52.35
45	YCCIRVFLA	2.68	55.20
51*	FLAARLWRA	17.89	97.95
55	RLWRATPLG	1.19	71.89
95	LMWYELSKI	1.40	76.25
Intermediate Control	~	13.54 ± 1.0	17.54
Positive Control	~	40.37‡ ± 3.1	100.00

TABLE 5.4: Estimated half-lives of peptide-HLA-A*0201 complexes for ORF28-derived peptides; KSHV control peptide (P95; highlighted lilac); and intermediate (highlighted yellow) and positive (highlighted green) controls. * indicates the five peptides that scored highest in the binding assay – these were also the five highest-scorers in the off-rate assay. ‡ indicates that the measurement interval of 24 hr was too short to calculate this value accurately.

the five peptides with the longest A*0201-peptide complex half-lives in the REVEAL™ off-rate assay. Also of interest was that P95, the KSHV control peptide, was one of the poorest scorers in the off-rate assay with an estimated A*0201-peptide complex half-life of 1.40h, despite being a middle-ranking scorer out of the 12 'passed' peptides in the binding assay and despite being previously identified as an epitope in *ex vivo* experiments by other groups (Table 5.1).

5.1.e Further *In Silico* Analysis of ORF28 Peptides

For the 12 'passed' peptides, further *in silico* analysis was performed. First, each peptide's sequence was examined for known A*0201-binding 'motif' aa residues. Each peptide's binding score and off-rate score (A*0201-peptide complex half-life) were then compared. Finally, each peptide was run through three 'epitope-prediction' computer algorithms to see how highly they scored in these. Three known very high-affinity A*0201-restricted T-cell epitopes (GILGFVFTL, the high-affinity control used in the binding and off-rate assays from the influenza matrix protein; SLYNTVATL from HIV GAG; and NLVPMVATV from CMV pp65) were included in these analyses for comparison. All these results are summarised in Table 5.5. The three algorithms used were Immune Epitope Database (IEDB; www.immuneepitope.org); SYFPEITHI (www.syfpeithi.de); and HLA_BIND (www-bimas.cit.nih.gov/molbio/hla_bind/). Each of the ORF28 peptide sequences was also entered into the NCBI PubMed search engine (www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) to ensure that none of these peptides had been previously used in a published study. No publications were found for any of the ORF28 peptides.

Peptide	Amino Acid Residue									REVEAL™ Binding Assay Ranking	REVEAL™ Off-rate Assay Ranking	Immune Epitope Database Score	SYFPEITHI Score	HLA_BIND Score	Combined Algorithm Score
	1	2	3	4	5	6	7	8	9						
Optimal HLA-A*0201 'Motif' Residues		L							L						
		M							V						
						V									
Influenza	G	I	L	G	F	V	F	T	L	2	2	18.8	30	550.9	+++
HIV gag	S	L	Y	N	T	V	A	T	L	Nt	Nt	16.0	31	157.2	+++
CMV pp65	N	L	V	P	M	V	A	T	V	Nt	Nt	32.1	30	160.0	+++
ORF28 P12	G	M	V	D	G	S	V	L	V	1	6	23.4	24	206.0	+++
ORF28 P51	F	L	A	A	R	L	W	R	A	3	4	3.7	21	394.2	+++
ORF28 P36	F	L	L	V	I	G	A	C	V	4	5	10.8	25	1183.8	+++
ORF28 P30	G	L	I	T	V	L	F	L	L	5	1	11.2	27	285.0	+++
ORF28 P21	R	M	A	T	K	P	P	V	I	6	3	257.8	20	<10	++
ORF8 P95	L	M	W	Y	E	L	S	K	I	7	11	18.6	24	244.5	+++
ORF28 P55	R	L	W	R	A	T	P	L	G	8	12	>5000.0	<15	<10	
ORF28 P29	I	G	L	I	T	V	L	F	L	9	13	>5000.0	19	11.1	++
ORF28 P34	V	L	F	L	L	V	I	G	A	10	9	93.9	22	71.9	+++
ORF28 P45	Y	C	C	I	R	V	F	L	A	11	7	3854.3	<15	<10	+
ORF28 P38	L	V	I	G	A	C	V	Y	C	12	10	>5000.0	<15	<10	
ORF28 P2	S	M	T	S	P	S	P	V	T	13	8	>5000.0	15	<10	+

TABLE 5.5: Amino-acid sequences, ranks in binding and denaturation assays, and computer algorithm scores of ORF28-derived peptides with high affinity for HLA-A*0201.

Although binding 'motifs' are neither sufficient nor necessary for an epitope to be presented *in vivo*, they are an indication of the best peptide binders to a specific HLA allele and thus help to identify candidate epitopes. For A*0201, a leucine (L) or a methionine (M) residue at the second position in conjunction with an L or a valine (V) residue at the ninth position creates an optimal binding motif, and a V at the sixth position also promotes binding (Table 5.5). HIV SLYNTVATL and CMV NLVPMVATV both contained an optimal A*0201-binding motif [an L at the second position with an L (SLYNTVATL) or a V (NLVPMVATV) at the ninth position], as well as both having a V at the sixth position. Influenza GILGFVFTL contained a V at the sixth position and an L at the ninth position, and also had an isoleucine (I) residue at the second position. Isoleucine is very similar in chemical formula and structure to leucine, and there is little change in protein structure or folding if these aa residues are substituted. Thus the presence of an I residue at the second or ninth position (where an L contributes to an optimal binding 'motif') may also improve a peptide's A*0201-binding affinity.

Three out of the 12 'passed' peptides contained an optimal binding 'motif'. P12 had an M at the second position and a V at the ninth position and was the highest scorer in the binding assay. P30 had two L residues at the second and ninth positions and was the highest scorer in the off-rate assay. P36 had an L at the second position and a V at the ninth position, and was within the top-five highest scorers in both the binding and the off-rate assay. Three peptides (P29, P34 and P45) contained a V at the sixth position. A further seven peptides (P51, P21, P29, P28, P34, P55 and P95) contained one residue from a binding 'motif'. Two peptides (P21 and P95) also

contained an isoleucine (I) residue at the ninth residue. The only peptide that did not contain any binding 'motif' residues was P38.

The three 'epitope-prediction' computer algorithms used for analysis each have different methods for scoring potential peptides and different cut-off points are used to distinguish peptides 'unlikely' to be epitopes. With IEDB, a low score is best and a commonly used cut-off score of greater than 5000 eliminates unlikely peptides (Westrop et al., 2009). With SYFPEITHI, a high score is best, and a common cut-off score is less than 15 (Rammensee et al., 1999). With HLA_BIND a high score is best and a commonly used cut-off score is less than 10 (Parker et al., 1994). Each peptide was given a combined algorithm score to indicate how many of the three algorithms scored the peptide within their 'likely' epitope range.

GILGFVFTL, SLYNTVATL, NLVPMATV and the P95 control KSHV peptide all performed well in all three computer algorithms. This is to be expected, as such algorithms are written based on documented peptide sequences. Only five of the 11 'passed' ORF28 peptides (P12, P51, P36, P30 and P34) were classed as 'likely' epitopes by all three algorithms. Four of these (P12, P51, P36 and P30) were also among the five peptides that scored most highly in both the binding and off-rate assays. The other peptide that scored in the top five in the binding assays, P21, was designated a 'likely' epitope by two of the three algorithms. Two of the 11 'passed' ORF28 peptides (P55 and P38) were not classified as likely epitopes by any of the three algorithms, and a further two (P45 and P2) were only classed as likely by one of the three algorithms.

The entire ORF28 aa sequence was also run through each of the algorithms to see if they identified any peptides likely to be epitopes that did not bind well to A*0201 in the binding assay. All peptides identified by one or more algorithms as being putative epitopes are shown in Table 5.6. Only two of the peptides (P31 and P32) eliminated by the binding assay were flagged as candidate epitopes by more than one algorithm. Of these, P32 was identified by two algorithms but performed very poorly in the binding assay. P31, however, was classed as a likely epitope by all three algorithms, and also performed quite well in the binding assay, with a relative binding score of 38.34% that was greater than that of the intermediate control (17.54% ± 2.7) although just under the pass score of 45%. P31 may well thus represent a weaker binding A*0201-restricted epitope and may warrant further investigation in the future.

Of the 14 peptides identified by one or more algorithms as a potential epitope but eliminated by the binding assay, one (P23) had a V at the sixth position (an optimal A*0201-binding residue), and also had one residue from within an optimal A*0201 binding 'motif' (an L at the ninth position) A further nine peptides also contained a single residue from within an optimal A*0201-binding 'motif' (P59, P67, P31, P27, P71, P26, P79, P1, P42).

The twelve peptides indicated as candidate epitopes by the binding and off-rate assays (including the KSHV control peptide, P95 [LMWYELSKI from ORF8]) were synthesised for use in further assays to test the recognition of these peptides by T cells from KSHV-positive individuals. Results are presented in section 5.2.

Peptide	Amino Acid Residue									REVEAL™ Binding Assay Score	Immune Epitope Database Score	SYFPEITHI Score	HLA_BIND Score	Combined Algorithm Score
	1	2	3	4	5	6	7	8	9					
Optimal HLA-A*0201 'Motif' Residues		L							L					
		M							V					
						V								
Influenza	G	I	L	G	F	V	F	T	L	100.00	18.8	30	550.9	+++
HIV gag	S	L	Y	N	T	V	A	T	L	Nt	16.0	31	157.2	+++
CMVpp65	N	L	V	P	M	V	A	T	V	Nt	32.1	30	160.0	+++
ORF28 P23	A	T	K	P	P	V	I	G	L	0.69	>5000.0	23	<10	+
ORF28 P59	A	T	P	L	G	R	A	T	V	0.00	>5000.0	22	<10	+
ORF28 P67	V	A	Y	Q	V	L	R	T	L	1.01	>5000.0	22	<10	+
ORF28 P31	L	I	T	V	L	F	L	L	V	38.34	308.0	21	30.9	+++
ORF28 P32	I	T	V	L	F	L	L	V	I	0.00	2560.8	20	<10	++
ORF28 P27	P	V	I	G	L	I	T	V	L	2.66	>5000.0	20	<10	+
ORF28 P81	S	H	A	P	P	T	V	G	I	1.05	>5000.0	20	<10	+
ORF28 P90	A	T	Q	E	P	Y	R	T	I	0.00	>5000.0	19	<10	+
ORF28 P71	V	L	R	T	L	G	P	Q	A	0.00	>5000.0	17	<10	+
ORF28 P47	C	I	R	V	F	L	A	A	R	28.84	>5000.0	16	<10	+
ORF28 P26	P	P	V	I	G	L	I	T	V	0.13	>5000.0	15	<10	+
ORF28 P79	A	G	S	H	A	P	P	T	V	26.72	>5000.0	15	<10	+
ORF28 P1	M	S	M	T	S	P	S	P	V	0.93	1856.8	<15	<10	+
ORF28 P42	A	C	V	Y	C	C	I	R	V	27.42	4259.8	<15	<10	+

TABLE 5.6: Amino-acid sequences, ranks in the binding assay, and computer algorithm scores of ORF28-derived peptides with low affinity for HLA-A*0201.

*5.2 Recognition of KSHV Epitopes by T cells from A*0201-positive, KSHV-positive Individuals*

5.2.a Identification of HLA-A*0201-positive, KSHV-positive Individuals

In the experiment investigating KSHV T-cell responses with lentiviral-transduced moDCs described in Chapter 4 of this thesis, HIV-positive individuals who had a history of KSHV-related neoplasia but were in remission were found to be the most useful available cohort for such immunological studies. This is because they are easily identifiable; attend clinics regularly; and showed comparatively high levels of KSHV-specific T-cell responses (see sections 4.1.a and 4.2.b). Thus this cohort was again selected to test the recognition of the candidate ORF28 peptides identified in section 5.1, and to investigate patterns of immunodominance in a selection of previously identified A*0201-restricted, KSHV-specific epitopes (indicated in Table 5.1). To do this, it was first necessary to identify further a number of HLA-A*0201-positive individuals that fitted this specification.

Small peripheral blood samples (3ml) were collected from 59 HIV-positive individuals attending an HIV/oncology clinic at Chelsea and Westminster Hospital for DNA extraction and HLA-typing. Samples were collected from those who either had a history of, or who currently had, a KSHV-related neoplasia (KS or MCD), so that those identified as HLA-A*0201-positive could be followed-up at a later date (preferably when in complete remission) by taking a larger blood sample for use in the proposed functional studies.

Of the 59 individuals HLA-typed, 27 were HLA-A*0201-positive. Thus 45.8% of this patient cohort are A*0201-positive. Within this cohort, 24 out of 47 individuals (51.2%) of Caucasian origin were A*0201-positive – a close match to the reported prevalence of A*0201 in the Caucasian population of North America (45.6%; source: HLA matchmaker; http:tpis.upmc.edu/tpis/HLAMatchmaker/). Only four out of 12 individuals (25.0%) of mixed, non-Caucasian origin were A*0201-positive, but this again fitted observations of HLA-type prevalence between different ethnic groups in North America (see section 5.1.a).

Further blood samples were obtained from 18 of these 27 HLA-A*0201-positive individuals for use in *ex vivo* assays to assess the recognition of KSHV-derived peptides.

5.2.b Study Participants

Individual characteristics of the 18 HIV-positive participants can be seen in Table 2.9 (Materials and Methods section 2.6.a). In summary, all participants were male, and their age at venesection ranged from 29 to 67 years. CD4 counts ranged from 48 to 1467 cells/mm^3 and HIV viral loads ranged from undetectable (<50 copies per ml) to 11164. Three individuals had active KS at the time of venesection (B257, B792 and S314), the rest were either in remission from KSHV-related neoplasia, or had quiescent (regressing) KS. In addition to the 18 HIV-positive individuals with a history of KSHV-related neoplasia, four HIV-negative, KSHV-negative healthy controls were included in the study to ensure the peptides did not show any cross-

reactivity with other antigens. All study participants' KSHV serostatus at the time of venesection was tested by ELISA. Results are shown in Table 5.7.

	Participant ID	KSHV Serostatus		Comments
		Positive	Negative	
HIV-negative healthy controls	HC-A		N	
	HC-B		N	
	HC-C		N	
	HC-D		N	
HIV-positive individuals with a history of KSHV-related neoplasia	B042		(N)	Borderline negative
	B066	P		
	B257	P		
	B792	P		
	C448	P		
	D592	P		
	D850	P		
	G386	P		
	H501	P		
	H980	P		
	J228	P		
	K331	P		
	M907	P		
	P896	P		
	P940	P		
	S314	P		
	S929	P		
	T541	P		

TABLE 5.7: KSHV serostatus of study participants at time of venesection for testing recognition of HLA-A*0201-restricted KSHV peptides. P = positive; N = negative; (N) = borderline negative.

Seventeen of the 18 HIV-positive participants were KSHV-seropositive, as would be expected given that they all had a history of KSHV-related neoplasia. However, one HIV-positive individual (B042) had an antibody titre that was borderline negative for KSHV seropositivity (0.055 ± 0.001 compared to a cut-off value for positivity of 0.060). This individual had entered remission from KS after

completing a course of systemic chemotherapy less than six months prior to venesection. Thus it was decided to include the individual in the study as a KSHV-positive individual, regardless of this serology result. Such serological assays are recognised to be limited in their sensitivity, particularly in HIV-positive individuals, whose B cells and helper T cells may be severely compromised thus affecting their antibody production. All four HIV-negative, healthy-control individuals were very clearly KSHV-seronegative.

5.2.c T-cell Responses to KSHV CD8 Peptides by IFNγ ELISpot

The 11 ORF28-derived peptides identified as potential HLA-A*0201-restricted epitopes in section 5.1 (see Table 5.3) and nine previously described HLA-A*0201-restricted KSHV CD8 epitopes (including the KSHV control peptide used in the binding assays, $Gb_{492\text{-}500;}$ indicated in Table 5.1) were tested for recognition by T cells in *ex vivo* IFNγ ELISpot assays. Results are shown in Figure 5.5.a, 5.5.b and 5.5.c. A positive response was designated as a response that was greater than 20 spot-forming cells (SFC) per million PBMCs above the background response to media alone and greater than five times the background response. Background responses ranged from 0.0 to 5.0 SFC per million PBMCs.

For the first five individuals tested (B257, B792, H980, K331 and P869; Figure 5.5.a), all KSHV peptides, control peptides [FEC (a pool of influenza EBV and CMV peptides), HIV SLYNTVATL and CMV NLVPMVATV] and a PHA control were used at a final concentration of 5μg/ml. Responses to PHA and FEC were observed in all individuals, a response to SLYNTVATL was seen in one individual

(B257) and responses to NLVPMVATV were observed in three (B257, K331 and P869) out of the four individuals tested (B792 did not respond and H980 was not tested). None of the KSHV peptides (new or previously identified) elicited responses in any of the study participants. KSHV-specific responses are reportedly weak compared to those against antigens from other viruses, and so the concentration of KSHV peptides was increased to 10μg/ml for the next individual tested (P940; Figure 5.5.a) while controls were kept at the same concentration. P940 responded to PHA and FEC, but not to SLYNTVATL, and there were still no responses observed to any of the KSHV peptides. Therefore, for the next 12 individuals tested (B042, B066, C448, D592, D850, G386, H501, J228, M907, S314, S929, T541; Figure 5.5.b.), KSHV peptides were used at 20μg/ml whilst controls were kept at the same concentration.

All 12 of these individuals responded to PHA, FEC and NLVPMVATV. One (G386) out of 11 tested gave a weak response to SLYNTVATL (30.0 SFC per million). Significantly, at this increased peptide concentration, nine of the 12 individuals (B042, B066, C448, D850, G386, H501, M907, S929, and T541) responded to one of the KSHV peptides – the previously identified peptide LVLILYLCV, derived from the late lytic ORF K8.1 that encodes glycoprotein 35/37. No responses were observed to any of the other KSHV peptides by any of the individuals tested. Responses to the K8.1 peptide were weak (21.7 to 113.3 SFC per million; mean = 47.6; median = 36.7), especially compared to responses to CMV NLVPMVATV by these 12 individuals, which ranged from 68.3 to 1383.3 SFC per million (mean = 640.4; median = 635.0). Nonetheless, these data clearly indicate that K8.1 LVLILYLCV represents an immunodominant HLA-A*0201-

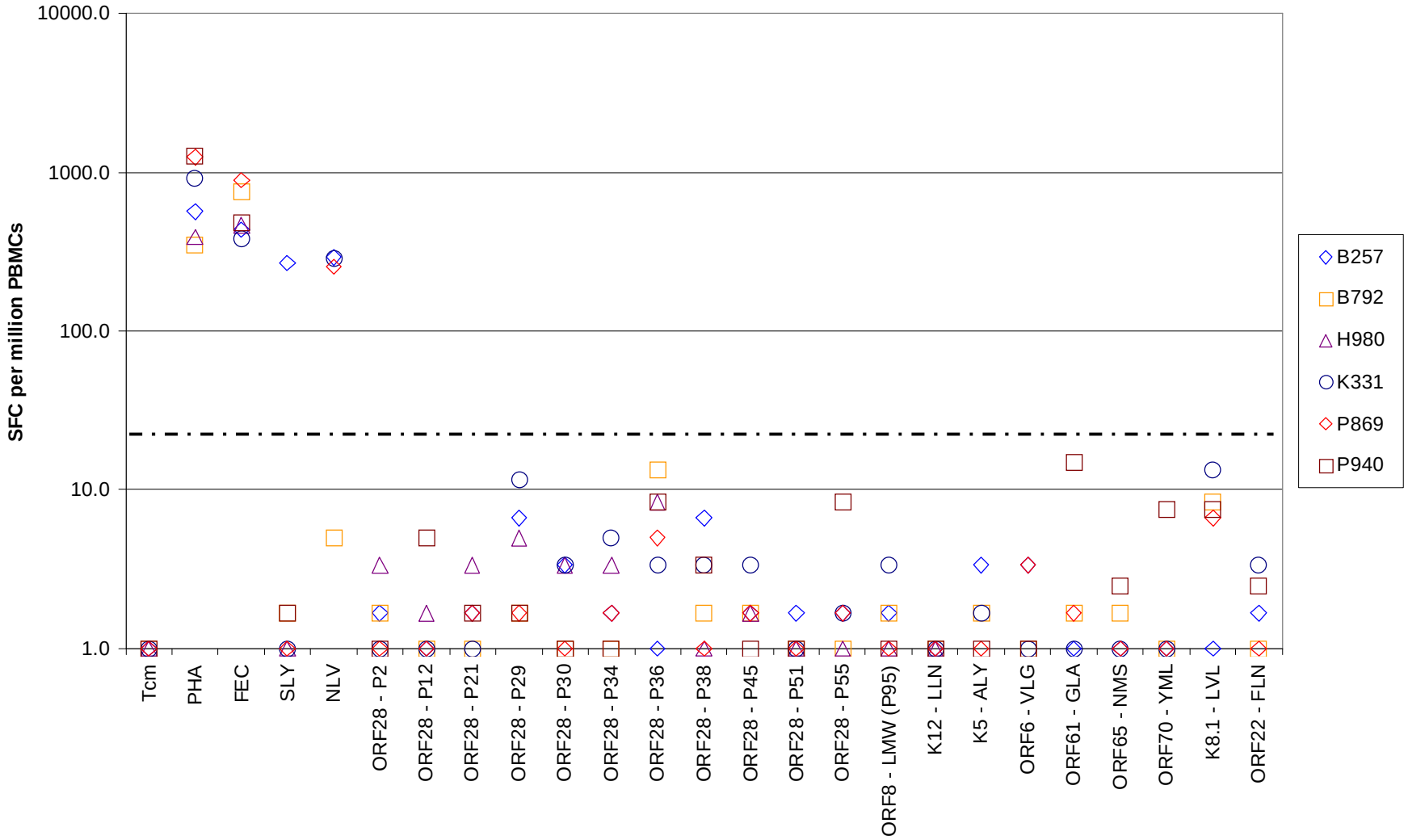


FIGURE 5.5.a: CD8 T-cell responses to A*0201-restricted peptides by HIV-positive, KSHV-positive individuals. PHA, control peptides (FEC, SLY, NLV) and KSHV peptides were all used at 5µg/ml (except for P940; KSHV peptides = 10µg/ml). Responses shown as SFC per million PBMCs with background response to T-cell media (Tcm) alone subtracted. Responses below 1.0 were set to 1.0 to enable plotting of data on a logarithmic scale. Dashed line indicates positive response cut-off value of 20 SFC per million PBMCs.

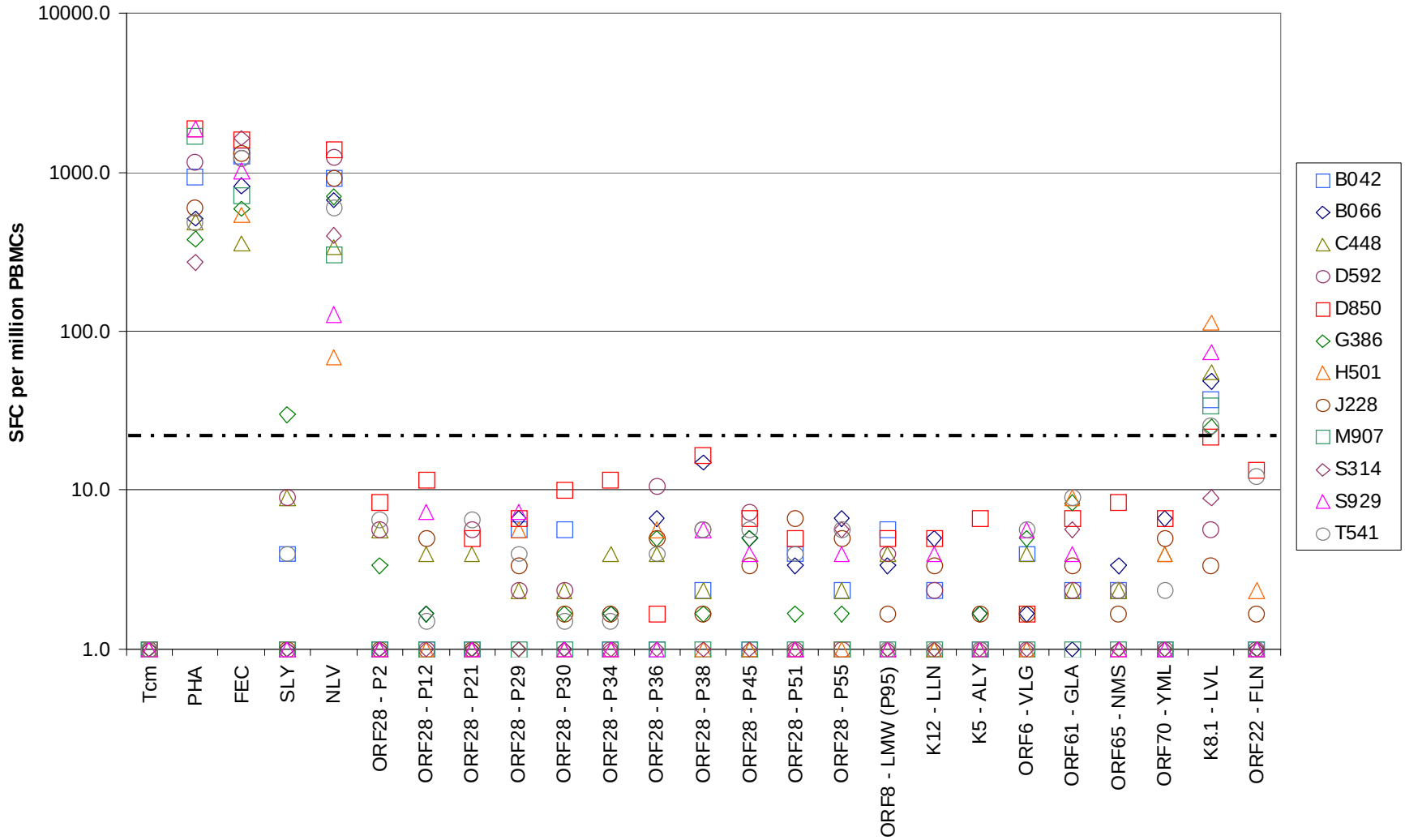


FIGURE 5.5.b: CD8 T-cell responses to A*0201-restricted peptides by HIV-positive, KSHV-positive individuals. PHA and control peptides (FEC, SLY and NLV) and were used at 5μg/ml and KSHV peptides were used at 20μg/ml. Responses shown as SFC per million PBMCs with background response to T-cell media alone (Tcm) subtracted. Responses below 1.0 are set to 1.0 to enable plotting of data on a logarithmic scale. Dashed line indicates positive response cut-off value of 20 SFC per million PBMCs.

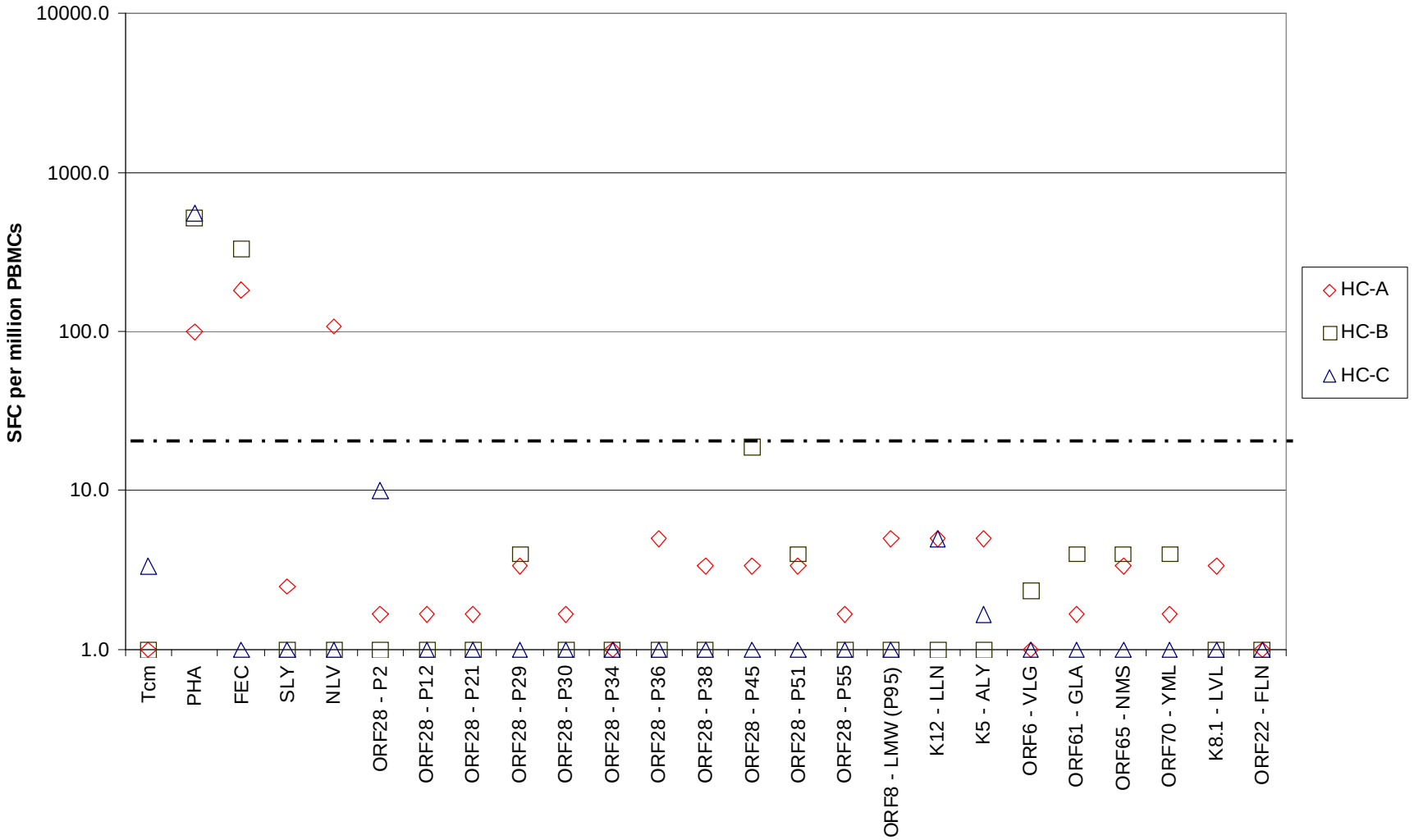


FIGURE 5.5.c: CD8 T-cell responses to A*0201-restricted peptides by HIV-negative, KSHV-negative healthy controls. PHA and control peptides (FEC, SLY and NLV) were used at 5μg/ml and KSHV peptides were used at 20μg/ml. Responses shown as SFC per million PBMCs with background response to T-cell media (Tcm) alone subtracted. Responses below 1.0 are set to 1.0 to enable plotting of data on a logarithmic scale. Dashed line indicates positive response cut-off value of 20 SFC per million PBMCs.

restricted KSHV CD8 epitope as, at a concentration of 20μg/ml, it was recognised by T cells from 75.0% of HIV-positive, KSHV-positive, HLA-A*0201-positive individuals.

Three HIV-negative, KSHV-negative individuals (HC-A, HC-B, and HC-C) were tested for responses to all peptides (control peptides at 5μg/ml and KSHV peptides at 20μg/ml) to ensure there was no cross-reactivity of peptides with other antigens (Figure 5.5.c). The exact HLA-types of these individuals were unknown, although they were all Caucasian and were anecdotally reported to be A*0201-positive from participation in previous immunological studies. All three individuals responded to PHA, and two (HC-A and HC-B) responded to FEC. One (HC-A) responded to CMV NLVPMVATV (108.3 SFC per million PBMCs), confirming that this individual was indeed A*0201-positive. No responses were seen to any of the KSHV peptides by T cells from any of these three individuals, although one (HC-B) had a response to one peptide (ORF28 - P45) that was just below the cut-off value for positivity (19.0 SFC per million PBMCs).

5.2.d Enhancing Responses to KSHV Peptides by using Peptide-pulsed Autologous moDCs as Stimulants in ELISpots

The lack of responses observed in the above ELISpot assays, particularly to published KSHV peptides that have reportedly been recognised by 13% to 32% of HIV-positive individuals in other studies (Table 5.1), was somewhat surprising. However, several other groups have reported difficulty in detecting weak KSHV-specific T-cell responses to peptides using such IFNγ ELISpot assays, particularly

in HIV-positive individuals (Guihot et al., 2006; Wang et al., 2002b). Autologous moDCs pulsed with KSHV peptides have thus been used previously as stimulants in place of peptide solutions to facilitate detection of elusive weak responses (Wang et al., 2002b). This technique was therefore tried with a selection of peptides [ORF28 - P36; ORF28 - P51; ORF8 - LMW (P95); and K8.1 – LVL] to see if any additional responses could be detected. ORF28 - P36 and ORF28 - P51 were selected as these were two of the highest-scoring ORF28 peptides in the MHC binding and off-rate assays. ORF8 - LMW was included as the best-documented A*0201-restricted KSHV epitope, and also as others have detected responses to this peptide using peptide-pulsed moDCs in a similar protocol to this one (Wang et al., 2002b). K8.1 - LVL was selected to examine whether weak responses observed to this peptide in solution could be replicated and enhanced using this protocol. Results from six individuals (D850, J228, M907, S314, T514 and HC-A) are shown in Figure 5.6.

Three of the six individuals (D850, M907, and T514) had shown weak positive responses to the K8.1 peptide in peptide-stimulated ELISpots (21.7, 31.7 and 25.0 SFC per million PBMCs, respectively). Using peptide-pulsed autologous moDCs as stimulants enhanced these responses to 78.3, 111.7 and 138.3 SFC per million PBMCs, respectively. The other three individuals (J228, S314 and HC-A) had not responded to the K8.1 peptide in the original assay, and did not respond to autologous moDCs pulsed with the K8.1 peptide. Likewise, none of the six individuals responded to any of the other peptides (ORF28 - P36, ORF28 - P51, ORF8 - LMW) in the original assay and they did not respond to moDCs pulsed with these peptides. This gives confidence that using this technique does not generate false positive responses. It was thus concluded that pulsing moDCs is a useful way

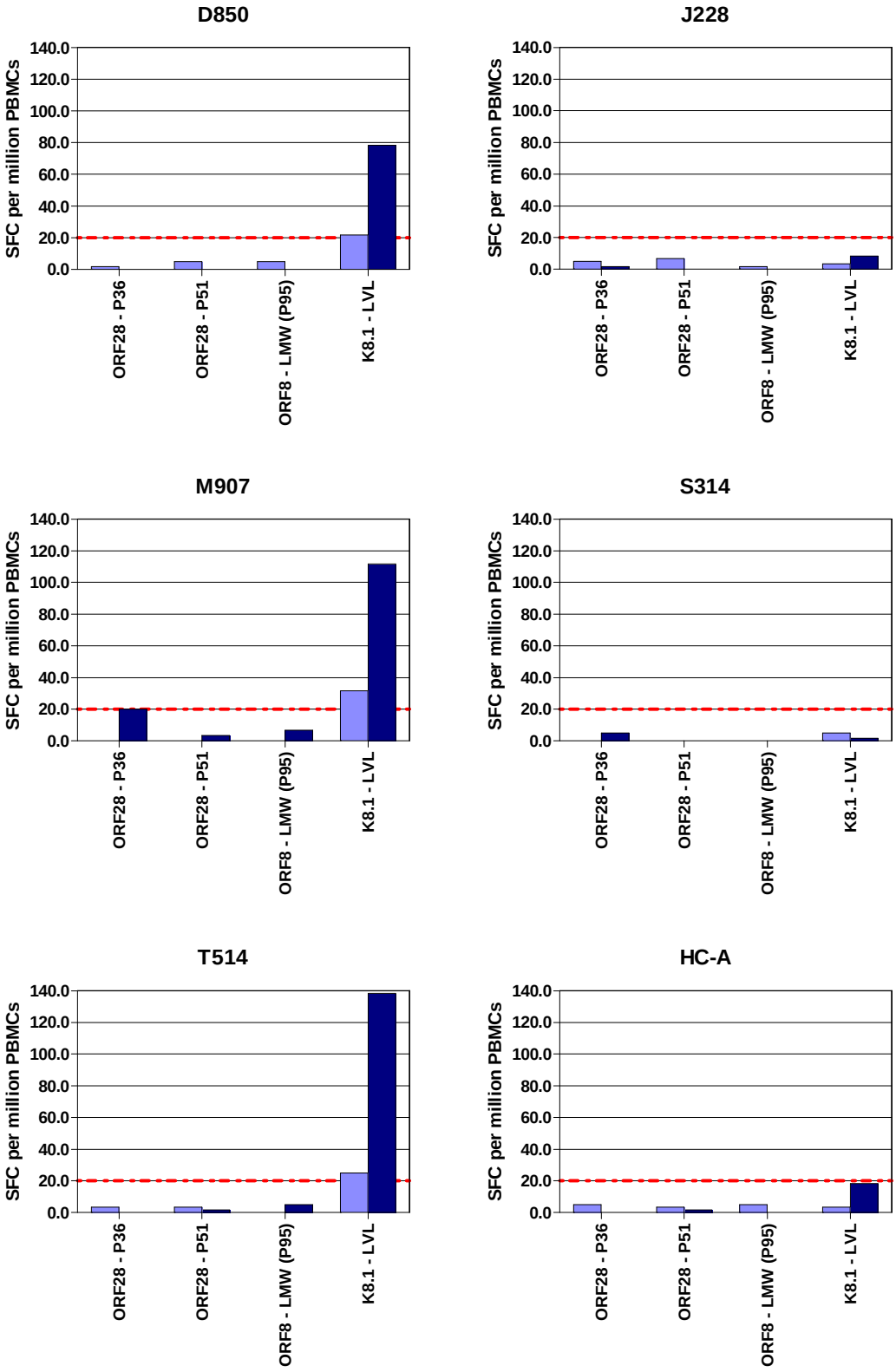


FIGURE 5.6: T-cell responses to HLA-A*0201-restricted peptides in IFNγ ELISpot assays using peptide solutions (pale blue bars) or peptide-pulsed autologous moDCs (dark blue bars) as stimulants. Responses shown are SFC per million PBMCs after the background response to T-cell media alone (peptide solution assays) or moDCs alone (peptide-pulsed moDC assays) had been subtracted. Dashed red line indicates positive cut-off value of 20 SFC per million PBMCs.

to enhance weak responses, and can confirm the validity of low positive responses (for example, those below 50 SFC per million PBMCs) observed in peptide-stimulated assays.

A limitation of this technique is, however, that far fewer peptides can be investigated in this manner, due to the number of PBMCs needed to generate sufficient numbers of moDCs for use with each different peptide. It is thus possible that, had it been feasible to test a broader range of peptides, some of those that elicited responses close to the positive cut-off value of 20 SFC per million may have proved to be positive. For example 16.7 and 15.0 SFC per million PBMCs were observed in response to ORF28 - P38 in D850 and B066 respectively, and ORF61 - GLA elicited a response of 15.0 SFC per million in P940.

5.2.e Peptides Derived from KSHV ORF36 and ORF37

As, disappointingly, no responses were observed to any of the ORF28 peptides in any of the individuals tested, it was decided to test a selection of additional peptides derived from ORF36 and ORF37, the other two ORFs within the late lytic gene pool that was frequently recognised in the lentivirus-based screen for immunogenic KSHV ORFs. The size and complexity of these two ORFs meant that performing MHC binding and denaturation assays on peptides spanning the entire ORF sequences (as was done for ORF28 in section 5.1) would have been impractical. The data and *in silico* analysis described in section 5.1 indicated that the use of computer algorithms to identify candidate T-cell epitopes from within an ORF is still very much a technology in its infancy, with different algorithms often returning

different results and no one algorithm providing definitive solutions. However, when multiple algorithms were used and potential epitopes were selected based on a consensus of results from all algorithms, this seemed to correlate well with peptides that performed well in MHC-binding and denaturation assays. Thus a combination of three computer algorithms (IEDB; SYFPEITHI and HLA_BIND; see section 5.1.e) was used to identify three good candidate HLA-A*0201-restricted epitopes from within ORF36 and ORF37. Because neither ORF36 nor ORF37 encode unique viral proteins with respect to their amino acid sequence, each potential epitope was also run through NCBI's 'blastp' tool, to ensure there was no sequence similarity to potential antigens from other viruses. The six selected candidate epitopes are shown in Table 5.8 with their scores from the three computer algorithms.

The six additional peptide were tested at a concentration of 20μg/ml for responses by T cells from nine of the HIV-positive, KSHV-positive, HLA-A*0201-positive individuals recruited to this study (B792, C448, D592, D850, G386, K331, P869, S314 and S929) and three HIV-negative, KSHV-negative healthy controls (HC-A, HC-C and HC-D). Results are shown in Figure 5.7. No responses were observed to any of the new peptides by any of the controls. Disappointingly, neither were any responses observed to any of the peptides by any of the KSHV-positive individuals.

Peptide	Amino Acid Residue									Immune Epitope Database Score	SYFPEITHI Score	HLA_BIND Score	Combined Algorithm Score
	1	2	3	4	5	6	7	8	9				
Optimal HLA-A*0201 'Motif' Residues		L							L				
		M							V				
						V							
Influenza	G	I	L	G	F	V	F	T	L	18.8	30	550.9	+++
HIV gag	S	L	Y	N	T	V	A	T	L	16.0	31	157.2	+++
CMVpp65	N	L	V	P	M	V	A	T	V	32.1	30	160.0	+++
ORF36 - YLG	Y	L	G	F	M	I	P	R	V	5.8	26	735.9	+++
ORF36 - GLM	G	L	M	A	A	V	S	F	L	10.9	26	999.9	+++
ORF36 - VMT	V	M	T	Q	I	L	S	A	V	34.3	23	196.4	+++
ORF37 - YLV	Y	L	V	D	T	L	D	G	L	6.7	28	454.1	+++
ORF37 - ALK	A	L	K	D	F	F	Y	S	I	14.0	24	10.8	+++
ORF37 - RLG	R	L	G	S	P	K	Y	Y	I	211.3	20	57.4	+++

TABLE 5.8: Candidate HLA-A*0201-restricted CD8 epitopes from within ORF36 and ORF37 identified using the combined results of three computer algorithms for epitope prediction.

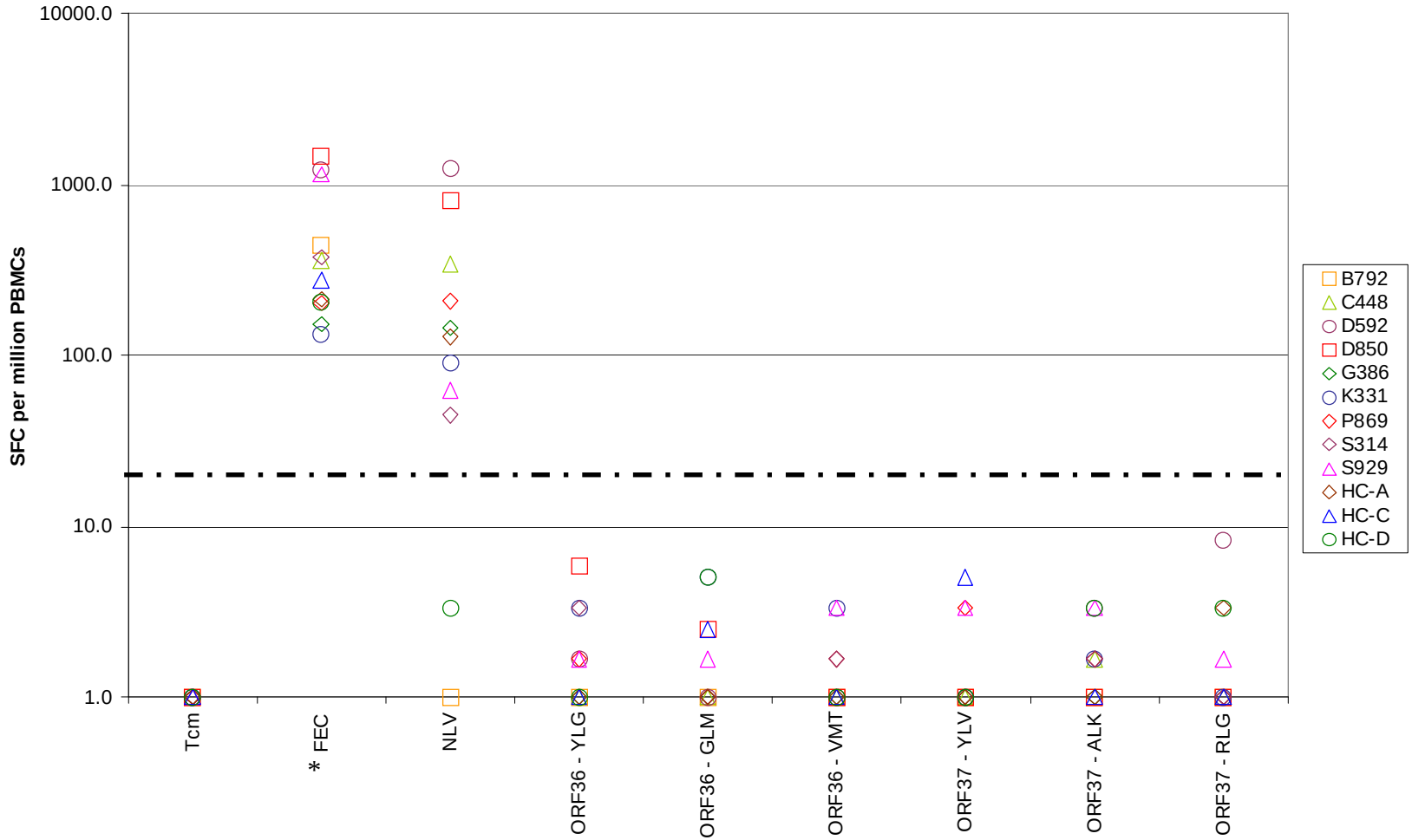


FIGURE 5.7: CD8 T-cell responses to ORF36 and ORF37 A*0201-restricted peptides by HIV-positive, KSHV-positive individuals and HIV-negative, KSHV-negative healthy controls. Control peptides FEC (* indicates that for HC-C, PHA was used in place of FEC as this individual did not respond to FEC in previous assays) and NLV were used at 5μg/ml and KSHV peptides were used at 20μg/ml. Responses shown as SFC per million PBMCs with background response to T-cell media (Tcm) alone subtracted. Responses below 1.0 are set to 1.0 to enable plotting of data on a logarithmic scale. Dashed line indicates positive response cut-off value of 20 SFC per million PBMCs.

5.3 The Use of Pentamers to Determine the Memory Phenotypes and Functions of Virus-specific T cells

One of the main goals of the studies described in sections 5.1 and 5.2 was to identify an immunodominant epitope (either a novel late lytic epitope, or one of several previously described epitopes) that would be a suitable target for investigations utilising pentamers to enumerate KSHV-specific T cells and to determine their memory phenotypes and functional properties. The results of these studies identified one clear immunodominant epitope: LVLILYLCV from the late lytic ORF, K8.1. Disappointingly, when synthesis of a pentamer containing this peptide was attempted, the peptide proved to be too hydrophobic for the pentamer to be synthesised. Unfortunately, as none of the other KSHV-derived peptides included in this study had elicited responses in any of the study participants, there were no other suitable KSHV peptides against which to attempt the synthesis of a corresponding pentamer. It was thus not possible to explore the phenotypes and functions of KSHV-specific CD8 T cells as had been hoped, although much work had been done to refine the technique and to establish suitable protocols using control pentamers directed against HIV SLYNTVATL and CMV NLVPMVATV. This work is briefly described below.

5.3.a Memory Phenotypes of Virus-specific CD8 T cells

Pentamers specific for the immunodominant HLA-A*0201-restricted CMV peptide NLVPMVATV (NLV) were used in conjunction with antibodies against the T-cell

differentiation markers CCR7 and CD45RA to enumerate NVL-specific cells and to determine the memory phenotype of NLV-specific CD8 T cells. An example of staining and gating strategy are shown in Figure 5.8.

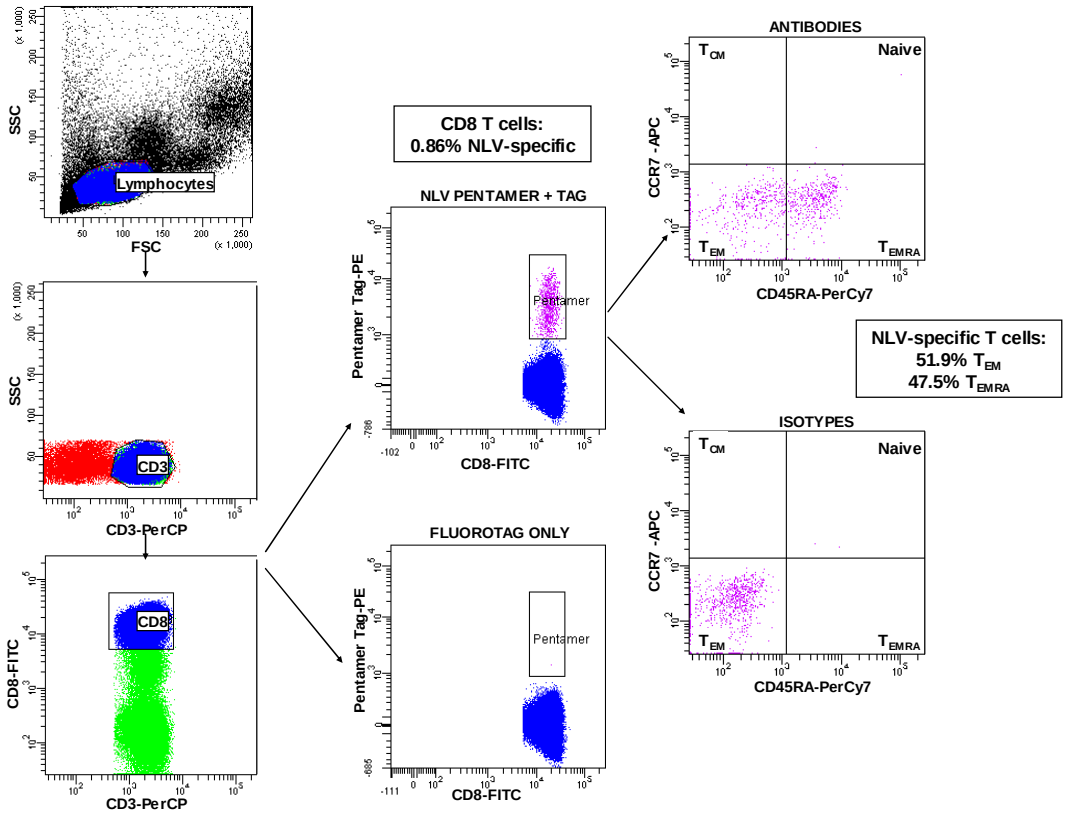


FIGURE 5.8: Example of pentamer staining used in conjunction with staining for cell-surface markers to elucidate the memory phenotype of virus-specific CD8 T cells. Shows staining performed on PBMCs from an HIV-positive individual (C448). Gates are set on the live lymphocyte population, then the CD3-positive and CD8-positive T cells, and finally on the NLV pentamer-specific population. Staining with antibodies against the lymphoid homing marker CCR7 and the 'RA' isoform of CD45 allows different CD8 T-cell memory sub-populations to be identified.

Enumeration and phenotyping of NLV-specific CD8 T cells was performed using PBMCs from seven individuals who responded to the NLV peptide in the ELISpot assays described in section 5.2. Six of these were HIV-positive (B257, C448, J228, K331, M907 and P869) and one was HIV-negative (HC-A). Results are summarised in Table 5.9.

	Participant ID	% CD8 T cells that are NLV-specific	% NLV-specific T cells that are	
			T_{EM}	T_{EMRA}
HIV-positive	B257	0.30	43.8	55.3
	C448	0.86	51.9	47.5
	J228	0.43	79.3	19.8
	K331	0.11	78.6	19.6
	M907	0.72	7.0	92.0
	P869	1.19	33.0	66.8
HIV-negative	HC-A	0.23	60.6	39.4
Mean		0.55	50.6	48.6
Median		0.43	51.9	47.5

TABLE 5.9: Enumeration of CD8 T cells specific for CMV NLVPMVATV and determination of memory phenotype of CMV-specific T cells.

The proportion of CD8 T cells that were NLV-positive ranged from 0.23% to 1.19%, with a mean of 0.55%. These values are similar to those reported by other groups (Stone et al., 2005). Although there was some variation between individuals, on average, 50.6% of NLV-specific T cells had a T_{EM} phenotype and 48.6% had a T_{EMRA} phenotype. Again, this is in keeping with observations by other groups (Champagne et al., 2001).

One of these seven individuals (B257) also showed responses to the immunodominant HLA-A*0201-resricted HIV epitope SLYNTVATL in the ELISpot assays described in section 5.2. It was therefore possible to make a comparison of the memory phenotypes of CMV- and HIV-specific CD8 T cells from this individual using pentamers directed against NLVPMVATV and SLYNTVATL. Results from this experiment are shown in Figure 5.9.

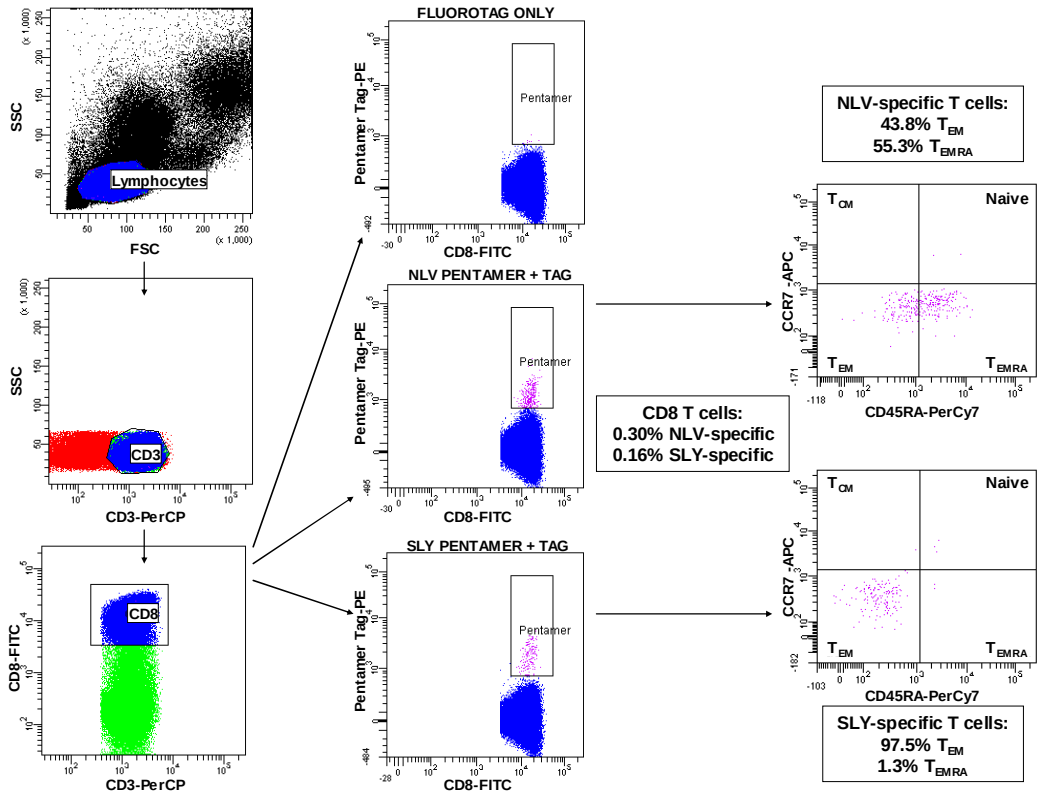


FIGURE 5.9: Comparison of memory phenotypes of CD8 T cells specific for CMV or for HIV. Results shown are from one individual, B257.

In this individual, CMV-specific CD8 T cells were 43.8% T_{EM} cells and 55.3% T_{EMRA} cells, whereas HIV-specific T cells were 97.5% T_{EM} cells and 1.3% T_{EMRA} cells. This is in keeping with reports that HIV-specific CD8 T cells show defective differentiation from T_{EM} cells to T_{EMRA} cells, a phenotype that is thought to contribute towards the pathogenesis of HIV (Champagne et al., 2001).

5.3.b Functions of Virus-specific CD8 T cells

The first step towards using pentamers to investigate the functions of virus-specific CD8 T cells was to establish a suitable antibody panel. A six-colour flow cytometer allowed simultaneous investigation of three cytokines (IFNγ, TNFα and IL2).

However, as antibodies against these cytokines are only commercially available conjugated to a limited number of fluorochromes (FITC, PE, APC and PeCy7) it was first necessary to find CD3 and CD8 antibodies conjugated to APCCy7 and PerCP that could be used in conjunction with the pentamers. Certain CD3 and CD8 antibody clones inhibit pentamer binding and none that had previously been tested were available conjugated to the correct fluorochromes. Thus an experiment was performed to ensure that the antibodies CD3-APCCy7 (clone SK7; BD Biosciences) and CD8-PerCP (clone HIT3; Abcam) did not inhibit pentamer binding. Results are shown in Figure 5.10.

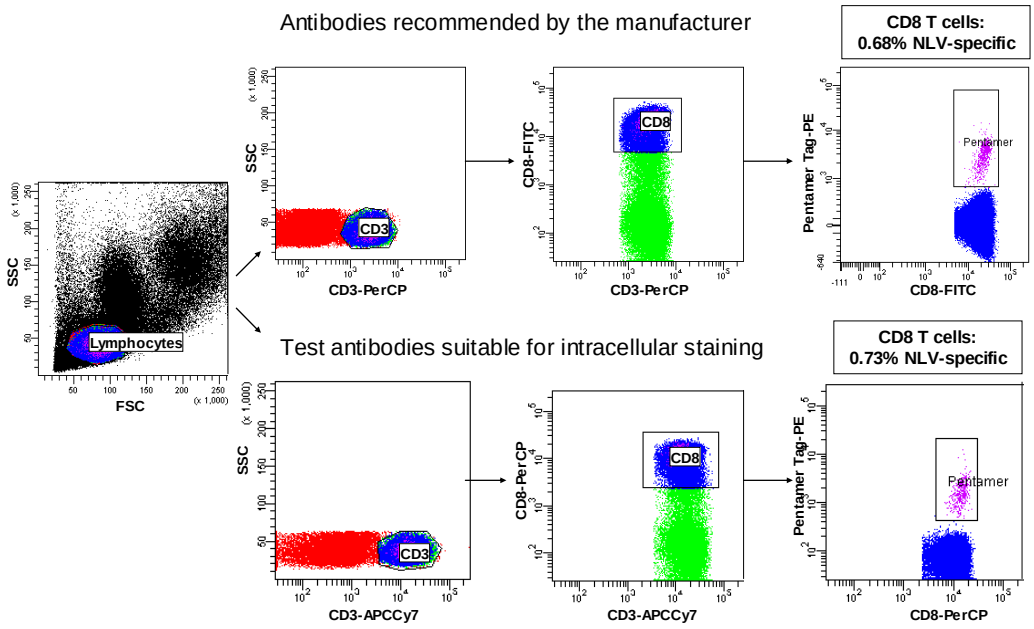


FIGURE 5.10: Identification of antibodies against CD3 and CD8 that were suitable for use in conjunction with intracellular staining and did not inhibit pentamer binding.

With a suitable antibody panel established, several attempts were made at performing pentamer staining in conjunction with staining for the production of the

intracellular cytokines IFNγ, TNFα and IL2. However, in each case no cells positive for the pentamer were observed after the 16-hour intracellular staining protocol, despite pentamer-positive cells being detectable at 0 hours using the standard cell-surface staining protocol. By sequentially omitting each of the steps involved in the intracellular protocol, it was established that the pentamer staining was lost upon permeabilisation of the cells, after the overnight stimulation for cytokine production and before staining for the expression of the cytokines. On the manufacturer's recommendation the permeabilisation and fixation steps were split from one step (using a Cytofix/Cytoperm kit available from BD) to two steps, using manually prepared fixation and permeabilisation buffers (see Materials and Methods section 2.8.b). Nonetheless, the same loss of pentamer staining was observed. An example is shown in Figure 5.11.

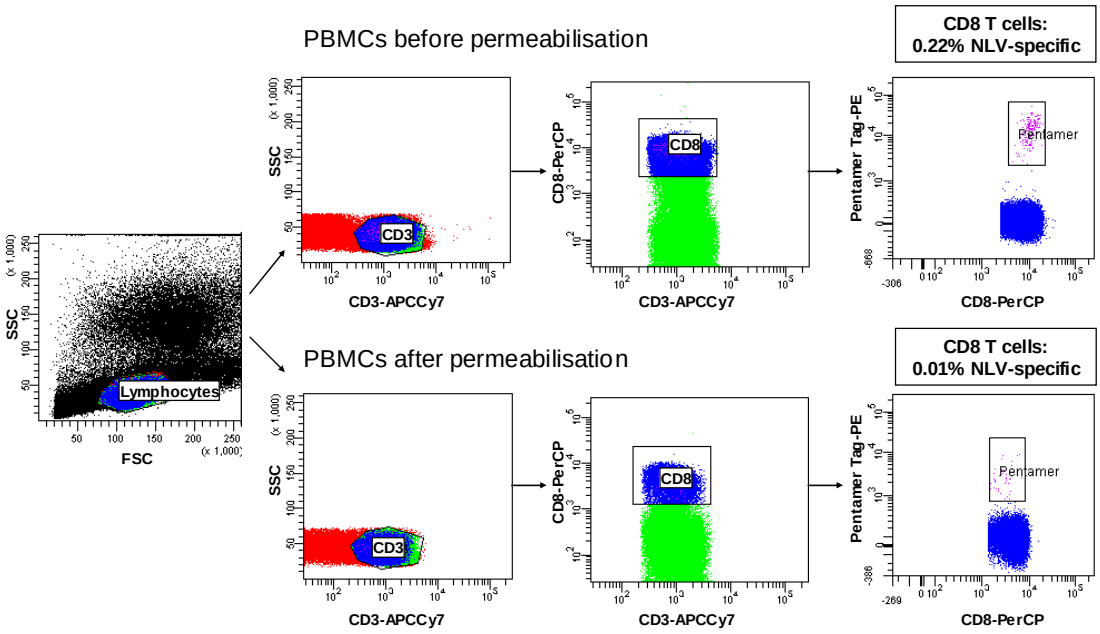


FIGURE 5.11: Loss of pentamer staining upon permeabilisation of PBMCs for intracellular staining.

It was thus concluded that, in my hands, this protocol for pentamer staining in conjunction with staining for the production of intracellular cytokines did not appear to be a very robust method for investigating the functions of virus-specific CD8 T cells.

5.4 T-cell Memory Phenotypes in KSHV Infection

In addition to different virus-specific T cells displaying distinct patterns of memory phenotype, different viral infections are also associated with varying representation of memory T-cell phenotypes (i.e. naïve, central memory, effector memory and terminally differentiated effector memory) in the whole circulating T-cell population. In some viral infections this varies further during the course of chronic infection and manifestation of disease. Thus, the phenotypes of the whole circulating T-cell population in individuals at different stages of KSHV infection were investigated using whole-blood staining.

5.4.a Study Participants

The representation of T-cell memory subsets in circulating T-cell populations was examined in 15 HIV-positive individuals in remission from KSHV-related neoplasm and nine HIV-positive individuals with active KS, and compared to representation of T-cell memory subsets in 11 HIV-negative, KSHV-negative control individuals. All individuals tested were male and aged between 24 and 73 years-old at venesection, with a mean age of 42 years. HIV-positive individuals had

CD4 counts from 159 to 811, with a mean of 484. Mean CD4 counts were lower in those with active KS than those in remission (397 compared to 537, respectively) although this did not reach statistical significance ($p = 0.08$; student T test). All HIV-positive individuals were on HAART, apart from one individual with active KS. HIV viral loads were generally undetectable with the exception of one individual in remission from KS and two with active KS who had HIV viral loads between 50 and 150, and two further individuals with active KS who had higher HIV viral loads of 384 and 12599. HIV-positive individuals in remission from KSHV-related neoplasm had had either KS (n = 8) or MCD (n = 7). None of the study participants were receiving chemotherapy at the time of venesection. Characteristics of HIV-positive individuals are shown in detail in Table 2.13 and are summarised in Table 5.10.

Group	Group Description	Number	Mean Age (Range)	Mean CD4 Count (Range)	HIV Viral Load Range
1	HIV-/KSHV- Healthy Controls	11	32 (24 – 60)	Nt	Nt
2	HIV+/KSHV+; history of KSHV-related neoplasia, in remission	15	47 (30 – 74)	555 (188 – 811)	<50 – 69
3	HIV+/KSHV+; Active KS	9	46 (34 – 71)	397 (159 – 575)	<50 – 12599

TABLE 5.10: Summary of characteristics of groups of study participants for whole-blood T-cell phenotyping. Nt = not tested**.**

5.4.b Differentiation of T-cell Memory Subsets in the Whole Circulating T-cell Population

The lymphoid homing marker, CCR7, and the 'RA' isoform of CD45 were used to distinguish naïve, central memory (T_{CM}) and effector memory (T_{EM}) CD8 and CD4 T-cell subpopulations, and the terminally differentiated effector memory CD8 T-cell subpopulation (T_{EMRA}). The representation of each of these subpopulations was assessed in each group of study participants. Gating strategy is shown in Figure 5.12.

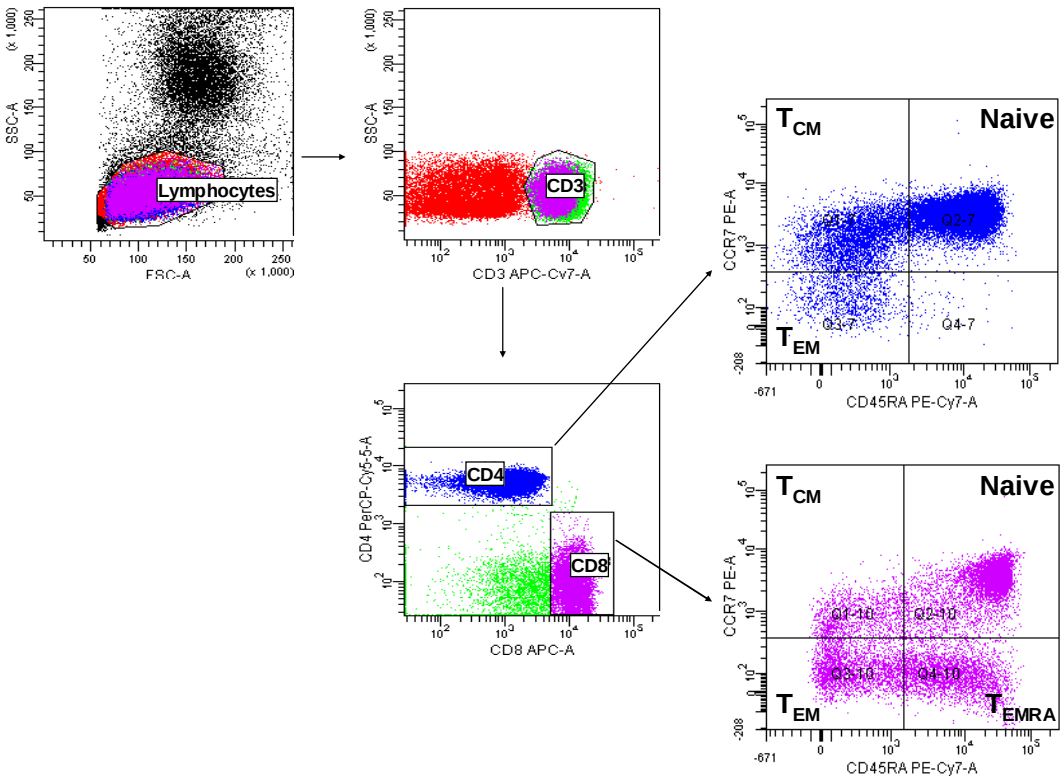


FIGURE 5.12: Gating strategy for whole-blood T-cell phenotyping. The lymphoid homing marker CCR7 and the RA isoform of CD45 were used to distinguish CD8 and CD4 memory T-cell subpopulations.

5.4.c Representation of T-cell Memory Subsets During Chronic KSHV Infection and KS

Figures 5.13.a and 5.13.b show the representation of CD8 and CD4 T-cell memory subsets in each group of study participants respectively. The proportion of naïve CD8 T cells was dramatically reduced in HIV-positive individuals compared to HIV-negative individuals (means of 12.3% and 38.2%, respectively; $p<0.05$; Mann-Whitney test; Figure 5.13.a), as has been described elsewhere (Chen et al., 2001). No differences were observed between the proportion of T_{CM} CD8 T cells between HIV-positive individuals and HIV-negative individuals. However, there was a slight enrichment in the proportion of T_{CM} CD8 T cells in HIV-positive individuals in remission from KSHV-related neoplasm (mean = 13.2%) as compared to those with active KS (mean = 6.7%) that reached statistical significance ($p<0.05$; Mann-Whitney Test; Figure 5.13.a). A small study of iatrogenic KS patients has previously shown that conversion of their immunosuppressive drug regime from calcineurin inhibitors to sirolimus led to increased proportions of circulating T_{CM} CD8 T cells, concurrent with KS regression [(Barozzi et al., 2008); see Introduction section 1.3.a *The CD8 T-cell Response Against KSHV*]. Thus, the restoration of a central memory CD8 T-cell subpopulation may play a role in control of KSHV infection in both individuals with HIV and transplant recipients on immunosuppressive drugs. No significant differences were observed in the representation of T_{EM} and T_{EMRA} CD8 T-cell subpopulations between any of the three groups of individuals (Figure 5.13.a).

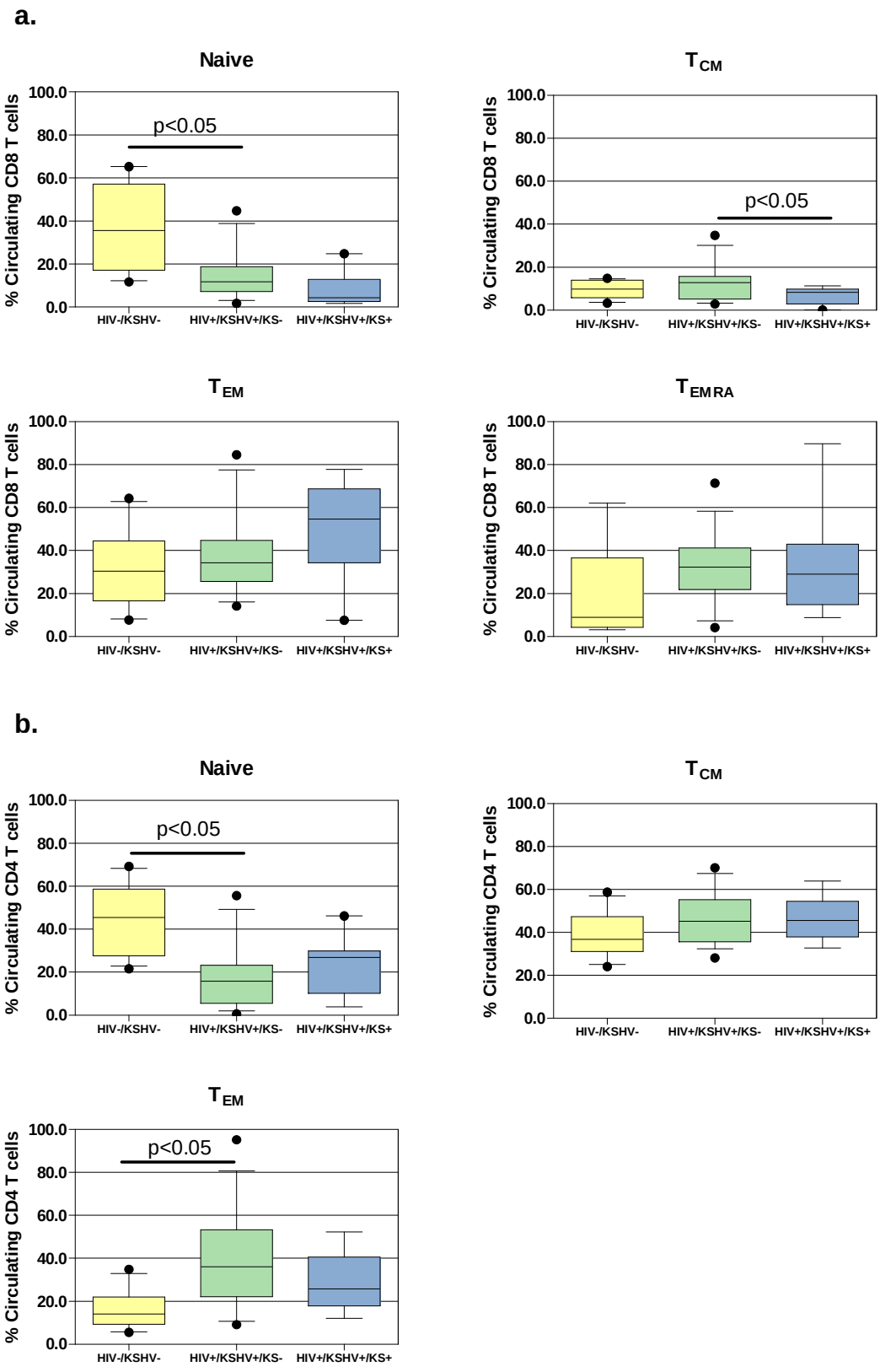


FIGURE 5.13: Representation of memory subsets in the whole circulating CD8 (a.) and CD4 (b.) T-cell populations in healthy controls (yellow bars), individuals with chronic KSHV infection (green bars) and individuals with KS (blue bars). Medians, inter-quartile range, and 10th and 90th percentiles are shown. Dots indicate out-liers.

The proportion of naïve CD4 T cells was also dramatically reduced in HIV-positive individuals as compared to HIV-negative individuals (means of 20.4% and 45.4%, respectively; $p<0.05$, Mann-Whitney test; Figure 5.13.b.), as has been previously described (Roederer et al., 1995). There was also a concurrent enrichment in the proportion of T_{EM} CD4 T cells in HIV-positive individuals (mean = 35.2%) as compared to HIV-negative individuals (mean = 16.1; $p<0.05$, Mann-Whitney test; Figure 5.13.b.), as described elsewhere (Roederer et al., 1995). No significant differences were observed in the proportion of T_{CM} CD4 T cells between any of the three groups of individuals. No significant differences were observed in the representation of any of the CD4 T-cell memory subsets in individuals in remission from KSHV-related neoplasm compared to individuals with KS (Figure 5.13.b.). CD4 effector memory T cells do not generally reexpress CD45RA (Sallusto et al., 2004), and so no CD4 T_{EMRA} population was observed.

5.5 Summary

MHC binding assays and computer algorithms were used to identify potential new HLA-A*0201-restricted epitopes from within the late lytic KSHV ORFs, ORF28, ORF36 and ORF37. These were then tested for recognition by T cells from 18 HIV-positive, KSHV-positive, HLA-A*0201-positive individuals alongside nine previously identified A*0201-restricted KSHV epitopes from ORFs expressed across the spectrum of the KSHV replication cycle. The immunodominant A*0201-restricted epitopes from HIV GAG (SLYNTVATL) and CMV pp65 (NLVPMVATV) were also included in the assays for comparison. Only two out of the 17 individuals tested (11.8%) responded to HIV SLYNTVATL, a result that

may be unexpected for an epitope widely classified as ‘immunodominant’. However, other groups have reported similarly low frequencies of recognition for this epitope, particularly among individuals who have been receiving HAART for extended periods of time (Lambert et al., 2006). A more robust immunodominant response was observed for CMV NLVPMVATV, which was recognised by 15 of the 16 individuals tested (93.8%), in keeping with reports by other groups (Lambert et al., 2006).

Disappointingly, none of the potential new A*0201 epitopes identified from within ORF28, ORF36 and ORF37 were recognised by T cells from any of the 18 individuals tested. Of note, however, no other responses were observed by any of these individuals to eight out of nine previously described KSHV epitopes, all of which could be expected to elicit responses in 13% to 32% of HIV-positive individuals based on findings from previous studies (Table 5.1). This was a surprising result, and is perhaps indicative of the difficulties associated with searching for T-cell responses to a virus know to elicit comparatively weak responses in individuals with compromised immune systems.

Interestingly one peptide – LVLILYLCV, from the late lytic, glycoprotein-encoding ORF, K8.1 – did elicit a response by T cells from nine of the 12 individuals tested (75.0%) when used at a concentration of 20μg/ml. This concurs with the original data collected by Bourboulia *et al.* which indicated that LVLILYLCV could be the most frequently recognised of all known A*0201-restricted KSHV epitopes, with responses seen in 50% of individuals tested [(Bourboulia et al., 2004); Table 5.1]. The limitations of this original study were a small sample number (eight

individuals), and a lack of comparison to responses to other known KSHV CD8 epitopes by the same individuals. The new data described in this thesis thus provides further evidence for K8.1 LVLILYLCV representing an immunodominant A*0201-restricted KSHV epitope, arguably the first such epitope to be described. This information may have important implications for future immunological studies of KSHV, and may have a clinical application, for example in the design of immunotherapies for KSHV-related malignancies or potentially even a KSHV vaccine.

Disappointingly, this one peptide that consistently and reliably elicited responses in KSHV-positive individuals proved to be too hydrophobic for a pentamer to be synthesised that would enable fluorescent labelling of KSHV-specific T cells. It was thus not possible to use pentamer technology to enumerate KSHV-specific T cells and to elucidate their memory phenotypes and functional properties, as had been initially proposed. Experiments that were performed to establish such assays are however described. Pentamers directed against CMV NLVPMVATV- and HIV SLYNTVATL-specific T cells were used in conjunction with staining for cell-surface markers to demonstrate a dramatic difference in the memory phenotypes of CMV- and HIV-specific CD8 T cells. NLVPMVATV-specific T cells were predominantly T_{EM} or T_{EMRA} cells and were, on the whole, fairly evenly distributed between these two subpopulations. By contrast, SLYNTVATL-specific T cells were overwhelmingly of a T_{EM} phenotype, illustrating the defective differentiation of HIV-specific T cells towards a T_{EMRA} phenotype that has been previously described (Champagne et al., 2001). Pentamer staining in conjunction with staining for intracellular cytokine production was less successful, as the permeabilisation step

required for this protocol appeared to interfere with either pentamer binding or stability. Thus this may not be the most useful technique for investigations into the functional properties of virus-specific T cells.

In the absence of a suitable pentamer for investigating the memory phenotypes of KSHV-specific T cells, the representation of T-cell memory subsets by the whole circulating T-cell population was examined in chronic KSHV infection and KS. No differences were observed in the representation of CD4 T-cell memory subpopulations. However, the central memory CD8 T-cell subpopulation was slightly reduced in individuals with KS compared with those in remission, indicating that restoration of this T-cell subpopulation may be important for the control of KSHV infection.

CHAPTER 6: Discussion

The aim of this thesis was to achieve a better understanding of the T-cell response against KSHV. To this end, a novel methodological approach was developed to investigate the targets of CD4 and CD8 KSHV-specific T cells. Monocyte-derived dendritic cells were transduced with a library of lentiviral expression vectors encoding KSHV ORFs and then cocultured with CFSE-stained autologous T cells. In this way, pools of KSHV ORFs that elicited T-cell proliferation responses were identified. Experiments were then performed to identify potential epitopes from within these ORFs and these potential epitopes were tested for recognition by T cells from KSHV-positive individuals alongside nine previously described KSHV epitopes in order to investigate patterns of immunodominance. Finally, the use of pentamers as tools for the elucidation of the memory phenotypes and functional properties of virus-specific T cells was investigated.

On embarking on this project, my hypothesis was that the KSHV gene products that elicit the strongest T-cell responses were those that are expressed early in the viral lytic cycle, as the T-cell response against EBV (the most closely related human herpesvirus to KSHV) is skewed towards immediate-early EBV gene products (Rickinson and Moss, 1997; Landais et al., 2005a; Pudney et al., 2005). It has been suggested that this pattern observed in the T-cell response against EBV is attributable to the fact that early in the viral lytic cycle is the time when it is most important for the host to keep the virus in check in order to prevent uncontrolled

viral replication. It seemed reasonable to hypothesise that the KSHV-specific T-cell response might display a similar pattern.

Preparation for and Design of a Lentiviral-Based Screen for Immunogenic KSHV ORFs

To test my hypothesis, a novel approach using a pre-existing library of lentiviral expression vectors to transduce moDCs and then stimulate autologous T cells was proposed. This approach offered a number of advantages. Lentiviral vectors have been shown to efficiently transduce moDCs and to integrate into the cellular genome resulting in sustained transgene (Chinnasamy et al., 2000; Schroers et al., 2000; Dyall et al., 2001). Lentiviral-transduced moDCs endogenously process the antigenic protein, and so no prior knowledge of the optimal epitope or HLA-restriction was required. Furthermore, the pre-existing library already included 26 KSHV ORFs, representing approximately 30% of the entire KSHV genome. This system therefore enabled simultaneous screening of a large number of KSHV ORFs for immunogenicity– far more than would have been possible using artificial peptides.

By including both known immunogenic ORFs and ORFs that had not been previously investigated for immunogenicity in the screen, it was hoped to determine the relative importance of documented antigens and also to identify some novel targets of both the CD8 and CD4 T-cell responses against KSHV. Therefore, it was decided first to clone five additional known or putative immunogenic KSHV ORFs into the pSIN lentiviral vector for inclusion in the screen alongside the 26 ORFs

already in the pre-existing library. The novel genes that were selected were ORF8, ORF57, ORF59, ORF61 and ORF65.

ORF8, ORF57, ORF61 and ORF65 are all documented targets of the KSHV-specific CD8 T-cell response (Wang et al., 2000; Wang et al., 2001b; Wang et al., 2002b; Bourboulia et al., 2004; Woodberry et al., 2005; Guihot et al., 2006; Lambert et al., 2006; Bihl et al., 2007b; Guihot et al., 2008). Thus, they were cloned in to the lentiviral vector and included in the library in order to investigate the immunodominance of these CD8 antigens and also to discover whether they additionally yielded CD4 T-cell epitopes.

ORF57 is an immediate-early lytic gene that is involved in controlling viral gene expression through post-transcriptional regulation (Gupta et al., 2000). It is a homologue of the EBV gene BMLF1, although the amino-acid sequences of these genes share only minimal sequence similarity. BMLF1 is one of the immunodominant targets of the EBV-specific CD8 T-cell response and has also been shown to elicit CD4 T-cell responses (Landais et al., 2005a; Hislop et al., 2007). ORF61 is an early lytic gene that codes for the large ribonucleotide reductase that is required for DNA replication. It is a homologue of the ORF61 gene of the mouse γ2-herpesvirus, murine herpesvirus 68 (MHV68), which is a target of the MHV68-specific T-cell response (Stevenson et al., 1999). ORF65 is also an early lytic gene that codes for the small capsomer-interacting protein that decorates the outside of KSHV capsids and is essential for capsid assembly (Nealon et al., 2001; Perkins et al., 2008). It lacks sequence similarity with its structural counterparts from other herpesviruses (Nealon et al., 2001) and is highly antigenic,

eliciting strong antibody responses frequently used in serological assays for the detection of KSHV infection (Simpson et al., 1996; Pau et al., 1998). ORF57, ORF61 and ORF65 were all successfully cloned into the lentiviral vector without the incorporation of any amino-acid-substituting mutations.

ORF8 is an early lytic gene that codes for glycoprotein B (gB). It is a homologue of gBs from other herpesviruses, including EBV and MHV68. However, KSHV gB has several unique features. Unlike EBV and MHV68 gBs, it is incorporated into both the virion envelope and the membrane of infected cells (Akula et al., 2001). KSHV gB also contains a unique arginine-glycine-aspartate (RGD) motif, which binds to the cell surface and promotes cell entry by activating intracellular signalling pathways involved in endocytosis and cytoskeleton restructuring (Wang et al., 2003; Sharma-Walia et al., 2004). The expression pattern and function of KSHV gB makes it an attractive target for host immunosurveillance, identifying infected cells and flagging up resurgences in active viral lytic replication. ORF8 was successfully cloned into the lentiviral vector, but contained a single point mutation that resulted in an amino acid substitution. This was at base pair 1199 and converted amino acid 400 from a valine to an alanine. This was carefully noted, but as it lay outside of the well-documented gB CD8 epitope (see Tables 1.2 and 5.1) and the RGD motif, and did not result in a truncated protein, it was considered to be acceptable for use in the proposed screen.

ORF59 is an early lytic gene that codes for the processivity factor of the viral DNA polymerase, and is required for extension during DNA replication (Lin et al., 1998). It is a homologue of the EBV gene BMRF1, which is an immunodominant target of

the EBV-specific CD8 T-cell response (Landais et al., 2005a; Hislop et al., 2007). To the best of my knowledge, T-cell responses against KSHV ORF59 have not been investigated, and so it was included in the screen as an unknown potential immunogen. ORF59 was successfully cloned into the lentiviral vector with complete sequence fidelity.

At this time, cloning of the CMV gene phosphoprotein 65 (pp65) into the lentiviral vector was also attempted, as this was proposed for use in experiments to refine the protocol for the screen for immunogenic KSHV ORFs, and also for subsequent use as a positive control in the screen. CMV is a β-herpesvirus that is found in 50% to 90% of immunocompetent individuals from different populations (Staras et al., 2006) and is a common opportunistic infection in individuals with AIDS. It is distinct enough from KSHV to ensure minimal cross-reactivity of T-cell responses. The tegument protein pp65 is the immunodominant protein target of the CD8 response against CMV (Wills et al., 1996), and a major target of the CD4 response against CMV (Kern et al., 2002). Unfortunately, despite extensive efforts CMVpp65 could not be inserted into the lentiviral vector, and so an alternative approach had to be found.

Preparations of an adenoviral vector encoding CMVpp65 (Adpp65) were a kind gift from Dr Magnus Essand of Uppsala University, Sweden. moDCs transduced with this construct were previously used to stimulate and promote proliferation of both CD8 and CD4 CMV-specific T cells from CMV-infected individuals (Carlsson et al., 2003). Adpp65 was therefore used in preliminary experiments to refine the protocol for the immunogenic screen and was also used later as a positive control,

with the caveat that transgene expression was through a different vector and therefore responses were not directly comparable to responses elicited by transgenes expressed through the lentiviral vector.

Another important step in the preparation for the immunogenic screen was to refine techniques for the culture and lentiviral transduction of primary moDCs. moDCs were derived from monocytes by an established method of CD14-positive monocyte isolation from peripheral blood followed by culture in the presence of IL4 and GMCSF (Sallusto and Lanzavecchia, 1994). A suitable serum supplement and maturation stimulus for the culture of moDCs for use in the proposed screen had to be selected. Early protocols for culturing moDCs used RPMI media supplemented with foetal calf serum (FCS). FCS is not, however, suitable for use in moDC preparations intended for clinical applications. A variety of alternative supplements have thus been investigated, including human plasma, serum albumin or various serum-free medias but these reportedly give rise to lower yields of moDCs and larger numbers of adherent cells (Jeras et al., 2005). For the purpose of the proposed immunogenic screen, FCS was also not suitable for moDC culture, as moDCs cultured in FCS prime a background response to bovine-derived antigens when cultured with autologous T cells (Jonuleit et al., 2001). Therefore, human AB serum was investigated as alternative media supplement for culturing moDCs.

Different maturation stimuli have also been found to affect the phenotype of moDCs, for example, by polarising moDCs to prime either Th1- or Th2-type CD4 T-cell responses (Jeras et al., 2005). It was proposed to not only use the lentiviral system to identify KSHV T-cell antigens but also to investigate cytokine release by

KSHV-specific T cells (for example, to determine the type of helper T-cell response elicited by KSHV CD4 antigens). Thus it was important to find a method for deriving moDCs that were not polarised, in order to have a neutral background in which to perform the immunogenic screen. Two different maturation stimuli were investigated: lipopolysaccharides (LPS) or a mixture of inflammatory cytokines comprising TNFα, IL1β, IL6 and PGE2 (referred to as a 'cytokine cocktail'). moDCs matured by stimulation with LPS produce IL12 and prime Th1-type CD4 T-cell responses. The 'cytokine cocktail' was trialled in the hope it would not polarise moDCs in this way. There has been concern that PGE2-treated moDCs promote a Th2-polarised response, as PGE2 inhibits bioactive IL12 production by moDCs (Schuler et al., 2003; Jeras et al., 2005). However, there is also evidence to dispute this, demonstrating that PGE2-treated moDCs are equally capable of promoting a Th1-type response (Jonuleit et al., 1997; Schuler-Thurner et al., 2002; Schuler et al., 2003).

Therefore, the viability, immunophenotype and allogenic stimulatory capacity of moDCs cultured in different serum supplements and matured by exposure to different stimuli were compared. A batch of AB serum (027K0432) was identified in which moDCs showed good viability, comparable to moDCs cultured conventionally in FCS. moDCs cultured under all conditions showed the expected expression of markers including the major histocompatibility complexes I (MHC-I) and II (HLA-DR); the costimulatory molecules CD40, CD80 and CD86; and the DC maturation marker CD83. As has been previously described (Jeras et al., 2005), moDCs cultured in AB serum were more phenotypically heterogeneous than those cultured in FCS, but this was less apparent after maturation. The 'cytokine cocktail'

was the most potent maturation stimulus, in terms of both the immunophenotype of the mature moDCs and the allogenic stimulatory capacity of these moDCs. After stimulation with the 'cytokine cocktail', moDCs cultured in AB serum were more than 80% double-positive for HLA-DR and CD80 (indicative of a mature phenotype), and showed comparable stimulatory capacity to mature moDCs cultured in FCS, especially when cultured at high ratios of moDCs to allogenic T cells (1 to 20 or 1 to 40). Furthermore, CD4 T cells stimulated by allogenic cytokine-matured moDCs appeared to produce less IFNγ than CD4 T cells stimulated by allogenic LPS-matured moDCs. This indicated that cytokine-matured moDCs do not prime polarised Th responses, although it must be noted that the difference in proportion of IFNγ-producing CD4 T cells after stimulation with either LPS- or cytokine-matured moDCs did not reach statistical significance. Nevertheless, the 'cytokine-cocktail' was selected as the best suited maturation stimulus for use in the proposed screen, as it was the most potent stimulus and, taken together, my data and the literature indicated that it would provide the most neutral background for the investigation of polarisation of Th responses to antigens.

With appropriate culture conditions for moDCs established, the transduction of moDCs using the lentiviral vector was investigated. A lentivirus encoding GFP (pCSGW) was used to demonstrate that moDCs could be successfully transduced with this lentiviral vector and to investigate the kinetics of transgene expression and the optimal multiplicity of infection (MOI) for this vector (Robey et al., 2009). In keeping with findings by other groups (Schroers et al., 2000; Dyall et al., 2001), GFP transgene expression was found to increase steadily over each day post-transduction. A six-day period was selected as a suitable length of time for culture

of transduced moDCs for the screen, representing a balance between good transgene expression and optimal moDC viability (since these primary cells are not suited to extended periods of culture). After six days, a transduction efficiency of 12.25 ± 2.5% was observed (mean ± s.d.). The MOI after transduction with different volumes of pCSGW was also investigated and compared to levels of GFP transgene expression. An optimal MOI of between three and eight for each transduction was selected for the screen, as with the GFP construct this achieved good transgene expression, with no notable improvement if the MOI was increased further. Furthermore, whilst there is a consensus that lentiviral transduction of moDCs at MOIs of less than 10 does not affect moDC viability, immunophenotype or antigen presenting function (Gruber et al., 2000; Dyall et al., 2001; Koya et al., 2003; Dullaers et al., 2004), the evidence regarding transduction of moDCs with higher MOIs is less clear (Chen et al., 2004). In keeping with this, transduction of moDCs with the lentiviral vector at an MOI of between three and eight was demonstrated not to affect the moDCs' immunophenotype or maturation, as demonstrated by expression of MHC-I and CD80.

Interestingly, a slight downregulation of GFP transgene expression was observed after maturation of moDCs. Other groups have reported that mature moDCs are harder to transduce than immature moDCs (Gruber et al., 2000; Schroers et al., 2000), but to the best of my knowledge there has been no previous documentation of decreased transgene expression when maturation is induced after transduction. This downregulation may be the result of the terminal differentiation of mature moDCs and the concurrent changes in cellular gene expression and promoter activity. Importantly, even after maturation with the 'cytokine cocktail' an average

of 7.5 ± 1.7% GFP-positive cells was achieved, a level comparable to those reported by other groups using a similar protocol (Gruber et al., 2000; Schroers et al., 2000; Dyall et al., 2001; Esslinger et al., 2002; Lizee et al., 2004).

Following the experiments with pCSGW, RT-PCR was used to ensure that all KSHV ORFs were expressed by moDCs after transduction and quantitative PCR was performed to titre all KSHV-ORF-encoding lentivirus preparations. Additionally, the transduction of moDCs with up to three different KSHV-ORF-encoding lentiviruses simultaneously was demonstrated to result in the expression of all three transgenes (Robey et al., 2009). Thus it was decided to perform the screen with pools of ORFs grouped according to their expression in PEL cell lines (Jenner et al., 2001; Jenner and Boshoff, 2002) in order to make the best use of clinical samples and to determine whether latent, immediate-early, early or late lytic gene products elicit the strongest T-cell responses.

Finally, an adenovirus encoding the immunodominant CMV gene pp65 (Adpp65) was used to refine methods for investigating antigen-specific memory T-cell responses. The protocol for transduction of moDCs with Adpp65 (including MOI and length of time for transduction) was performed as previously described (Carlsson et al., 2003). In cultures with autologous T cells, cytokine-matured, Adpp65-transduced moDCs elicited strong proliferative responses. These were assumed to be antigen-specific, as non-transduced moDCs did not elicit similar responses. Non-transduced moDCs cultured in FCS did, however, stimulate a substantial background response, presumably directed against bovine-derived antigens as has been previously reported (Jonuleit et al., 2001). There was little

response to non-transduced moDCs cultured in AB serum, confirming that this was the most appropriate serum supplement for culturing moDCs for use in the screen for immunogenic KSHV ORFs. A ratio of moDCs to T cells of 1 to 40 was selected, representing a balance between a strong antigen-specific response to transduced moDCs and a low background response to non-transduced moDCs. Unfortunately, an empty adenoviral vector was not available for comparison, so it was not possible to determine whether the antigen-specific responses was to the CMV transgene or to adenoviral proteins encoded in the vector. However, the paper in which the use of Adpp65-transduced moDCs was originally described provided substantial evidence that they stimulated both CD8 and CD4 CMV-specific memory T cells (Carlsson et al., 2003). CD4 T cells stimulated with cytokine-matured, Adpp65-transduced moDCs produced cytokines indicative of a Th1-polarised response, in keeping with the expected response elicited by pp65 (Gamadia et al., 2004). This was also dramatically different to cytokine production by CD4 T cells stimulated with allogenic cytokine-matured, non-transduced moDCs, further indicating that the 'cytokine cocktail' was an appropriate stimulus for use in experiments investigating cytokine secretion in response to antigen stimulation.

Based on the results discussed above, a protocol for a lentiviral-based screen to investigate the targets of CD8 and CD4 KSHV-specific T cells was established, and is shown schematically in Figure 4.1.

Investigation of the Targets of the CD8 and CD4 T-cell Responses against KSHV Using a Lentiviral-Based Screen

The screen for immunogenic KSHV ORFs was performed in 14 KSHV-seropositive (12 HIV-positive) and seven KSHV-seronegative (four HIV-positive) individuals. Out of the 14 KSHV-seropositive individuals tested, six responded to more than one pool of KSHV ORFs (total of CD8- and CD4-mediated responses) and were classified as 'good responders', and eight were classified as 'poor responders' (i.e. gave one or no total CD8 and CD4 responses). Although this study was too small to draw definite conclusions regarding the differences in phenotypes of KSHV-seropositive individuals who made several responses to KSHV antigens using this protocol and those who did not, some interesting observations were made.

Significantly, one HIV-positive individual with no history of KSHV-related neoplasia (H419) who was originally intended for inclusion in the screen as an HIV-positive, KSHV-negative control (as he was considered to be at low risk of KSHV infection) gave several strong CD8 and CD4 responses to KSHV antigens. It was only upon retrospective analysis of the individual's KSHV-serostatus using plasma frozen at the time of venesection that the individual was discovered to be KSHV-seropositive and therefore an asymptomatic carrier of KSHV. This finding gave extra confidence in the specificity of the KSHV responses observed using this assay, as it was the presence of these responses that first indicated this individual was a carrier of KSHV. Furthermore, the number and strength of responses seen in this individual fitted with previous reports that KSHV T-cell responses are of greater frequency and magnitude in asymptomatic carriers of the virus than in those

with KS (Guihot et al., 2006; Lambert et al., 2006). Likewise, two individuals with active KS at the time of venesection (S346 and W692) were poor responders.

Interestingly, although the screen was not performed in any individuals with active MCD, the two individuals that were in remission from MCD (P940 and V689) were both good responders, in line with previous reports that individuals with active MCD show strong CD8 T-cell responses to KSHV antigens (Guihot et al., 2008; Barozzi et al., 2009) that, in one study, were comparable to those seen in asymptomatic carriers of KSHV (Guihot et al., 2008). Finally, all individuals who had received HAART alone as treatment for their KS were poor responders, whereas all good responders had received HAART in combination with cancer-specific cytotoxic therapy. This was surprising, as individuals whose KS regresses spontaneously on initiation of HAART might be expected to have higher levels of KSHV-specific T cells than individuals who require chemotherapy in addition to HAART to treat their KS. Furthermore, some forms of chemotherapy may cause collateral damage to cells of the immune system, including T cells. A study of 33 HIV clade C-infected individuals with KS randomised to two treatment arms (HAART alone versus combined HAART and chemotherapy) observed no differences in the levels of T-cell responses between the two groups at five and 11 months after the initiation of therapy (Bihl et al., 2007a). The authors did, however, observe reduced KSHV viraemia and better clinical outcome in those treated with combined HAART and chemotherapy. This indicates that chemotherapy may not, in the short term, inhibit reconstitution of KSHV-specific T-cell responses and it may be that, in the long run, dissemination of KS lesions by chemotherapy

increases systemic exposure to KSHV antigens, thus enhancing the generation of a memory T-cell response against KSHV.

Overall, in this cohort of 14 KSHV-seropositive individuals, a distinct skewing of both CD8 and CD4 responses towards early lytic and late lytic gene products was observed (Robey et al., 2009). This was an unexpected result as it appears to contrast with observations of the T-cell response against EBV, which preferentially targets immediate-early lytic EBV gene products. It has been suggested that immediate-early gene products might be frequent targets of herpesvirus-specific T-cell responses as this enables the activation of the host immune response before the expression of viral genes involved in immune evasion strategies. However, it has recently been reported that the CD8 T-cell response against the murine γ2-herpesvirus, MHV68, is also directed against early lytic and late lytic gene products (Gredmark-Russ et al., 2008). A broad repertoire of epitopes derived from late lytic structural proteins and early lytic proteins involved in DNA replication were identified in MHV68, but no epitopes derived from immediate-early proteins were found. Similarly, 86% of CD8 and 90% of CD4 T-cell responses against CMV were reported to target early lytic and late lytic gene products (Sylwester et al., 2005). Thus, protein abundance and, to a lesser degree, function, may be important factors affecting the immunogenicity of a gene product. Furthermore, a possible explanation for the immunodominance of early lytic and late lytic KSHV (and other herpesvirus) antigens may be the role they play in the establishment of homeostasis between host and virus. The activation of viral immune evasion strategies such as the downregulation of MHC molecules before the expression of immunodominant antigens may result in a blunting of the immune response, enabling the virus to

avoid elimination and to establish controlled chronic infection. Such persistent, asymptomatic infection is a hallmark of herpesvirus infections and is the usual manifestation of KSHV infection in immunocompetent individuals.

The three gene pools that were most frequently recognised by CD8 T cells from KSHV-seropositive individuals were [ORF8/ORF49/ORF61]; [ORF59/ORF65/K4.1]; and [ORF28/ORF36/ORF37]. As discussed above, ORF8, ORF61 and ORF65 are all documented CD8 antigens (Wang et al., 2000; Wang et al., 2001b; Wang et al., 2002b; Bourboulia et al., 2004; Woodberry et al., 2005; Guihot et al., 2006; Lambert et al., 2006; Guihot et al., 2008), and thus the data from the screen corroborated these findings as well as indicating for the first time the relative importance of these antigens compared to other documented latent and immediate-early lytic antigens included in the screen (for example K12, ORF73, K5 and ORF57; see Table 1.2). Interestingly, however, none of the gene products within the most frequently recognised CD8 target pool ([ORF28/ORF36/ORF37], which was recognised by seven out of 14 individuals) have previously been investigated for immunogenicity. The three ORFs within this late lytic gene pool are therefore good candidates for further investigations into the dominant antigens of the KSHV-specific CD8 T-cell response.

Overall, less CD4 T-cell responses were observed to KSHV antigens than CD8 T-cell responses. This may be due to preferential processing and presentation of endogenously expressed antigens (i.e. lentiviral transgenes) through the MHC-I pathway. Lentiviral-transduced moDCs have been demonstrated by others to present MHC-II-restricted epitopes and to elicit CD4 T-cell responses, although

presentation was less efficient than that observed in moDCs pulsed with MHC-II-restricted peptide epitopes (He et al., 2005). This is likely the result of decreased access of endogenously processed antigens to the MHC-II processing pathway. In future experiments, KSHV-ORF-encoding lentiviral vectors could be specifically designed with the addition of signals that target expression of transgenes to endosomal or lysosomal compartments in order to increase processing of antigens through the MHC-II pathway and thereby enhance presentation of endogenously expressed MHC-II-restricted epitopes (Wu et al., 1995; Dullaers et al., 2004).

Interestingly, two of the most common CD8 targets, [ORF8/ORF49/ORF61] and [ORF59/ORF65/K4.1], were also two of the most common CD4 targets (recognised by six and four out of 14 individuals respectively) alongside another pool of late lytic KSHV ORFs [ORF33/K1/K8.1] (which was recognised by CD4 T cells from four individuals). As discussed in section 1.3.b *The CD4 T-cell Response Against KSHV*, very little is known about the CD4 response against KSHV. Three KSHV CD4 antigens have been previously identified: two peptides have been identified, one from within K12 and one from within K15, which each elicited a response in one KSHV-positive individual out of 52 tested (Guihot et al., 2006); and overlapping peptides spanning the entire sequences of K12 and K8.1 were shown to elicit CD4 responses in one and two out of three iatrogenic KS patients respectively (Barozzi et al., 2008). The data from the present study also indicate that K8.1 could be an important CD4 antigen. The [K11.1/K12/K15] gene pool, however, was only recognised by CD4 T cells from one individual tested using this methodological approach (H419; HIV-positive asymptomatic carrier of KSHV). This is perhaps not surprising given the low number of individuals that gave CD4 responses to K12 and

K15 in the studies discussed above, and may indicate that these are not important targets of the KSHV-specific CD4 response. Overall, the data from the screen indicate that the major targets of the KSHV-specific CD4 response are likely to lie within the same early lytic and late lytic gene products that elicit the strongest CD8 T-cell responses. This fits with observations in other herpesviruses, for example, in the T-cell response against CMV, 53% of the most recognised ORFs are common to both the CD8 and CD4 response (Sylwester et al., 2005). Similarly, the major EBV CD8 targets BZLF1 and BMLF1 (immediate-early lytic) and EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C and LMP2 (latent) also elicit CD4 responses (Landais et al., 2005a; Hislop et al., 2007).

Thus the lentiviral-based screen proved to be a useful system for achieving a broad overview of the targets of the CD8 and CD4 T-cell responses against KSHV and identifying antigenic hotspots within the KSHV genome. In particular, these data showed a clear skewing of responses towards viral proteins expressed later in the viral lytic cycle than was hypothesised, and identified some candidate ORFs in which to focus future immunological investigations. However, this methodological approach was also found to have limitations. In particular, a relatively high background response was observed in T cells from some individuals to autologous moDCs transduced with the empty lentiviral vector alone. This was a surprising result, as lentiviral vectors are generally held to be the least immunogenic of available viral vectors and reportedly do not elicit significant background responses (Collins and Cerundolo, 2004; Palmowski et al., 2004). This is largely due to the 'gutless' structure of lentiviral vectors, whereby the vector does not encode, and therefore does not express, any HIV-derived genes (it is only the physical structure

of the vector that is comprised of HIV-derived proteins). However, one group has reported the induction of primary CD8 and CD4 T-cell responses against HIV using moDCs transduced with such gutless, self-inactivating lentiviral vectors at very high MOIs (50 to 100 – more than 10 times the MOIs used in the present screen) (Chen et al., 2006). It is possible, therefore, that in this HIV-positive cohort, some of the background response to moDCs transduced with the empty lentiviral vector was directed against HIV proteins contained within the vector, such as HIV-GAG.

Notably, however, a background response of comparable magnitude to moDCs transduced with the empty lentiviral vector was observed in T cells from both HIV-positive and HIV-negative individuals. One explanation could be cross-reactivity with endogenous retroviruses. Alternatively, these responses could be due to contamination of the lentiviral preparations from two possible sources. Lentiviral preparations were prepared from filtered supernatant harvested from transfected 293T cells. By necessity, this supernatant contained FCS as 293T cells were found not to survive sufficiently in human AB serum for the preparation of lentivirus (data not shown). Although the volume of lentivirus preparations used for transduction of moDCs was small in comparison to the total volume of moDC media, it is none the less possible that, despite filtering, some bovine antigens were present in the lentiviral preparations and were taken up and presented by moDCs. The data presented in Chapter 3 of this thesis and by others (Jonuleit et al., 2001) have shown that bovine antigens can elicit background responses in systems such as this one. Secondly, the 293T cell line is derived from the 293 cell line, which was originally derived from human embryonic kidney cells by transformation of the cells through exposure to fragments of adenovirus type-5 (Ad5) DNA (Graham et al., 1977).

Subsequent analysis revealed that the transformation had been brought about by the insertion of 4.5 kilobases of Ad5 DNA (as a single linear insertion of viral DNA with no rearrangements) into human chromosome 19 (Louis et al., 1997). 293T cells therefore express Ad5 proteins which may additionally be present in the lentiviral preparations. Given the high levels of pre-existing immunity to Ad5 in the general population (Kostense et al., 2004), it seems likely that the T-cell responses observed to moDCs transduced with the empty lentiviral vector were, at least in part, directed against Ad5 antigens. In future experiments using lentiviral vectors for such immunological investigations, prior purification of the preparations should be considered, for example through a sucrose gradient. This procedure has been shown to greatly reduce the immunogenicity of lentiviral preparations; however, it is a cumbersome process and has also been shown to greatly reduce lentivirus titres (Baekelandt et al., 2003).

The major significance of the observed background response was that strict criteria had to be used to designate positive responses, which may have resulted in weaker responses not being detected. This might partly explain why one or no responses were detected in the 'poor responders'. Furthermore, it was felt that the background response would be difficult to distinguish from KSHV-specific responses when examining cytokine production, and thus this system was not appropriate for the investigation of the functional properties of KSHV-specific T cells. Therefore, an alternative strategy was proposed, to use pentamer technology to elucidate the memory phenotypes and functions of virus-specific T cells.

Identification of an Immunodominant Late Lytic KSHV Epitope

Pentamer technology requires the prior identification of an immunodominant (or at least a frequently recognised) epitope which can be targeted. From the findings in the lentiviral-based screen for immunogenic KSHV ORFs, a search for novel epitopes from within the late lytic pool of ORFs [ORF28/ORF36/ORF37] seemed warranted. It was decided to focus on HLA*0201-restricted epitopes, since HLA*0201 is one of the most common HLA-types in people of all ethnic backgrounds, and so such epitopes were felt to have the greatest relevance for future immunological studies as well as in the potential design of immunotherapies and vaccines. MHC binding assays and computer algorithms were used to identify candidate epitopes from within ORF28, ORF36 and ORF37. These peptides were then tested for recognition by T cells from 18 HIV-positive, KSHV-positive, HLA-A*0201-positive individuals alongside nine previously described HLA-A*0201-restricted KSHV epitopes (Brander et al., 2001; Micheletti et al., 2002; Wang et al., 2002b; Bourboulia et al., 2004; Lambert et al., 2006; Ribechini et al., 2006). Disappointingly, none of our candidate epitopes were recognised by any individual. This could mean that the responses observed to this pool of ORFs in the lentiviral-based screen were not HLA-A*0201-restricted, or that the candidate epitopes identified by the MHC binding assays and computer algorithms were not the HLA-A*0201 epitopes that were endogenously processed and presented by the moDCs in the screen. Of note, however, eight of the nine previously identified KSHV epitopes were also not recognised by any of the 18 KSHV-positive individuals. This was surprising, as reports in the literature indicated that these eight epitopes were recognised by 16% to 100% of all individuals tested (total of HIV-positive and

HIV-negative) or 13% to 32% of HIV-positive individuals tested in previous studies (see Table 5.1).

Two of these epitopes (K5 - ALY and ORF70 - YML) had not previously been tested in HIV-positive individuals, but only in one study of HIV-negative, asymptomatic carriers of KSHV where they were tested in seven and five individuals respectively (Ribechini et al., 2006). Responses to these epitopes were detected by chromium-release and ELISpot assays after 14- or 21-day stimulations with peptide-pulsed T2 cells in the presence of IL2 (Ribechini et al., 2006). Three further epitopes (K12 - LLN, ORF8 - LMW and ORF22 - FLN) were also originally identified using similar protocols for memory T-cell expansion (several weeks of culture with peptide-pulsed antigen-presenting cells in the presence of IL2) before detection of T-cell responses by cytotoxicity, lysis or ELISpot assays (Brander et al., 2001; Micheletti et al., 2002; Wang et al., 2002b). Recognition of these three epitopes (K12 - LLN, ORF8 - LMW and ORF22 - FLN) have been confirmed by additional studies using *ex vivo* ELISpot protocols similar to the ones used in this thesis (Bourboulia et al., 2004; Guihot et al., 2006; Guihot et al., 2008). However, the vast majority of HIV-positive, KSHV-positive individuals used in these studies were either asymptomatic carriers of KSHV or were individuals with MCD [who reportedly show KSHV-specific CD8 responses equivalent to asymptomatic carriers (Guihot et al., 2008)]. In fact, responses to these three epitopes have only been detected *ex vivo* by ELISpot in four HIV-positive individuals with a history of KS: three with quiescent KS or KS in remission (Bourboulia et al., 2004) and one with active KS (Guihot et al., 2006).

The three remaining epitopes (ORF6 - VLG; ORF61 - NMS; and ORF65 - NMS) had been previously identified by one study only (Lambert et al., 2006), although with a large cohort of study participants (46 to 48 individuals for each epitope, including 23 or 24 HIV-positive individuals). Responses to these epitopes were detected by tetramer staining, using the mean frequency + 2 s.d. of tetramer-positive cells in 18 HLA-A2-negative, KSHV-positive individuals as a cut-off value for positivity (Lambert et al., 2006).

The differences in techniques and study participants used by these studies and in this thesis may help to explain my inability to detect responses to these previously documented KSHV epitopes. In particular, the frequent use of prolonged cultures to expand memory T cells before responses against peptides can be detected reflects the extremely low frequency of cells specific for these epitopes *in vivo* and, arguably, the limited biological relevance of these epitopes in the control of KSHV infection. One attempt was made at replicating one of these memory T-cell expansion protocols (Wang et al., 2002b) during the course of this thesis, in the hope of detecting elusive weak responses to these peptides. However, the background response observed to non-peptide-pulsed autologous moDCs by T cells expanded in the same way (in the presence of IL2) was so high that distinguishing peptide-specific responses to peptide-pulsed moDCs was deemed not possible (data not shown).

Additionally, the lack of responses observed in this cohort of HIV-positive, KSHV-positive individuals with a history of KSHV-related neoplasia could be an indication of the limitation of this cohort for detection of such weak T-cell

responses by *ex vivo* ELISpot. Although restoration of KSHV-specific T-cell responses have been documented in HIV-positive individuals in remission from KS (Bourboulia et al., 2004; Bihl et al., 2007a), it seems that these are not equivalent to responses observed in true controllers of KSHV infection, i.e. asymptomatic carriers of KSHV (either HIV-positive or HIV-negative). It is therefore possible that, if further investigations were performed in an asymptomatic cohort of KSHV-positive individuals, then one or more of the candidate epitopes identified from the late lytic ORF pool may yet prove to be a weak T-cell epitope that elicits responses equivalent to responses to most of the epitopes that have already been identified.

Significantly, just one of the nine previously identified KSHV epitopes (K8.1 - LVL) elicited responses in this cohort of KSHV-positive individuals. When used at a concentration of 20μg/ml, this late lytic epitope elicited responses in nine of the 12 individuals tested (75%). This was the only epitope that had been originally identified using an *ex vivo* ELISpot assay similar to the one used in this thesis (Bourboulia et al., 2004). In this original paper, Bourboulia *et al.* observed responses to K8.I - LVL by T cells from four out of eight KSHV-positive individuals who had been on HAART for more than two years at the time of venesection. Together, this data and the data from the present study provide evidence of an immunodominant role for this epitope. In the present study, three individuals who had active KS at the time of venesection (B257, B792, and S314) did not respond to the K8.1 - LVL peptide (although two of these, B257 and B792, were tested at a lower concentration of 5μg/ml), which may further indicate a protective effect for CD8 T cells specific for this peptide in the control of KSHV infection.

The finding that, out of nine known KSHV epitopes derived from proteins expressed at various stages of the virus life cycle, one late lytic peptide displayed a clear pattern of immunodominance is in keeping with the findings from the lentiviral-based screen for immunogenic KSHV ORFs, which also indicated that KSHV-specific T-cell responses are skewed towards early lytic and late lytic antigens. The results from the two methodological approaches can not be easily compared. The lentiviral system allows endogenous processing of an antigenic protein and requires no prior knowledge of an optimal epitope or HLA-restriction and therefore the responses observed in this system can not be considered equivalent to responses observed to pre-determined artificial peptides. Nonetheless, it is worth noting that the gene pool containing K8.1 ([ORF33/K1/K8.1]) was recognised by CD8 T cells from four out of 14 KSHV-seropositive individuals, making it the fourth most frequently recognised CD8 target in the screen. Out of the four participants in the screen who were known to be A*0201-postive (P940, V689, A556 and T541), one (A556) responded to [ORF33/K1/K8.1]. Of the remaining three, two (P940 and T541) were tested for responses to peptides in the ELISpot assay. P940 did not respond to the K8.1 peptide, in keeping with the results from the screen. T541 did respond to the K8.1 peptide, but only very weakly (25 spot-forming cells per million PBMCs). Thus this weak response may have been missed in the lentiviral-based screen due to the strict criteria that were necessary to distinguish a positive response.

Disappointingly, the K8.1 peptide proved to be too hydrophobic for the synthesis of a pentamer directed against T cells specific for this peptide, and so the proposed experiments to use pentamers as tools to investigate the memory phenotypes and

functions of KSHV-specific T cells could not be performed. Preliminary experiments using pentamers directed against CMV- and HIV-specific T cells demonstrated their use for enumerating and determining the memory phenotypes of virus-specific T cells. However, I was not able to use these pentamers to investigate the functional properties of virus-specific CD8 T cells, as I found that pentamer staining was lost upon permeabilisation of PBMCs during the protocol for staining for intracellular cytokine production.

Conclusions and Future Directions

The major finding of the work described in this thesis was that, in contrast to my hypothesis, the KSHV-specific CD8 and CD4 T-cell responses are skewed towards early lytic and late lytic KSHV antigens. Evidence for this was provided by a lentiviral-based screen for immunogenic KSHV ORFs, and also through *ex vivo* ELISpot assays testing recognition of known KSHV epitopes. Although this finding was unexpected, it was supported by findings reported during the course of the research for this thesis that the CD8 T-cell response against the murine γ2-herpesvirus MHV68 also preferentially targets early lytic and late lytic antigens (Gredmark-Russ et al., 2008). This information may have important implications for future immunological investigations of KSHV, and potentially the development of immunotherapies for KSHV-related malignancies or even a vaccine against KSHV.

This thesis has also provided the first evidence of an immunodominant late lytic CD8 KSHV epitope. It would have been interesting to explore the memory

phenotypes and functional properties of CD8 T cells specific for this epitope through the use of pentamer technology. Unfortunately, this was not possible due to the hydrophobic nature of this peptide epitope. Furthermore, as discussed, I experienced severe technical difficulties when using pentamer staining in conjunction with staining for intracellular cytokines. Thus, an alternative strategy to investigate the functional properties of KSHV-specific CD8 T cells could be to use moDCs pulsed with the K8.1-derived peptide epitope LVLILYLCV as stimulants in ELISpot assays to detect release of other cytokines such as TNFα, IL4 or IL2. This may reveal more about the role of T-cells specific for this epitope in the control of KSHV infection.

A major challenge in the research presented in this thesis was finding an appropriate methodological approach for the investigation of the KSHV-specific T-cell response, particularly in the available cohort of KSHV-positive individuals, who were also HIV-coinfected and had a history of KSHV-related neoplasia. This cohort clearly had disadvantages, with respect to eliciting sub-optimal KSHV-specific T-cell responses. Nonetheless, it could be argued that with a long-term goal of developing better treatments for, or a prophylaxis against, symptomatic KSHV infection, investigations in this cohort of individuals remain the most relevant. With this in mind, the major philosophy behind the lentiviral-based screen remains intact – that using antigen-loaded moDCs to detect responses to endogenously processed antigenic proteins offers several advantages over the use of pre-determined artificial peptides. This was highlighted by the lack of responses observed to either candidate late lytic epitopes or known KSHV epitopes in peptide-based ELISpot assays. Furthermore, the use of moDCs to maximise responses to antigens was

demonstrated by both the lentiviral-based screen as well as in ELISpot assays that compared the use of peptide-pulsed moDCs or peptide solutions as stimulants. However, the major disadvantage of the lentiviral-based system was that preparations of the empty lentiviral vector proved to be immunogenic, highlighting some of the unknown entities and complications associated with the use of these vectors.

For future investigations, I suggest that the use of antigen-loaded moDCs still seems to be a powerful tool, although an alternative antigen-delivery strategy needs to be found. Novel, non-viral delivery methods may provide an efficient solution and could circumvent many of the problems associated with the lentiviral vectors. One such delivery method is the recently described SMoC technology (Okuyama et al., 2007). SMoCs are artificially synthesized small-molecule mimics of the alpha-helical peptide protein transduction domain (PTD) of the HIV TAT protein. SMoCs have been shown to efficiently deliver dye molecules and functional proteins to a variety of cell types (including those of a haematopoietic lineage) and to various subcellular locations. If SMoCs proved to be efficient at delivering cargo to moDCs, they could be extremely useful. Late lytic ORFs could be delivered to the nucleus for further investigation of the KSHV-specific CD8 T-cell response (for example which of ORF28, ORF36 and ORF37 is the most immunogenic). This may also enable exploration of cytokine release by proliferating cells in response to antigen-presenting moDCs, as was originally proposed for the lentiviral-based experiments. For further investigation of the CD4 response, an alternative strategy delivering recombinant early lytic and late lytic proteins instead of ORFs to moDCs could be investigated, with SMoCs targeting these proteins to endosomal or

lysosomal compartments for processing and presentation through the MHC-II pathway. Since the uptake of SMoCs by cells appears to be via the clathrin-mediated endocytotic pathway (Okuyama et al., 2007), it is possible that using this technology to deliver proteins to antigen-presenting cells may prove to be ideal for optimal antigen presentation through MHC-II and stimulation of CD4 T-cell responses. Furthermore, SMoCs would be easier to translate to a clinical setting, as they are likely to pose less safety concerns than the use of lentiviral vectors.

In light of recent advances in the development of dendritic-cell-based immunotherapies and vaccines for a variety of tumours (Ballestrero et al., 2008), it is tempting to speculate that such therapies may one day be realised for the treatment of KSHV infection and its related malignancies – even if this is a long way in the future, and first requires a yet deeper understanding of the KSHV-specific T-cell response.

REFERENCES

Akula,S.M., Pramod,N.P., Wang,F.Z., and Chandran,B. (2001). Human herpesvirus 8 envelope-associated glycoprotein B interacts with heparan sulfate-like moieties. Virology *284*, 235-249.

Altman,J.D., Moss,P.A.H., Goulder,P.J.R., Barouch,D.H., Heyzer-Williams,M.G., Bell,J.I., McMichael,A.J., and Davis,M.M. (1996). Phenotypic analysis of antigen-specific T lymphocytes. Science *274*, 94-96.

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

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

Appay,V., Nixon,D.F., Donahoe,S.M., Gillespie,G.M.A., Dong,T., King,A., Ogg,G.S., Spiegel,H.M.L., Conlon,C., Spina,C.A., Havlir,D.V., Richman,D.D., Waters,A., Easterbrook,P., McMichael,A.J., and Rowland-Jones,S.L. (2000). HIV-specific CD8+ T cells produce antiviral cytokines but are impaired in cytolytic function. J. Exp. Med. *192*, 63-76.

Appay,V., Dunbar,P.R., Callan,M., Klenerman,P., Gillespie,G.M.A., Papagno,L., Ogg,G.S., King,A., Lechner,F., Spina,C.A., Little,S., Havlir,D.V., Richman,D.D., Gruener,N., Pape,G., Waters,A., Easterbrook,P., Salio,M., Cerundolo,V., McMichael,A.J., and Rowland-Jones,S.L. (2002). Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nature Med. *8*, 379-385.

Araki,K., Turner,A.P., Shaffer,V.O., Gangappa,S., Keller,S.A., Bachmann,M.F., Larsen,C.P., and Ahmed,R. (2009). mTOR regulates memory CD8 T-cell differentiation. Nature *460*, 108-112.

Arthur,J.F., Butterfield,L.H., Roth,M.D., Bui,L.A., Kiertscher,S.M., Lau,R., Dubinett,S., Glaspy,J., McBride,W.H., and Economou,J.S. (1997). A comparison of gene transfer methods in human dendritic cells. Cancer Gene Ther. *4*, 17-25.

Baekelandt,V., Eggermont,K., Michiels,M., Nuttin,B., and Debyser,Z. (2003). Optimized lentiviral vector production and purification procedure prevents immune response after transduction of mouse brain. Gene Ther. *10*, 1933-1940.

Ballestas,M.E., Chatis,P.A., and Kaye,K.M. (1999). Efficient persistence of extrachromosomal KSHV DNA mediated by latency-associated nuclear antigen. Science *284*, 641-644.

Ballestrero,A., Boy,D., Moran,E., Cirmena,G., Brossart,P., and Nencioni,A. (2008). Immunotherapy with dendritic cells for cancer. Adv. Drug Deliv. Rev. *60*, 173-183.

Banchereau,J. and Steinman,R.M. (1998). Dendritic cells and the control of immunity. Nature *392*, 245-252.

Barcy,S., De Rosa,S.C., Vieira,J., Diem,K., Ikoma,M., Casper,C., and Corey,L. (2008). Gamma-delta+ T cells involvement in viral immune control of chronic human herpesvirus 8 infection. J. Immunol. *180*, 3417-3425.

Barozzi,P., Bonini,C., Potenza,L., Masetti,M., Cappelli,G., Gruarin,P., Whitby,D., Gerunda,G.E., Mondino,A., Riva,G., Vallerini,D., Quadrelli,C., Bosco,R., Ciceri,F., Bordignon,C., Schulz,T.F., Torelli,G., and Luppi,M. (2008). Changes in the immune responses against human herpesvirus-8 in the disease course of posttransplant Kaposi sarcoma. Transplantation *86*, 738-744.

Barozzi,P., Riva,G., Vallerini,D., Bosco,R., Quadrelli,C., Zanetti,E., Potenza,L., Forghieri,F., Torelli,G., and Luppi,M. (2009). Indirect antitumor effects of mammalian target of rapamycin inhibitors against Kaposi sarcoma in transplant patients. Transplantation *88*, 597-598.

Bedi,G.C., Westra,W.H., Farzadegan,H., Pitha,P.M., and Sidransky,D. (1995). Microsatellite instability in primary neoplasms from HIV + patients. Nature Med. *1*, 65-68.

Belanger,C., Gravel,A., Tomoiu,A., Janelle,M.E., Gosselin,J., Tremblay,M.J., and Flamand,L. (2001). Human herpesvirus 8 viral FLICE-inhibitory protein inhibits Fas-mediated apoptosis through binding and prevention of procaspase-8 maturation. J. Hum. Virol. *4*, 62-73.

Beral,V., Peterman,T.A., Berkelman,R.L., and Jaffe,H.W. (1990). Kaposi's sarcoma among persons with AIDS: a sexually transmitted infection? Lancet *335*, 123-128.

Beral,V. (1991). Epidemiology of Kaposi's sarcoma. Cancer Surv. *10*, 5-22.

Betts,M.R., Nason,M.C., West,S.M., De Rosa,S.C., Migueles,S.A., Abraham,J., Lederman,M.M., Benito,J.M., Goepfert,P.A., Connors,M., Roederer,M., and Koup,R.A. (2006). HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. Blood *107*, 4781-4789.

Bickham,K., Munz,C., Tsang,M.L., Larsson,M., Fonteneau,J.F., Bhardwaj,N., and Steinman,R. (2001). EBNA1-specific CD4+ T cells in healthy carriers of Epstein-Barr virus are primarily Th1 in function. J. Clin. Invest. *107*, 121-130.

Bihl,F., Mosam,A., Henry,L.N., Chisholm,J.V.III., Dollard,S., Gumbi,P., Cassol,E., Page,T., Mueller,N., Kiepiela,P., Martin,J.N., Coovadia,H.M., Scadden,D.T., and Brander,C. (2007a). 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., Narayan,M., Chisholm,J.V.III., Henry,L.M., Suscovich,T.J., Brown,E.E., Welzel,T.M., Kaufmann,D.E., Zaman,T.M., Dollard,S., Martin,J.N., Wang,F., Scadden,D.T., Kaye,K.M., and Brander,C. (2007b). 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.

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

Blackbourn,D.J., Lennette,E.T., Ambroziak,J., Mourich,D.V., and Levy,J.A. (1998). Human herpesvirus 8 detection in nasal secretions and saliva. J. Infect. Dis. *177*, 213-216.

Blackbourn,D., Osmond,D., Levy,J., and Lennette,E. (1999). Increased human herpesvirus 8 seroprevalence in young homosexual men who have multiple sex contacts with different partners. J. Infect. Dis. *179*, 237-239.

Blasig,C., Zietz,C., Haar,B., Neipel,F., Esser,S., Brockmeyer,N.H., Tschachler,E., Colombini,S., Ensoli,B., and Sturzl,M. (1997). Monocytes in Kaposi's sarcoma lesions are productively infected by human herpesvirus 8. J. Virol. *71*, 7963-7968.

Boshoff,C., Schulz,T.F., Kennedy,M.M., Graham,A.K., Fisher,C., Thomas,A., McGee,J.O., Weiss,R.A., and O'Leary,J.J. (1995). Kaposi's sarcoma-associated herpesvirus infects endothelial and spindle cells. Nature Med. *1*, 1274-1278.

Boshoff,C. and Weiss,R.A. (2001). Epidemiology and pathogenesis of Kaposi's sarcoma-associated herpesvirus. Philos. Trans. R. Soc. Lond B Biol. Sci. *356*, 517-534.

Boshoff,C. and Weiss,R. (2002). AIDs-related malignancies. Nature Rev. Cancer *2*, 373-382.

Bourboulia,D., Whitby,D., Boshoff,C., Newton,R., Beral,V., Carrara,H., Lane,A., and Sitas,F. (1998). Serologic evidence for mother-to-child transmission of kaposi sarcoma-associated herpesvirus infection. JAMA *280*, 31-32.

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

Brander,C., O'Connor,P., Suscovich,T., Jones,N., Lee,Y., Kedes,D., Ganem,D., Martin,J., Osmond,D., Southwood,S., Sette,A., Walker,B., and Scadden,D. (2001). Definition of an optimal cytotoxic T lymphocyte epitope in the latently expressed Kaposi's sarcoma–associated herpesvirus kaposin protein. J. Infect. Dis. *184*, 119-126.

Breckpot,K., Dullaers,M., Bonehill,A., van,M.S., Heirman,C., de Greef,C., van der Bruggen,P., and Thielemans,K. (2003). Lentivirally transduced dendritic cells as a tool for cancer immunotherapy. J. Gene Med. *5*, 654-667.

Breckpot,K., Heirman,C., De Greef,C., van der Bruggen,P., and Thielemans,K. (2004). Identification of new antigenic peptide presented by HLA-Cw7 and encoded by several MAGE genes using dendritic cells transduced with lentiviruses. J. Immunol. *172*, 2232-2237.

Breckpot,K., Aerts,J.L., and Thielemans,K. (2007). Lentiviral vectors for cancer immunotherapy: transforming infectious particles into therapeutics. Gene Ther. *14*, 847-862.

Brenchley,J.M., Karandikar,N.J., Betts,M.R., Ambrozak,D.R., Hill,B.J., Crotty,L.E., Casazza,J.P., Kuruppu,J., Migueles,S.A., Connors,M., Roederer,M., Douek,D.C., and Koup,R.A. (2003). Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. Blood *101*, 2711-2720.

Burton,C.T., Gotch,F., and Imami,N. (2006). Rapid qualitative and quantitative analysis of T-cell responses in HIV-1-infected individuals receiving successful HAART and HIV-1 sero-negative controls: Concomitant assessment of perforin, IFN-gamma and IL-4 secretion. J. Immunol. Meth. *308*, 216-230.

Burysek,L., Yeow,W.S., and Pitha,P.M. (1999). Unique properties of a second human herpesvirus 8-encoded interferon regulatory factor (vIRF-2). J. Hum. Virol. *2*, 19-32.

Cai,X., Lu,S., Zhang,Z., Gonzalez,C.M., Damania,B., and Cullen,B.R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. Proc. Natl. Acad. Sci. U.S.A. *102*, 5570-5575.

Callan,M.F., Steven,N., Krausa,P., Wilson,J.D., Moss,P.A., Gillespie,G.M., Bell,J.I., Rickinson,A.B., and McMichael,A.J. (1996). Large clonal expansions of CD8+ T cells in acute infectious mononucleosis. Nature Med. *2*, 906-911.

Campbell,T., Borok,M., Ndemera,B., Fiorillo,S., White,I., Zhang,X., Machekano,R., Katzenstein,D., and Gwanzura,L. (2009). Lack of evidence for frequent heterosexual transmission of human herpesvirus 8 in Zimbabwe. Clin. Infect. Dis. *48*, 1601-1608.

Carbone,A., Cesarman,E., Spina,M., Gloghini,A., and Schulz,T.F. (2009). HIV-associated lymphomas and gamma-herpesviruses. Blood *113*, 1213-1224.

Carding,S.R. and Egan,P.J. (2002). Gamma delta T cells: functional plasticity and heterogeneity. Nature Rev. Immunol. *2*, 336-345.

Carlsson,B., Cheng,W.S., Totterman,T.H., and Essand,M. (2003). Ex vivo stimulation of cytomegalovirus (CMV)-specific T cells using CMV pp65-modified dendritic cells as stimulators. Br. J. Haematol. *121*, 428-438.

Carroll,P.A., Brazeau,E., and Lagunoff,M. (2004). Kaposi's sarcoma-associated herpesvirus infection of blood endothelial cells induces lymphatic differentiation. Virology *328*, 7-18.

Catalina,M.D., Sullivan,J.L., Brody,R.M., and Luzuriaga,K. (2002). Phenotypic and functional heterogeneity of EBV epitope-specific CD8+ T cells. J. Immunol. *168*, 4184-4191.

Cattani,P., Cerimele,F., Porta,D., Graffeo,R., Ranno,S., Marchetti,S., Ricci,R., Capodicasa,N., Fuga,L., Amico,R., Cherchi,G., Gazzilli,M., Zanetti,S., and Fadda,G. (2003). Age-specific seroprevalence of human herpesvirus 8 in Mediterranean regions. Clin. Microbiol. Infect. *9*, 274-279.

Cattani,P., Capuano,M., Graffeo,R., Ricci,R., Cerimele,F., Cerimele,D., Nanni,G., and Fadda,G. (2001). Kaposi's sarcoma associated with previous human herpesvirus 8 infection in kidney transplant recipients. J. Clin. Microbiol. *39*, 506-508.

Cattelan,A.M., Calabro,M.L., Aversa,S.M., Zanchetta,M., Meneghetti,F., De,R.A., and Chieco-Bianchi,L. (1999). Regression of AIDS-related Kaposi's sarcoma following antiretroviral therapy with protease inhibitors: biological correlates of clinical outcome. Eur. J. Cancer *35*, 1809-1815.

CDC Report (1981). Kaposi's sarcoma and Pneumocystis pneumonia among homosexual men--New York City and California. MMWR Morb. Mortal. Wkly. Rep. *30*, 305-308.

Cesarman,E., Chang,Y., Moore,P.S., Said,J.W., and Knowles,D.M. (1995). Kaposi's sarcoma-associated herpesvirus-like DNA sequences in AIDS-related body-cavity-based lymphomas. N. Engl. J. Med. *332*, 1186-1191.

Champagne,P., Ogg,G.S., King,A.S., Knabenhans,C., Ellefsen,K., Nobile,M., Appay,V., Rizzardi,G.P., Fleury,S., Lipp,M., Forster,R., Rowland-Jones,S., Sekaly,R.P., McMichael,A.J., and Pantaleo,G. (2001). Skewed maturation of memory HIV-specific CD8 T lymphocytes. Nature *410*, 106-111.

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

Chang,Y., Moore,P.S., Talbot,S.J., Boshoff,C.H., Zarkowska,T., Godden,K., Paterson,H., Weiss,R.A., and Mittnacht,S. (1996). Cyclin encoded by KS herpesvirus. Nature *382*, 410.

Chaudhary,P.M., Jasmin,A., Eby,M.T., and Hood,L. (1999). Modulation of the NF-kappa B pathway by virally encoded death effector domains-containing proteins. Oncogene *18*, 5738-5746.

Chen,G., Shankar,P., Lange,C., Valdez,H., Skolnik,P.R., Wu,L., Manjunath,N., and Lieberman,J. (2001). CD8 T cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection. Blood *98*, 156-164.

Chen,X., He,J., and Chang,L.J. (2004). Alteration of T cell immunity by lentiviral transduction of human monocyte-derived dendritic cells. Retrovirology. *1*, 37.

Chen,X., Wang,B., and Chang,L.J. (2006). Induction of primary anti-HIV CD4 and CD8 T cell responses by dendritic cells transduced with self-inactivating lentiviral vectors. Cellular Immunol. *243*, 10-18.

Chinnasamy,N., Chinnasamy,D., Toso,J.F., Lapointe,R., Candotti,F., Morgan,R.A., and Hwu,P. (2000). Efficient gene transfer to human peripheral blood monocyte-derived dendritic cells using human immunodeficiency virus type 1-based lentiviral vectors. Hum. Gene Ther. *11*, 1901-1909.

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

Cirone,M., Lucania,G., Bergamo,P., Trivedi,P., Frati,L., and Faggioni,A. (2007). Human herpesvirus 8 (HHV-8) inhibits monocyte differentiation into dendritic cells and impairs their immunostimulatory activity. Immunol. Lett. *113*, 40-46.

Collins,M.K. and Cerundolo,V. (2004). Gene therapy meets vaccine development. Trends Biotech. *22*, 623-626.

Cook-Mozaffari,P., Newton,R., Beral,V., and Burkitt,D.P. (1998). The geographical distribution of Kaposi's sarcoma and of lymphomas in Africa before the AIDS epidemic. Br. J. Cancer *78*, 1521-1528.

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

Cotter,M.A. and Robertson,E.S. (1999). The Latency-Associated Nuclear Antigen Tethers the Kaposi's Sarcoma-Associated Herpesvirus Genome to Host Chromosomes in Body Cavity-Based Lymphoma Cells. Virology *264*, 254-264.

Couty,J.P. and Gershengorn,M.C. (2004). Insights into the viral G protein-coupled receptor encoded by human herpesvirus type 8 (HHV-8). Biol. Cell *96*, 349-354.

Cronin,J., Zhang,X.Y., and Reiser,J. (2005). Altering the tropism of lentiviral vectors through pseudotyping. Curr. Gene Ther. *5*, 387-398.

Dairaghi,D.J., Fan,R.A., McMaster,B.E., Hanley,M.R., and Schall,T.J. (1999). HHV8-encoded vMIP-I selectively engages chemokine receptor CCR8. Agonist and antagonist profiles of viral chemokines. J. Biol. Chem. *274*, 21569-21574.

Davidovici,B., Karakis,I., Bourboulia,D., Ariad,S., Zong,J.C., Benharroch,D., Dupin,N., Weiss,R., Hayward,G., Sarov,B., and Boshoff,C. (2001). Seroepidemiology and molecular epidemiology of Kaposi's sarcoma-associated herpesvirus among Jewish population groups in Israel. J. Natl. Cancer Inst. *93*, 194-202.

Davis,M.A., Sturzl,M.A., Blasig,C., Schreier,A., Guo,H.G., Reitz,M., Opalenik,S.R., and Browning,P.J. (1997). Expression of human herpesvirus 8-encoded cyclin D in Kaposi's sarcoma spindle cells. J. Natl. Cancer Inst. *89*, 1868-1874.

de Thé,G., Bestetti,G., van Beveren,M., and Gessain,A. (1999). Prevalence of human herpesvirus 8 infection before the acquired immunodeficiency disease syndrome-related epidemic of Kaposi's sarcoma in East Africa. J. Natl. Cancer Inst. *91*, 1888-1889.

De Maria,A., Ferrazin,A., Ferrini,S., Ciccone,E., Terragna,A., and Moretta,L. (1992). Selective increase of a subset of T cell receptor gamma delta T lymphocytes in the peripheral blood of patients with human immunodeficiency virus type 1 infection. J. Infect. Dis. *165*, 917-919.

De Paoli,P., Gennari,D., Martelli,P., Basaglia,G., Crovatto,M., Battistin,S., and Santini,G. (1991). A subset of gamma delta lymphocytes is increased during HIV-1 infection. Clin. Exp. Immunol. *83*, 187-191.

Dechanet,J., Merville,P., Pitard,V., Lafarge,X., and Moreau,J.F. (1999). Human gamma delta T cells and viruses. Microbes Infection *1*, 213-217.

Decker,L.L., Shankar,P., Khan,G., Freeman,R.B., Dezube,B.J., Lieberman,J., and Thorley-Lawson,D.A. (1996). The Kaposi sarcoma-associated herpesvirus (KSHV) is present as an intact latent genome in KS tissue but replicates in the peripheral blood mononuclear cells of KS patients. J. Exp. Med *184*, 283-288.

Delenda,C. (2004). Lentiviral vectors: optimization of packaging, transduction and gene expression. J. Gene Med. *6 Suppl 1*, S125-S138.

Demaison,C., Parsley,K., Brouns,G., Scherr,M., Battmer,K., Kinnon,C., Grez,M., and Thrasher,A.J. (2002). High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. Hum. Gene Ther. *13*, 803-813.

Deusch,K., Luling,F., Reich,K., Classen,M., Wagner,H., and Pfeffer,K. (1991). A major fraction of human intraepithelial lymphocytes simultaneously expresses the gamma/delta T cell receptor, the CD8 accessory molecule and preferentially uses the V delta 1 gene segment. Eur. J. Immunol. *21*, 1053-1059.

Diamond,C., Brodie,S.J., Krieger,J.N., Huang,M.L., Koelle,D.M., Diem,K., Muthui,D., and Corey,L. (1998). Human herpesvirus 8 in the prostate glands of men with Kaposi's sarcoma. J. Virol. *72*, 6223-6227.

Direkze,S. and Laman,H. (2004). Regulation of growth signalling and cell cycle by Kaposi's sarcoma-associated herpesvirus genes. Int. J. Exp. Pathol. *85*, 305-319.

Djerbi,M., Screpanti,V., Catrina,A.I., Bogen,B., Biberfeld,P., and Grandien,A. (1999). The inhibitor of death receptor signaling, FLICE-inhibitory protein defines a new class of tumor progression factors. J. Exp. Med. *190*, 1025-1032.

Dukers,N.H.T.M., Renwick,N., Prins,M., Geskus,R.B., Schulz,T.F., Weverling,G.J., Coutinho,R.A., and Goudsmit,J. (2000). Risk factors for human herpesvirus 8 seropositivity and seroconversion in a cohort of homosexual men. Am. J. Epidemiol. *151*, 213-224.

Dullaers,M., Breckpot,K., Van Meirvenne,S., Bonehill,A., Tuyaerts,S., Michiels,A., Straetman,L., Heirman,C., De Greef,C., van der Bruggen,P., and Thielemans,K. (2004). Side-by-side comparison of lentivirally transduced and mRNA-electroporated dendritic cells: implications for cancer immunotherapy protocols. Mol. Ther. *10*, 768-779.

Dullaers,M. and Thielemans,K. (2006). From pathogen to medicine: HIV-1-derived lentiviral vectors as vehicles for dendritic cell based cancer immunotherapy. J. Gene Med. *8*, 3-17.

Dupin,N., Fisher,C., Kellam,P., Ariad,S., Tulliez,M., Franck,N., van Marck,E., Salmon,D., Gorin,I., Escande,J.P., Weiss,R.A., Alitalo,K., and Boshoff,C. (1999). Distribution of human herpesvirus-8 latently infected cells in Kaposi's sarcoma, multicentric Castleman's disease, and primary effusion lymphoma. Proc. Natl. Acad. Sci. U.S.A. *96*, 4546-4551.

Dupin,N., Diss,T.L., Kellam,P., Tulliez,M., Du,M.Q., Sicard,D., Weiss,R.A., Isaacson,P.G., and Boshoff,C. (2000). HHV-8 is associated with a plasmablastic variant of Castleman disease that is linked to HHV-8-positive plasmablastic lymphoma. Blood *95*, 1406-1412.

Duprez,R., Lacoste,V., Briere,J., Couppie,P., Frances,C., Sainte-Marie,D., Kassa-Kelembho,E., Lando,M.J., Essame Oyono,J.L., Nkegoum,B., Hbid,O., Mahe,A., Lebbe,C., Tortevoye,P., Huerre,M., and Gessain,A. (2007). Evidence for a multiclonal origin of multicentric advanced lesions of Kaposi sarcoma. J. Natl. Cancer Inst. *99*, 1086-1094.

Dupuy,F.P., Mouly,E., Mesel-Lemoine,M., Morel,C., Abriol,J., Cherai,M., Baillou,C., Negre,D., Cosset,F.L., Klatzmann,D., and Lemoine,F.M. (2005). Lentiviral transduction of human hematopoietic cells by HIV-1- and SIV-based vectors containing a bicistronic cassette driven by various internal promoters. J. Gene Med. *7*, 1158-1171.

Dyall,J., Latouche,J.B., Schnell,S., and Sadelain,M. (2001). Lentivirus-transduced human monocyte-derived dendritic cells efficiently stimulate antigen-specific cytotoxic T lymphocytes. Blood *97*, 114-121.

Ellis,M., Peng Chew,Y., Fallis,L., Freddersdorf,S., Boshoff,C., Weiss,R.A., Lu,X., and Mittnacht,S. (1999). Degradation of p27Kip cdk inhibitor triggered by Kaposi's sarcoma virus cyclin-cdk6 complex. EMBO J. *18*, 644-653.

Ensoli,B., Sgadari,C., Barillari,G., Sirianni,M.C., Sturzl,M., and Monini,P. (2001). Biology of Kaposi's sarcoma. Eur. J. Cancer *37*, 1251-1269.

Esslinger,C., Romero,P., and MacDonald,H.R. (2002). Efficient transduction of dendritic cells and induction of a T-cell response by third-generation lentivectors. Hum. Gene Ther. *13*, 1091-1100.

Field,N., Low,W., Daniels,M., Howell,S., Daviet,L., Boshoff,C., and Collins,M. (2003). KSHV vFLIP binds to IKK-gamma to activate IKK. J. Cell Sci. *116*, 3721-3728.

Fielding,C.A., McLoughlin,R.M., Colmont,C.S., Kovaleva,M., Harris,D.A., Rose-John,S., Topley,N., and Jones,S.A. (2005). Viral IL-6 blocks neutrophil infiltration during acute inflammation. J. Immunol. *175*, 4024-4029.

Fiorelli,V., Gendelman,R., Caterina Sirianni,M., Chang,H.K., Colombini,S., Markham,P.D., Monini,P., Sonnabend,J., Pintus,A., Gallo,R.C., and Ensoli,B. (1998). Interferon-gamma produced by CD8+ T cells infiltrating Kaposi's sarcoma induces spindle cells with angiogenic phenotype and synergy with human immunodeficiency virus-1 tat protein: an immune response to human herpesvirus-8 infection? Blood *91*, 956-967.

Firat,H., Zennou,V., Garcia-Pons,F., Ginhoux,F., Cochet,M., Danos,O., Lemonnier,F.A., Langlade-Demoyen,P., and Charneau,P. (2002). Use of a lentiviral flap vector for induction of CTL immunity against melanoma. Perspectives for immunotherapy. J. Gene Med. *4*, 38-45.

Fisch,P., Meuer,E., Pende,D., Rothenfusser,S., Viale,O., Kock,S., Ferrone,S., Fradelizi,D., Klein,G., Moretta,L., Rammensee,H.G., Boon,T., Coulie,P., and van der Bruggen,P. (1997). Control of B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human Vgamma9/Vdelta2 T cells in tumor immunity. Eur. J. Immunol. *27*, 3368-3379.

Follenzi,A., Ailles,L.E., Bakovic,S., Geuna,M., and Naldini,L. (2000). Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. Nature Genet. *25*, 217-222.

Foster-Cuevas,M., Wright,G.J., Puklavec,M.J., Brown,M.H., and Barclay,A.N. (2004). Human herpesvirus 8 K14 protein mimics CD200 in down-regulating macrophage activation through CD200 receptor. J. Virol. *78*, 7667-7676.

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

Gamadia,L.E., Rentenaar,R.J., van Lier,R.A.W., and Ten Berge,I.J.M. (2004). Properties of CD4+ T cells in human cytomegalovirus infection. Hum. Immunol. *65*, 486-492.

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

Gao,S.J., Boshoff,C., Jayachandra,S., Weiss,R.A., Chang,Y., and Moore,P.S. (1997). KSHV ORF K9 (vIRF) is an oncogene which inhibits the interferon signaling pathway. Oncogene *15*, 1979-1985.

Gessain,A., Mauclere,P., van Beveren,M., Plancoulaine,S., Ayouba,A., Essame-Oyono,J.L., Martin,P.M., and de Thé,G. (1999). Human herpesvirus 8 primary infection occurs during childhood in Cameroon, Central Africa. Int. J. Cancer *81*, 189-192.

Gessain,A. and Duprez,R. (2005). Spindle cells and their role in Kaposi's sarcoma. Int. J. Biochem. Cell Biol. *37*, 2457-2465.

Gill,P.S., Tsai,Y.C., Rao,A.P., Spruck,C.H.III., Zheng,T., Harrington,W.A., Jr., Cheung,T., Nathwani,B., and Jones,P.A. (1998). Evidence for multiclonality in multicentric Kaposi's sarcoma. Proc. Natl. Acad. Sci. U.S.A. *95*, 8257-8261.

Gill,J., Bourboulia,D., Wilkinson,J., Hayes,P., Cope,A., Marcelin,A.G., Calvez,V., Gotch,F., Boshoff,C., and Gazzard,B. (2002). Prospective study of the effects of antiretroviral therapy on Kaposi sarcoma--associated herpesvirus infection in patients with and without Kaposi sarcoma. J. Acquir. Immune. Defic. Syndr. *31*, 384-390.

Girardi,M. (2006). Immunosurveillance and immunoregulation by gamma delta T Cells. J. Invest. Dermatol. *126*, 25-31.

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

Godden-Kent,D., Talbot,S.J., Boshoff,C., Chang,Y., Moore,P., Weiss,R.A., and Mittnacht,S. (1997). The cyclin encoded by Kaposi's sarcoma-associated herpesvirus stimulates cdk6 to phosphorylate the retinoblastoma protein and histone H1. J. Virol. *71*, 4193-4198.

Godfrey,A., Anderson,J., Papanastasiou,A., Takeuchi,Y., and Boshoff,C. (2005). Inhibiting primary effusion lymphoma by lentiviral vectors encoding short hairpin RNA. Blood *105*, 2510-2518.

Gonzalez,C.M., Wong,E.L., Bowser,B.S., Hong,G.K., Kenney,S., and Damania,B. (2006). Identification and characterization of the Orf49 protein of Kaposi's sarcoma-associated herpesvirus. J. Virol. *80*, 3062-3070.

Gradoville,L., Gerlach,J., Grogan,E., Shedd,D., Nikiforow,S., Metroka,C., and Miller,G. (2000). Kaposi's sarcoma-associated herpesvirus open reading frame 50/Rta protein activates the entire viral lytic cycle in the HH-B2 primary effusion lymphoma cell line. J. Virol. *74*, 6207-6212.

Graham,F.L., Smiley,J., Russell,W.C., and Nairn,R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. J. Gen. Virol. *36*, 59-72.

Grandadam,M., Dupin,N., Calvez,V., Gorin,I., Blum,L., Kernbaum,S., Sicard,D., Buisson,Y., Agut,H., Escande,J.P., and Huraux,J.M. (1997). Exacerbations of clinical symptoms in human immunodeficiency virus type 1-infected patients with multicentric Castleman's disease are associated with a high increase in Kaposi's sarcoma herpesvirus DNA load in peripheral blood mononuclear cells. J. Infect. Dis. *175*, 1198-1201.

Grayson,W. and Pantanowitz,L. (2008). Histological variants of cutaneous Kaposi sarcoma. Diagn. Pathol. *3*, 31.

Gredmark-Russ,S., Cheung,E.J., Isaacson,M.K., Ploegh,H.L., and Grotenbreg,G.M. (2008). The CD8 T-cell response against murine gammaherpesvirus 68 is directed toward a broad repertoire of epitopes from both early and late antigens. J. Virol. *82*, 12205-12212.

Green,I., Espiritu,E., Ladanyi,M., Chaponda,R., Wieczorek,R., Gallo,L., and Feiner,H. (1995). Primary lymphomatous effusions in AIDS: a morphological, immunophenotypic, and molecular study. Mod. Pathol. *8*, 39-45.

Gruber,A., Kan-Mitchell,J., Kuhen,K.L., Mukai,T., and Wong-Staal,F. (2000). Dendritic cells transduced by multiply deleted HIV-1 vectors exhibit normal phenotypes and functions and elicit an HIV-specific cytotoxic T-lymphocyte response in vitro. Blood *96*, 1327-1333.

Grundhoff,A. and Ganem,D. (2004). Inefficient establishment of KSHV latency suggests an additional role for continued lytic replication in Kaposi sarcoma pathogenesis. J. Clin. Invest. *113*, 124-136.

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

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

Gupta,A.K., Ruvolo,V., Patterson,C., and Swaminathan,S. (2000). The human herpesvirus 8 homolog of Epstein-Barr virus SM protein (KS-SM) is a posttranscriptional activator of gene expression. J. Virol. *74*, 1038-1044.

Hamza,M.S., Reyes,R.A., Izumiya,Y., Wisdom,R., Kung,H.J., and Luciw,P.A. (2004). ORF36 protein kinase of Kaposi's sarcoma herpesvirus activates the c-Jun N-terminal kinase signaling pathway. J. Biol. Chem. *279*, 38325-38330.

He,Y., Zhang,J., Mi,Z., Robbins,P., and Falo,L.D.Jr. (2005). Immunization with lentiviral vector-transduced dendritic cells induces strong and long-lasting T cell responses and therapeutic immunity. J. Immunol. *174*, 3808-3817.

Heller,K.N., Arrey,F., Steinherz,P., Portlock,C., Chadburn,A., Kelly,K., and Munz,C. (2008). Patients with Epstein Barr virus-positive lymphomas have decreased CD4(+) T-cell responses to the viral nuclear antigen 1. Int. J. Cancer *123*, 2824-2831.

Hengge,U.R., Ruzicka,T., Tyring,S.K., Stuschke,M., Roggendorf,M., Schwartz,R.A., and Seeber,S. (2002). Update on Kaposi's sarcoma and other HHV8 associated diseases. Part 1: epidemiology, environmental predispositions, clinical manifestations, and therapy. The Lancet Infect. Dis. *2*, 281-292.

Hislop,A.D., Gudgeon,N.H., Callan,M.F.C., Fazou,C., Hasegawa,H., Salmon,M., and Rickinson,A.B. (2001). EBV-Specific CD8+ T cell memory: relationships between epitope specificity, cell phenotype, and immediate effector function. J. Immunol. *167*, 2019-2029.

Hislop,A.D., Annels,N.E., Gudgeon,N.H., Leese,A.M., and Rickinson,A.B. (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.

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

Hladik,W., Dollard,S.C., Downing,R.G., Kataaha,P., Pellett,P.E., Karon,J.M., Mermin,J., and Lackritz,E.M. (2003). Kaposi's sarcoma in Uganda: risk factors for human herpesvirus 8 infection among blood donors. J. Acquir. Immune. Defic. Syndr. *33*, 206-210.

Holtmeier,W. and Kabelitz,D. (2005). Gamma delta T cells link innate and adaptive immune responses. Chem. Immunol. Allergy *86*, 151-183.

Hong,Y.K., Foreman,K., Shin,J.W., Hirakawa,S., Curry,C.L., Sage,D.R., Libermann,T., Dezube,B.J., Fingeroth,J.D., and Detmar,M. (2004). Lymphatic reprogramming of blood vascular endothelium by Kaposi sarcoma-associated herpesvirus. Nature Genet. *36*, 683-685.

Howard,M.R., Whitby,D., Bahadur,G., Suggett,F., Boshoff,C., Tenant-Flowers,M., Schulz,T.F., Kirk,S., Matthews,S., Weller,I.V., Tedder,R.S., and Weiss,R.A. (1997). Detection of human herpesvirus 8 DNA in semen from HIV-infected individuals but not healthy semen donors. AIDS *11*, F15-F19.

Huang,Y.Q., Li,J.J., Kaplan,M.H., Poiesz,B., Katabira,E., Zhang,W.C., Feiner,D., and Friedman-Kien,A.E. (1995). Human herpesvirus-like nucleic acid in various forms of Kaposi's sarcoma. Lancet *345*, 759-761.

Jaffe,H.W. (2008). The early days of the HIV-AIDS epidemic in the USA. Nature Immunol. *9*, 1201-1203.

Jenne,L., Schuler,G., and Steinkasserer,A. (2001). Viral vectors for dendritic cell-based immunotherapy. Trends Immunol. *22*, 102-107.

Jenner,R.G., Alba,M.M., Boshoff,C., and Kellam,P. (2001). Kaposi's sarcoma-associated herpesvirus latent and lytic gene expression as revealed by DNA arrays. J. Virol. *75*, 891-902.

Jenner,R.G. and Boshoff,C. (2002). The molecular pathology of Kaposi's sarcoma-associated herpesvirus. Biochim. Biophys. Acta *1602*, 1-22.

Jeras,M., Bergant,M., and Repnik,U. (2005). In vitro preparation and functional assessment of human monocyte-derived dendritic cells--potential antigen-specific modulators of in vivo immune responses. Transpl. Immunol. *14*, 231-244.

Jones,J.L., Hanson,D.L., Dworkin,M.S., and Jaffe,H.W. (2000). Incidence and trends in Kaposi's sarcoma in the era of effective antiretroviral therapy. J. Acquir. Immune. Defic. Syndr. *24*, 270-274.

Jonuleit,H., Kuhn,U., Muller,G., Steinbrink,K., Paragnik,L., Schmitt,E., Knop,J., and Enk,A.H. (1997). Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions. Eur. J. Immunol. *27*, 3135-3142.

Jonuleit,H., Giesecke-Tuettenberg,A., Tuting,T., Thurner-Schuler,B., Stuge,T.B., Paragnik,L., Kandemir,A., Lee,P.P., Schuler,G., Knop,J., and Enk,A.H. (2001). A comparison of two types of dendritic cell as adjuvants for the induction of melanoma-specific T-cell responses in humans following intranodal injection. Int. J. Cancer *93*, 243-251.

Judde,J.G., Lacoste,V., Briere,J., Kassa-Kelembho,E., Clyti,E., Couppie,P., Buchrieser,C., Tulliez,M., Morvan,J., and Gessain,A. (2000). Monoclonality or oligoclonality of human herpesvirus 8 terminal repeat sequences in Kaposi's sarcoma and other diseases. J. Natl. Cancer Inst. *92*, 729-736.

Katano,H., Sato,Y., Kurata,T., Mori,S., and Sata,T. (2000). Expression and localization of human herpesvirus 8-encoded proteins in primary effusion lymphoma, Kaposi's sarcoma, and multicentric Castleman's disease. Virology *269*, 335-344.

Kedes,D.H., Operskalski,E., Busch,M., Kohn,R., Flood,J., and Ganem,D. (1996). The seroepidemiology of human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus): distribution of infection in KS risk groups and evidence for sexual transmission. Nature Med. *2*, 918-924.

Kern,F., Bunde,T., Faulhaber,N., Kiecker,F., Khatamzas,E., Rudawski,I.M., Pruss,A., Gratama,J.W., Volkmer-Engert,R., Ewert,R., Reinke,P., Volk,H.D., and Picker,L.J. (2002). Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. J. Infect. Dis. *185*, 1709-1716.

Kirk,C.J. and Mule,J.J. (2000). Gene-modified dendritic cells for use in tumor vaccines. Hum. Gene Ther. *11*, 797-806.

Kledal,T.N., Rosenkilde,M.M., Coulin,F., Simmons,G., Johnsen,A.H., Alouani,S., Power,C.A., Luttichau,H.R., Gerstoft,J., Clapham,P.R., Clark-Lewis,I., Wells,T.N., and Schwartz,T.W. (1997). A broad-spectrum chemokine antagonist encoded by Kaposi's sarcoma-associated herpesvirus. Science *277*, 1656-1659.

Knowles,D.M., Inghirami,G., Ubriaco,A., and Dalla-Favera,R. (1989). Molecular genetic analysis of three AIDS-associated neoplasms of uncertain lineage demonstrates their B-cell derivation and the possible pathogenetic role of the Epstein-Barr virus. Blood *73*, 792-799.

Koelle,D.M., Huang,M.L., Chandran,B., Vieira,J., Piepkorn,M., and Corey,L. (1997). Frequent detection of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) DNA in saliva of human immunodeficiency virus-infected men: clinical and immunologic correlates. J. Infect. Dis. *176*, 94-102.

Komanduri,K.V., Viswanathan,M.N., Wieder,E.D., Schmidt,D.K., Bredt,B.M., Jacobson,M.A., and McCune,J.M. (1998). Restoration of cytomegalovirus-specific CD4+ T-lymphocyte responses after ganciclovir and highly active antiretroviral therapy in individuals infected with HIV-1. Nature Med. *4* , 953-956.

Kostense,S., Koudstaal,W., Sprangers,M., Weverling,G.J., Penders,G., Helmus,N., Vogels,R., Bakker,M., Berkhout,B., Havenga,M., and Goudsmit,J. (2004). Adenovirus types 5 and 35 seroprevalence in AIDS risk groups supports type 35 as a vaccine vector. AIDS *18*, 1213-1216.

Koya,R.C., Kasahara,N., Favaro,P.M., Lau,R., Ta,H.Q., Weber,J.S., and Stripecke,R. (2003). Potent maturation of monocyte-derived dendritic cells after CD40L lentiviral gene delivery. J. Immunother. *26*, 451-460.

Lacoste,V., Mauclre,P., Dubreuil,G., Lewis,J., Georges-Courbot,M.C., and Gessain,A. (2000). KSHV-like herpesviruses in chimps and gorillas. Nature *407*, 151-152.

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

Lagos,D., Vart,R.J., Gratrix,F., Westrop,S.J., Emuss,V., Wong,P.P., Robey,R., Imami,N., Bower,M., Gotch,F., Boshoff,C. (2008). Toll-like receptor 4 mediates innate immunity to Kaposi's sarcoma herpesvirus. Cell Host Microbe. *13*, 470-83.

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

Lan,K., Kuppers,D.A., Verma,S.C., and Robertson,E.S. (2004). Kaposi's sarcoma-associated herpesvirus-encoded latency-associated nuclear antigen inhibits lytic replication by targeting Rta: a potential mechanism for virus-mediated control of latency. J. Virol. *78*, 6585-6594.

Landais,E., Saulquin,X., and Houssaint,E. (2005a). The human T cell immune response to Epstein-Barr virus. Int. J. Dev. Biol. *49*, 285-292.

Landais,E., Saulquin,X., Bonneville,M., and Houssaint,E. (2005b). Long-term MHC class II presentation of the EBV lytic protein BHRF1 by EBV latently infected B cells following capture of BHRF1 antigen. J. Immunol. *175*, 7939-7946.

Lebbé,C., de Crémoux,P., Millot,G., Podgorniak,M.P., Verola,O., Berger,R., Morel,P., and Calvo,F. (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.

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

Lee,B.S., Alvarez,X., Ishido,S., Lackner,A.A., and Jung,J.U. (2000). Inhibition of intracellular transport of B cell antigen receptor complexes by Kaposi's sarcoma-associated herpesvirus K1. J. Exp. Med. *192*, 11-22.

Lennette,E.T., Blackbourn,D.J., and Levy,J.A. (1996). Antibodies to human herpesvirus type 8 in the general population and in Kaposi's sarcoma patients. Lancet *348*, 858-861.

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

Li,M., Lee,H., Yoon,D.W., Albrecht,J.C., Fleckenstein,B., Neipel,F., and Jung,J.U. (1997). Kaposi's sarcoma-associated herpesvirus encodes a functional cyclin. J. Virol. *71*, 1984-1991.

Lichterfeld,M., Yu,X.G., Waring,M.T., Mui,S.K., Johnston,M.N., Cohen,D., Addo,M.M., Zaunders,J., Alter,G., Pae,E., Strick,D., Allen,T.M., Rosenberg,E.S., Walker,B.D., and Altfeld,M. (2004). HIV-1-specific cytotoxicity is preferentially mediated by a subset of CD8+ T cells producing both interferon-gamma and tumor necrosis factor-alpha. Blood *104*, 487-494.

Lim,C., Sohn,H., Gwack,Y., and Choe,J. (2000). Latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus (human herpesvirus-8) binds ATF4/CREB2 and inhibits its transcriptional activation activity. J. Gen. Virol. *81*, 2645-2652.

Lin,K., Dai,C.Y., and Ricciardi,R.P. (1998). Cloning and functional analysis of Kaposi's sarcoma-associated herpesvirus DNA polymerase and its processivity factor. J. Virol. *72*, 6228-6232.

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

Lizee,G., Gonzales,M.I., and Topalian,S.L. (2004). Lentivirus vector-mediated expression of tumor-associated epitopes by human antigen presenting cells. Hum. Gene Ther. *15*, 393-404.

Lopes,L., Fletcher,K., Ikeda,Y., and Collins,M. (2006). Lentiviral vector expression of tumour antigens in dendritic cells as an immunotherapeutic strategy. Cancer Immunol. Immunother. *55*, 1011-1016.

Louis,N., Evelegh,C., and Graham,F.L. (1997). Cloning and sequencing of the cellular-viral junctions from the human adenovirus type 5 transformed 293 cell line. Virology *233*, 423-429.

Lukac,D.M., Renne,R., Kirshner,J.R., and Ganem,D. (1998). Reactivation of Kaposi's sarcoma-associated herpesvirus infection from latency by expression of the ORF 50 transactivator, a homolog of the EBV R protein. Virology *252*, 304-312.

Lynne,J.E., Schmid,I., Matud,J.L., Hirji,K., Buessow,S., Shlian,D.M., and Giorgi,J.V. (1998). Major expansions of select CD8+ subsets in acute Epstein-Barr virus infection: comparison with chronic human immunodeficiency virus disease. J. Infect. Dis. *177*, 1083-1087.

Lyons,A.B. and Parish,C.R. (1994). Determination of lymphocyte division by flow cytometry. J. Immunol. Meth. *171*, 131-137.

Lyons,A.B. (2000). Analysing cell division in vivo and in vitro using flow cytometric measurement of CFSE dye dilution. J. Immunol. Meth. *243*, 147-154.

Maeurer,M.J., Martin,D., Walter,W., Liu,K., Zitvogel,L., Halusczcak,K., Rabinowich,H., Duquesnoy,R., Storkus,W., and Lotze,M.T. (1996). Human intestinal Vdelta1+ lymphocytes recognize tumor cells of epithelial origin. J. Exp. Med. *183*, 1681-1696.

Mangi,R.J., Niederman,J.C., Kelleher,J.E., Jr., Dwyer,J.M., Evans,A.S., and Kantor,F.S. (1974). Depression of cell-mediated immunity during acute infectious mononucleosis. N. Engl. J. Med. *291*, 1149-1153.

Mann,D.J., Child,E.S., Swanton,C., Laman,H., and Jones,N. (1999). Modulation of p27Kip1 levels by the cyclin encoded by Kaposi's sarcoma-associated herpesvirus. EMBO J. *18*, 654-663.

Martin,J.N., Ganem,D.E., Osmond,D.H., Page-Shafer,K.A., Macrae,D., and Kedes,D.H. (1998). Sexual transmission and the natural history of human herpesvirus 8 infection. N. Engl. J. Med. *338*, 948-954.

Mayama,S., Cuevas,L.E., Sheldon,J., Omar,O.H., Smith,D.H., Okong,P., Silvel,B., Hart,C.A., and Schulz,T.F. (1998). Prevalence and transmission of Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in Ugandan children and adolescents. Int. J. Cancer *77*, 817-820.

McDonald,A.C., Ragin,C.C., Jenkins,F.J., Weissfeld,J., Wilson,J., Wheeler,V.W., Wilson,J.B., Bunker,C.H., and Taioli,E. (2009). Human herpesvirus 8 seroprevalence among Tobago women and the role of sexual lifestyle behavior. J. Med. Virol. *81*, 264-270.

Micheletti,F., Monini,P., Fortini,C., Rimessi,P., Bazzaro,M., Andreoni,M., Giuliani,M., Traniello,S., Ensoli,B., and Gavioli,R. (2002). Identification of cytotoxic T lymphocyte epitopes of human herpesvirus 8. Immunology *106*, 395-403.

Miyake,K., Suzuki,N., Matsuoka,H., Tohyama,T., and Shimada,T. (1998). Stable integration of human immunodeficiency virus-based retroviral vectors into the chromosomes of nondividing cells. Hum. Gene Ther. *9*, 467-475.

Miyoshi,H., Blomer,U., Takahashi,M., Gage,F.H., and Verma,I.M. (1998). Development of a self-inactivating lentivirus vector. J. Virol. *72*, 8150-8157.

Moore,P.S., Gao,S.J., Dominguez,G., Cesarman,E., Lungu,O., Knowles,D.M., Garber,R., Pellett,P.E., McGeoch,D.J., and Chang,Y. (1996a). Primary characterization of a herpesvirus agent associated with Kaposi's sarcomae. J. Virol. *70*, 549-558.

Moore,P.S., Kingsley,L.A., Holmberg,S.D., Spira,T., Gupta,P., Hoover,D.R., Parry,J.P., Conley,L.J., Jaffe,H.W., and Chang,Y. (1996b). Kaposi's sarcoma-associated herpesvirus infection prior to onset of Kaposi's sarcoma. AIDS *10*, 175-180.

Moore,P.S. and Chang,Y. (2003). Kaposi's sarcoma-associated herpesvirus immunoevasion and tumorigenesis: two sides of the same coin? Annu. Rev. Microbiol. *57*, 609-639.

Morgan,D.G., Miller,G., Niederman,J.C., Smith,H.W., and Dowaliby,J.M. (1979). Site of Epstein-Barr virus replication in the oropharynx. Lancet *314*, 1154-1157.

Muralidhar,S., Veytsmann,G., Chandran,B., Ablashi,D., Doniger,J., and Rosenthal,L.J. (2000). Characterization of the human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus) oncogene, kaposin (ORF K12). J. Clin. Virol. *16*, 203-213.

Nagy,S., Gyulai,R., Kemeny,L., Szenohradszky,P., and Dobozy,A. (2000). Iatrogenic Kaposi's sarcoma: HHV8 positivity persists but the tumors regress almost completely without immunosuppressive therapy. Transplantation *69*, 2230-2231.

Naldini,L., Blomer,U., Gallay,P., Ory,D., Mulligan,R., Gage,F.H., Verma,I.M., and Trono,D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science *272*, 263-267.

Nealon,K., Newcomb,W.W., Pray,T.R., Craik,C.S., Brown,J.C., and Kedes,D.H. (2001). Lytic replication of Kaposi's sarcoma-associated herpesvirus results in the formation of multiple capsid species: isolation and molecular characterization of A, B, and C capsids from a gammaherpesvirus. J. Virol. *75*, 2866-2878.

Neipel,F., Albrecht,J.C., and Fleckenstein,B. (1997). Cell-homologous genes in the Kaposi's sarcoma-associated rhadinovirus human herpesvirus 8: determinants of its pathogenicity? J. Virol. *71*, 4187-4192.

Nilsen,T.W. (2007). Mechanisms of microRNA-mediated gene regulation in animal cells. Trends Genet. *23*, 243-249.

Oettle,A. (1962). Geographical and racial differences in the frequency of Kaposi's sarcoma as evidence of environmental or genetic causes. Acta Unio. Int. Contra. Cancrum. *18*, 330-363.

Oksenhendler,E., Duarte,M., Soulier,J., Cacoub,P., Welker,Y., Cadranel,J., Cazals-Hatem,D., Autran,B., Clauvel,J.P., and Raphael,M. (1996). Multicentric Castleman's disease in HIV infection: a clinical and pathological study of 20 patients. AIDS *10*, 61-67.

Okuyama,M., Laman,H., Kingsbury,S.R., Visintin,C., Leo,E., Eward,K.L., Stoeber,K., Boshoff,C., Williams,G.H., and Selwood,D.L. (2007). Small-molecule mimics of an alpha-helix for efficient transport of proteins into cells. Nature Meth. *4*, 153-159.

Olsen,S.J., Chang,Y., Moore,P.S., Biggar,R.J., and Melbye,M. (1998). Increasing Kaposi's sarcoma-associated herpesvirus seroprevalence with age in a highly Kaposi's sarcoma endemic region, Zambia in 1985. AIDS *12*, 1921-1925.

Olweny,C.L., Kaddumukasa,A., Atine,I., Owor,R., Magrath,I., and Ziegler,J.L. (1976). Childhood Kaposi's sarcoma: clinical features and therapy. Br. J. Cancer *33*, 555-560.

Osman,M., Kubo,T., Gill,J., Neipel,F., Becker,M., Smith,G., Weiss,R., Gazzard,B., Boshoff,C., and Gotch,F. (1999). Identification of human herpesvirus 8-specific cytotoxic T-cell responses. J. Virol. *73*, 6136-6140.

Palmer,B.E., Blyveis,N., Fontenot,A.P., and Wilson,C.C. (2005). Functional and phenotypic characterization of CD57+CD4+ T cells and their association with HIV-1-induced T cell dysfunction. J. Immunol. *175*, 8415-8423.

Palmowski,M.J., Lopes,L., Ikeda,Y., Salio,M., Cerundolo,V., and Collins,M.K. (2004). Intravenous injection of a lentiviral vector encoding NY-ESO-1 induces an effective CTL response. J. Immunol. *172*, 1582-1587.

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

Parker,K.C., Bednarek,M.A., and Coligan,J.E. (1994). Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains. J. Immunol. *152*, 163-175.

Parkin,D.M., Sitas,F., Chirenje,M., Stein,L., Abratt,R., and Wabinga,H. (2008). Part I: Cancer in indigenous Africans--burden, distribution, and trends. Lancet Oncol. *9*, 683-692.

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

Parravicini,C., Chandran,B., Corbellino,M., Berti,E., Paulli,M., Moore,P.S., and Chang,Y. (2000). Differential viral protein expression in Kaposi's sarcoma-associated herpesvirus-infected diseases : Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease. Am. J. Pathol. *156*, 743-749.

Pau,C.P., Lam,L.L., Spira,T.J., Black,J.B., Stewart,J.A., Pellett,P.E., and Respess,R.A. (1998). Mapping and serodiagnostic application of a dominant epitope within the human herpesvirus 8 ORF 65-encoded protein. J. Clin. Microbiol. *36*, 1574-1577.

Pellett,P.E., Wright,D.J., Engels,E.A., Ablashi,D.V., Dollard,S.C., Forghani,B., Glynn,S.A., Goedert,J.J., Jenkins,F.J., Lee,T.H., Neipel,F., Todd,D.S., Whitby,D., Nemo,G.J., and Busch,M.P. (2003). Multicenter comparison of serologic assays and estimation of human herpesvirus 8 seroprevalence among US blood donors. Transfusion *43*, 1260-1268.

Perez-Blas,M., Regueiro,J.R., Ruiz-Contreras,J.R., and Arnaiz-Villena,A. (1992). T lymphocyte anergy during acute infectious mononucleosis is restricted to the clonotypic receptor activation pathway. Clin. Exp. Immunol. *89*, 83-88.

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

Plancoulaine,S., Abel,L., van Beveren,M., Tregouet,D.A., Joubert,M., Tortevoye,P., de Thé,G., and Gessain,A. (2000). Human herpesvirus 8 transmission from mother to child and between siblings in an endemic population. Lancet *356*, 1062-1065.

Precopio,M.L., Sullivan,J.L., Willard,C., Somasundaran,M., and Luzuriaga,K. (2003). Differential kinetics and specificity of EBV-specific CD4+ and CD8+ T cells during primary infection. J. Immunol. *170*, 2590-2598.

Pudney,V.A., Leese,A.M., Rickinson,A.B., and Hislop,A.D. (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.

Rabkin,C.S., Janz,S., Lash,A., Coleman,A.E., Musaba,E., Liotta,L., Biggar,R.J., and Zhuang,Z. (1997). Monoclonal origin of multicentric Kaposi's sarcoma lesions. N. Engl. J. Med. *336*, 988-993.

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

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

Rappocciolo,G., Jenkins,F.J., Hensler,H.R., Piazza,P., Jais,M., Borowski,L., Watkins,S.C., and Rinaldo,C.R.Jr. (2006). DC-SIGN is a receptor for human herpesvirus 8 on dendritic cells and macrophages. J. Immunol. *176*, 1741-1749.

Regamey,N., Tamm,M., Wernli,M., Witschi,A., Thiel,G., Cathomas,G., and Erb,P. (1998). Transmission of human herpesvirus 8 infection from renal-transplant donors to recipients. N. Engl. J. Med. *339*, 1358-1363.

Renne,R., Barry,C., Dittmer,D., Compitello,N., Brown,P.O., and Ganem,D. (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.

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

Ribechini,E., Fortini,C., Marastoni,M., Traniello,S., Spisani,S., Monini,P., and Gavioli,R. (2006). Identification of CD8+ T cell epitopes within lytic antigens of human herpesvirus 8. J. Immunol. *176*, 923-930.

Rickinson,A.B. and Moss,D.J. (1997). Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection. Annu. Rev. Immunol. *15*, 405-431.

Robey,R.C., Lagos,D., Gratrix,F., Henderson,S., Matthews,N.C., Vart,R.J., Bower,M., Boshoff,C., and Gotch,F.M. (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.

Roederer,M., Dubs,J.G., Anderson,M.T., Raju,P.A., Herzenberg,L.A., and Herzenberg,L.A. (1995). CD8 naive T cell counts decrease progressively in HIV-infected adults. J. Clin. Invest. *95*, 2061-2066.

Romani,N., Reider,D., Heuer,M., Ebner,S., Kampgen,E., Eibl,B., Niederwieser,D., and Schuler,G. (1996). Generation of mature dendritic cells from human blood. An improved method with special regard to clinical applicability. J. Immunol. Meth. *196*, 137-151.

Rouas,R., Uch,R., Cleuter,Y., Jordier,F., Bagnis,C., Mannoni,P., Lewalle,P., Martiat,P., and Van den Broeke,A. (2002). Lentiviral-mediated gene delivery in human monocyte-derived dendritic cells: optimized design and procedures for highly efficient transduction compatible with clinical constraints. Cancer Gene Ther. *9*, 715-724.

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

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

Sallusto,F. and Lanzavecchia,A. (1994). Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J. Exp. Med. *179*, 1109-1118.

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

Sallusto,F., Geginat,J., and Lanzavecchia,A. (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu. Rev. Immunol *22*, 745-763.

Samols,M.A., Hu,J., Skalsky,R.L., and Renne,R. (2005). Cloning and identification of a microRNA cluster within the latency-associated region of Kaposi's sarcoma-associated herpesvirus. J. Virol. *79*, 9301-9305.

Samols,M.A., Skalsky,R.L., Maldonado,A.M., Riva,A., Lopez,M.C., Baker,H.V., and Renne,R. (2007). Identification of cellular genes targeted by KSHV-encoded microRNAs. PLoS Pathog. *3*, e65.

Sarid,R., Sato,T., Bohenzky,R.A., Russo,J.J., and Chang,Y. (1997). Kaposi's sarcoma-associated herpesvirus encodes a functional bcl-2 homologue. Nature Med. *3*, 293-298.

Sarid,R., Flore,O., Bohenzky,R.A., Chang,Y., and Moore,P.S. (1998). Transcription mapping of the Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) genome in a body cavity-based lymphoma cell line (BC-1). J. Virol. *72*, 1005-1012.

Schroers,R., Sinha,I., Segall,H., Schmidt-Wolf,I.G.H., Rooney,C.M., Brenner,M.K., Sutton,R.E., and Chen,S.Y. (2000). Transduction of human PBMC-derived dendritic cells and macrophages by an HIV-1-based lentiviral vector system. Mol. Ther. *1*, 171-179.

Schuler-Thurner,B., Schultz,E.S., Berger,T.G., Weinlich,G., Ebner,S., Woerl,P., Bender,A., Feuerstein,B., Fritsch,P.O., Romani,N., and Schuler,G. (2002). Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells. J. Exp. Med. *195*, 1279-1288.

Schuler,G., Schuler-Thurner,B., and Steinman,R.M. (2003). The use of dendritic cells in cancer immunotherapy. Curr. Opin. Immunol. *15*, 138-147.

Sharma-Walia,N., Naranatt,P.P., Krishnan,H.H., Zeng,L., and Chandran,B. (2004). Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 envelope glycoprotein gB induces the integrin-dependent focal adhesion kinase-Src-phosphatidylinositol 3-kinase-rho GTPase signal pathways and cytoskeletal rearrangements. J. Virol. *78*, 4207-4223.

Shortman,K. and Liu,Y.J. (2002). Mouse and human dendritic cell subtypes. Nature Rev. Immunol. *2*, 151-161.

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

Sirianni,M.C., Vincenzi,L., Fiorelli,V., Topino,S., Scala,E., Uccini,S., Angeloni,A., Faggioni,A., Cerimele,D., Cottoni,F., Aiuti,F., and Ensoli,B. (1998). Interferon gamma production in peripheral blood mononuclear cells and tumor infiltrating lymphocytes from Kaposi's sarcoma patients: correlation with the presence of human herpesvirus-8 in peripheral blood mononuclear cells and lesional macrophages. Blood *91*, 968-976.

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

Spiller,O.B., Robinson,M., O'Donnell,E., Milligan,S., Morgan,B.P., Davison,A.J., and Blackbourn,D.J. (2003). Complement regulation by Kaposi's sarcoma-associated herpesvirus ORF4 protein. J. Virol. *77*, 592-599.

Staras,S.A.S., Dollard,S.C., Radford,K.W., Flanders,W.D., Pass,R.F., and Cannon,M.J. (2006). Seroprevalence of cytomegalovirus infection in the United States, 1988-1994. Clin. Infect. Dis. *43*, 1143-1151.

Stebbing,J., Portsmouth,S., and Bower,M. (2003a). Insights into the molecular biology and sero-epidemiology of Kaposi's sarcoma. Curr. Opin. Infect. Dis. *16*, 25-31.

Stebbing,J., Bourboulia,D., Johnson,M., Henderson,S., Williams,I., Wilder,N., Tyrer,M., Youle,M., Imami,N., Kobu,T., Kuon,W., Sieper,J., Gotch,F., and Boshoff,C. (2003b). Kaposi's sarcoma-associated herpesvirus cytotoxic T lymphocytes recognize and target darwinian positively selected autologous K1 Epitopes. J. Virol. *77*, 4306-4314.

Steigerwald-Mullen,P., Kurilla,M.G., and Braciale,T.J. (2000). Type 2 cytokines predominate in the human CD4+ T-lymphocyte response to Epstein-Barr virus nuclear antigen 1. J. Virol. *74*, 6748-6759.

Stevenson,P.G., Belz,G.T., Altman,J.D., and Doherty,P.C. (1999). Changing patterns of dominance in the CD8+ T cell response during acute and persistent murine gamma-herpesvirus infection. Eur. J. Immunol. *29*, 1059-1067.

Stine,J.T., Wood,C., Hill,M., Epp,A., Raport,C.J., Schweickart,V.L., Endo,Y., Sasaki,T., Simmons,G., Boshoff,C., Clapham,P., Chang,Y., Moore,P., Gray,P.W., and Chantry,D. (2000). KSHV-encoded CC chemokine vMIP-III is a CCR4 agonist, stimulates angiogenesis, and selectively chemoattracts TH2 cells. Blood *95*, 1151-1157.

Stone,S.F., Price,P., Khan,N., Moss,P.A., and French,M.A. (2005). HIV patients on antiretroviral therapy have high frequencies of CD8 T cells specific for immediate early protein-1 of cytomegalovirus. AIDS *19*, 555-562.

Sun,Q., Zachariah,S., and Chaudhary,P.M. (2003). The human herpes virus 8-encoded viral FLICE-inhibitory protein induces cellular transformation via NF-kappaB activation. J. Biol. Chem. *278*, 52437-52445.

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

Sylwester,A.W., Mitchell,B.L., Edgar,J.B., Taormina,C., Pelte,C., Ruchti,F., Sleath,P.R., Grabstein,K.H., Hosken,N.A., Kern,F., Nelson,J.A., and Picker,L.J. (2005). Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. J. Exp. Med. *202*, 673-685.

Tan,P.H., Beutelspacher,S.C., Xue,S.A., Wang,Y.H., Mitchell,P., McAlister,J.C., Larkin,D.F., McClure,M.O., Stauss,H.J., Ritter,M.A., Lombardi,G., and George,A.J.T. (2005). Modulation of human dendritic-cell function following transduction with viral vectors: implications for gene therapy. Blood *105*, 3824-3832.

Taraboletti,G., Benelli,R., Borsotti,P., Rusnati,M., Presta,M., Giavazzi,R., Ruco,L., and Albini,A. (1999). Thrombospondin-1 inhibits Kaposi's sarcoma (KS) cell and HIV-1 Tat-induced angiogenesis and is poorly expressed in KS lesions. J. Pathol. *188*, 76-81.

Taylor,G.S., Long,H.M., Haigh,T.A., Larsen,M., Brooks,J., and Rickinson,A.B. (2006). A fole for intercellular antigen transfer in the recognition of EBV-transformed B cell lines by EBV nuclear antigen-specific CD4+ T cells. J. Immunol. *177*, 3746-3756.

Taylor,G.S. and Rickinson,A.B. (2007). Antigens and autophagy: the path less travelled? Autophagy. *3*, 60-62.

Torseth,J.W., Cohen,G.H., Eisenberg,R.J., Berman,P.W., Lasky,L.A., Cerini,C.P., Heilman,C.J., Kerwar,S., and Merigan,T.C. (1987). Native and recombinant herpes simplex virus type 1 envelope proteins induce human immune T-lymphocyte responses. J. Virol. *61*, 1532-1539.

Unutmaz,D., KewalRamani,V.N., Marmon,S., and Littman,D.R. (1999). Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J. Exp. Med. *189*, 1735-1746.

van Baarle,D., Kostense,S., van Oers,M.H.J., Hamann,D., and Miedema,F. (2002). Failing immune control as a result of impaired CD8+ T-cell maturation: CD27 might provide a clue. Trends Immunol. *23*, 586-591.

Vart,R.J., Nikitenko,L.L., Lagos,D., Trotter,M.W.B., Cannon,M., Bourboulia,D., Gratrix,F., Takeuchi,Y., and Boshoff,C. (2007). Kaposi's sarcoma-associated herpesvirus-encoded interleukin-6 and G-protein-coupled receptor regulate angiopoietin-2 expression in lymphatic endothelial cells. Cancer Res. *67*, 4042-4051.

Verschuren,E.W., Klefstrom,J., Evan,G.I., and Jones,N. (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., Huang,M.L., Koelle,D.M., and Corey,L. (1997). Transmissible Kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in saliva of men with a history of Kaposi's sarcoma. J. Virol. *71*, 7083-7087.

Wabinga,H.R., Parkin,D.M., Wabwire-Mangen,F., and Nambooze,S. (2000). Trends in cancer incidence in Kyadondo County, Uganda, 1960-1997. Br. J. Cancer *82*, 1585-1592.

Wallace,M.E., Keating,R., Heath,W.R., and Carbone,F.R. (1999). The cytotoxic T-cell response to herpes simplex virus type 1 infection of C57BL/6 mice is almost entirely directed against a single immunodominant determinant. J. Virol. *73*, 7619-7626.

Wang,Q., Jenkins,F., Jacobson,L., Meng,Y.X., Pellett,P., Kingsley,L., Kousoulas,K., Baghian,A., and Rinaldo,C.R.Jr. (2000). CD8+ Cytotoxic T lymphocyte responses to lytic proteins of human herpesvirus 8 in human immunodeficiency virus type 1 infected and uninfected individuals. J. Infect. Dis. *182*, 928-932.

Wang,F.Z., Akula,S.M., Pramod,N.P., Zeng,L., and Chandran,B. (2001a). Human herpesvirus 8 envelope glycoprotein K8.1A interaction with the target cells involves heparan sulfate. J. Virol. *75*, 7517-7527.

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

Wang,H.W., Sharp,T.V., Koumi,A., Koentges,G., and Boshoff,C. (2002a). Characterization of an anti-apoptotic glycoprotein encoded by Kaposi's sarcoma-associated herpesvirus which resembles a spliced variant of human survivin. EMBO J. *21*, 2602-2615.

Wang,Q.J., Huang,X.L., Rappocciolo,G., Jenkins,F.J., Hildebrand,W.H., Fan,Z., Thomas,E.K., and Rinaldo,C.R.Jr. (2002b). 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,F.Z., Akula,S.M., Sharma-Walia,N., Zeng,L., and Chandran,B. (2003). Human herpesvirus 8 envelope glycoprotein B mediates cell adhesion via its RGD sequence. J. Virol. *77*, 3131-3147.

Wang,H.W., Trotter,M.W., Lagos,D., Bourboulia,D., Henderson,S., Makinen,T., Elliman,S., Flanagan,A.M., Alitalo,K., and Boshoff,C. (2004). Kaposi sarcoma herpesvirus-induced cellular reprogramming contributes to the lymphatic endothelial gene expression in Kaposi sarcoma. Nature Genet. *36*, 687-693.

Weber,K.S., Grone,H.J., Rocken,M., Klier,C., Gu,S., Wank,R., Proudfoot,A.E., Nelson,P.J., and Weber,C. (2001). Selective recruitment of Th2-type cells and evasion from a cytotoxic immune response mediated by viral macrophage inhibitory protein-II. Eur. J. Immunol. *31*, 2458-2466.

Weninger,W., Partanen,T.A., Breiteneder-Geleff,S., Mayer,C., Kowalski,H., Mildner,M., Pammer,J., Sturzl,M., Kerjaschki,D., Alitalo,K., and Tschachler,E. (1999). Expression of vascular endothelial growth factor receptor-3 and podoplanin suggests a lymphatic endothelial cell origin of Kaposi's sarcoma tumor cells. Lab. Invest. *79*, 243-251.

Westrop,S.J., Grageda,N., and Imami,N. (2009). Novel approach to recognition of predicted HIV-1 Gag B3501-restricted CD8 T-cell epitopes by HLA-B3501(+) patients: confirmation by quantitative ELISpot analyses and characterisation using multimers. J. Immunol. Meth. *341*, 76-85.

Whitby,D., Howard,M.R., Tenant-Flowers,M., Brink,N.S., Copas,A., Boshoff,C., Hatzioannou,T., Suggett,F.E., Aldam,D.M., Denton,A.S., and . (1995). Detection of Kaposi sarcoma associated herpesvirus in peripheral blood of HIV-infected individuals and progression to Kaposi's sarcoma. Lancet *346*, 799-802.

Wilkinson,J., Cope,A., Gill,J., Bourboulia,D., Hayes,P., Imami,N., Kubo,T., Marcelin,A., Calvez,V., Weiss,R., Gazzard,B., Boshoff,C., and Gotch,F. (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.

Wills,M.R., Carmichael,A.J., Mynard,K., Jin,X., Weekes,M.P., Plachter,B., and Sissons,J.G. (1996). The human cytotoxic T-lymphocyte (CTL) response to cytomegalovirus is dominated by structural protein pp65: frequency, specificity, and T- cell receptor usage of pp65-specific CTL. J. Virol. *70*, 7569-7579.

Woodberry,T., Suscovich,T.J., Henry,L.M., Martin,J.N., Dollard,S., O'Connor,P.G., Davis,J.K., Osmond,D., Lee,T.H., Kedes,D.H., Khatri,A., Lee,J., Walker,B.D., Scadden,D.T., and Brander,C. (2005). Impact of Kaposi sarcoma-associated herpesvirus (KSHV) burden and HIV coinfection on the detection of T cell responses to KSHV ORF73 and ORF65 proteins. J. Infect. Dis. *192*, 622-629.

Wu,T.C., Guarnieri,F.G., Staveley-O'Carroll,K.F., Viscidi,R.P., Levitsky,H.I., Hedrick,L., Cho,K.R., August,J.T., and Pardoll,D.M. (1995). Engineering an intracellular pathway for major histocompatibility complex class II presentation of antigens. Proc. Natl. Acad. Sci. U.S.A. *92*, 11671-11675.

Wu,F.Y., Ahn,J.H., Alcendor,D.J., Jang,W.J., Xiao,J., Hayward,S.D., and Hayward,G.S. (2001). Origin-independent assembly of Kaposi's sarcoma-associated herpesvirus DNA replication compartments in transient cotransfection assays and association with the ORF-K8 protein and cellular PML. J. Virol. *75*, 1487-1506.

Yam,P.Y., Li,S., Wu,J., Hu,J., Zaia,J.A., and Yee,J.K. (2002). Design of HIV vectors for efficient gene delivery into human hematopoietic cells. Mol. Ther. *5*, 479-484.

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

Zarei,S., Abraham,S., Arrighi,J.F., Haller,O., Calzascia,T., Walker,P.R., Kundig,T.M., Hauser,C., and Piguet,V. (2004). Lentiviral transduction of dendritic cells confers protective antiviral immunity in vivo. J. Virol. *78*, 7843-7845.

Zhu,F.X., King,S.M., Smith,E.J., Levy,D.E., and Yuan,Y. (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.

Zhu,F.X., Chong,J.M., Wu,L., and Yuan,Y. (2005). Virion proteins of Kaposi's sarcoma-associated herpesvirus. J. Virol. *79*, 800-811.

Ziegelbauer,J.M., Sullivan,C.S., and Ganem,D. (2009). Tandem array-based expression screens identify host mRNA targets of virus-encoded microRNAs. Nature Genet. *41*, 130-134.

Zufferey,R., Dull,T., Mandel,R.J., Bukovsky,A., Quiroz,D., Naldini,L., and Trono,D. (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. J. Virol. *72*, 9873-9880.

Zufferey,R., Donello,J.E., Trono,D., and Hope,T.J. (1999). Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element Enhances Expression of Transgenes Delivered by Retroviral Vectors. J. Virol. *73*, 2886-2892.

SUPPLEMENTARY INFORMATION

S1: Summary of All Study Participants

Table S1 below summarises all study participants who contributed blood samples for use in the research described in this thesis. Each participant is cross-referenced to all the assays in which their samples were used, as well as each of the Tables in the main body of the thesis text in which the participant appears. Each participant's KSHV serostatus and HIV serostatus is summarised, along with their KSHV-related disease history and their KSHV-related disease status at the time of venesection. Further clinical phenotypes of participants can be found in Tables 2.6, 2.9, 2.13, 4.1 and 5.10, as indicated. Those participants for whom full low- and high-resolution HLA-types were determined are also indicated in Table S1 below.

Code	KSHV serostatus	HIV serostatus	KSHV-related Disease History	KSHV-related Disease Status	Assays	Table(s)	HLA-Typed?
HC1	NEG	NEG	None	N/A	Lentivirus screen (Ch. 4)	4.1 and 4.2	N
HC2	NEG	NEG	None	N/A	Lentivirus screen (Ch. 4)	4.1 and 4.2	N
HC3	NEG	NEG	None	N/A	Lentivirus screen (Ch. 4)	4.1 and 4.2	N
C122	NEG	POS	None	N/A	Lentivirus screen (Ch. 4)	2.6; 4.1; and 4.2	N
D403	NEG	POS	None	N/A	Lentivirus screen (Ch. 4)	2.6; 4.1; and 4.2	N
D919	NEG	POS	None	N/A	Lentivirus screen (Ch. 4)	2.6; 4.1; and 4.2	N
S810	NEG	POS	None	N/A	Lentivirus screen (Ch. 4)	2.6; 4.1; and 4.2	N
S346	POS	NEG	KS	Active	Lentivirus screen (Ch. 4)	4.1 and 4.2	N
W946	POS	NEG	KS	Active	Lentivirus screen (Ch. 4)	4.1 and 4.2	N
H419	POS (Asymptomatic)	POS	None	N/A	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; 4.3; and 5.2	Y
A556	POS	POS	KS	Remission	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; 4.3; and 5.2	Y
B196	POS	POS	KS	Remission	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; 4.3; and 5.2	Y*
B625	POS	POS	KS	Remission	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; and 4.3	N
D208	POS	POS	KS	Remission	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; and 4.3	N
K058	POS	POS	PEL	Remission	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; and 4.3	N
M620	POS	POS	KS	Remission	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; 4.3; and 5.2	Y
P940	POS	POS	KS and MCD	Remission	Lentivirus screen (Ch. 4) Peptide screen (Section 5.2)	2.6; 4.1; 4.2; 4.3; and 5.2 2.9	Y
S079	POS	POS	KS	Remission	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; and 4.3	N
T328	POS	POS	KS	Remission	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; and 4.3	N
T541	POS	POS	KS	Remission	Lentivirus screen (Ch. 4) Peptide screen (Section 5.2)	2.6; 4.1; 4.2; 4.3; and 5.2 2.9	Y
V689	POS	POS	KS and MCD	Remission	Lentivirus screen (Ch. 4)	2.6; 4.1; 4.2; 4.3; and 5.2	Y

TABLE S1: Summary of all study participants; cross-referenced with assays and tables in main body of thesis. POS = positive; NEG = negative; N = no; Y = yes; N/A = not applicable; * = HLA-A and HLA-B subtypes not determined due to insufficient DNA available.

Code	KSHV serostatus	HIV serostatus	KSHV-related Disease History	KSHV-related Disease Status	Assays	Table(s)	HLA-Typed?
HC-A	NEG	NEG	None	N/A	Peptide screen (Section 5.2) T-cell phenotyping (Section 5.3)	5.7 5.9	N
HC-B	NEG	NEG	None	N/A	Peptide screen (Section 5.2)	5.7	N
HC-C	NEG	NEG	None	N/A	Peptide screen (Section 5.2)	5.7	N
HC-D	NEG	NEG	None	N/A	Peptide screen (Section 5.2)	5.7	N
B042	NEG**	POS	KS	Remission	Peptide screen (Section 5.2)	2.9 and 5.7	Y
B066	POS	POS	KS	Remission	Peptide screen (Section 5.2)	2.9 and 5.7	Y
C448	POS	POS	KS	Remission	Peptide screen (Section 5.2) T-cell phenotyping (Section 5.3 & 5.4)	2.9 and 5.7 2.13 and 5.9	Y
D592	POS	POS	MCD	Remission	Peptide screen (Section 5.2)	2.9 and 5.7	Y
D850	POS	POS	MCD	Remission	Peptide screen (Section 5.2) T-cell phenotyping (Section 5.4)	2.9 and 5.7 2.13	Y
G386	POS	POS	MCD	Remission	Peptide screen (Section 5.2)	2.9 and 5.7	Y
H501	POS	POS	KS and MCD	Remission	Peptide screen (Section 5.2) T-cell phenotyping (Section 5.4)	2.9 and 5.7 2.13	Y
H980	POS	POS	KS	Remission	Peptide screen (Section 5.2)	2.9 and 5.7	Y
J228	POS	POS	MCD	Remission	Peptide screen (Section 5.2) T-cell phenotyping (Section 5.3 & 5.4)	2.9 and 5.7 2.13 and 5.9	Y
K331	POS	POS	KS	Remission	Peptide screen (Section 5.2) T-cell phenotyping (Section 5.3 & 5.4)	2.9 and 5.7 2.13 and 5.9	Y
M907	POS	POS	KS	Remission	Peptide screen (Section 5.2) T-cell phenotyping (Section 5.3)	2.9 and 5.7 5.9	Y
P869	POS	POS	KS and MCD	Remission	Peptide screen (Section 5.2) T-cell phenotyping (Section 5.3)	2.9 and 5.7 5.9	Y
S929	POS	POS	KS	Remission	Peptide screen (Section 5.2)	2.9 and 5.7	Y

TABLE S1 continued: Summary of all study participants; cross-referenced with assays and tables in main body of thesis. POS = positive; NEG = negative; N = no; Y = yes; N/A = not applicable; ** indicates KSHV-seronegative, although borderline with cut-off for positive (section 5.2.b).

Code	KSHV serostatus	HIV serostatus	KSHV-related Disease History	KSHV-related Disease Status	Assays	Table(s)	HLA-Typed?
B257	POS	POS	KS	Active	Peptide screen (Section 5.2) T-cell phenotyping (Section 5.3 & 5.4)	2.9 and 5.7 2.13 and 5.9	Y
B792	POS	POS	KS	Active	Peptide screen (Section 5.2)	2.9 and 5.7	Y
S314	POS	POS	KS	Active	Peptide screen (Section 5.2)	2.9 and 5.7	Y
B862	POS	POS	MCD	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
C473	POS	POS	KS	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
C991	POS	POS	KS	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
E043	POS	POS	KS	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
F667	POS	POS	KS	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
N047	POS	POS	MCD	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
S343	POS	POS	KS	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
S433	POS	POS	MCD	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
S583	POS	POS	MCD	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
V685	POS	POS	KS	Remission	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
E881	POS	POS	KS	Active	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
N484	POS	POS	KS	Active	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
N494	POS	POS	KS	Active	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
R632	POS	POS	KS	Active	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
V313	POS	POS	KS	Active	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
W375	POS	POS	KS	Active	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
W481	POS	POS	KS	Active	T-cell phenotyping (Section 5.4)	2.13 and 5.10	Y
O629	POS	POS	KS	Remission	Peptide screen (S2)	None	Y
T107	POS	POS	KS	Remission	Peptide screen & T-cell phenotype (S2)	None	Y

TABLE S1 continued: Summary of all study participants; cross-referenced with assays and tables in main body of thesis. POS = positive; NEG = negative; N = no; Y = yes; N/A = not applicable.

S2: A Novel Late Lytic KSHV Epitope

Two further peripheral blood samples were obtained from two KSHV-positive, HIV-positive, HLA-A*0201 study participants (O629 and T107). PBMCs were isolated and tested for recognition of the 11 putative epitopes derived from ORF28 and the nine previously identified epitopes, as described in section 5.2. Participants ages were: O629 = 45 years; T107 = 35 years. Their CD4 counts were: O629 = 96 cells/mm^3; T107 = 484 cells/mm^3. Both participants were male and receiving HAART and had an undetectable HIV viral load (<50 copies/mm^3). Both participants had a history of KS but were currently in remission having completed a course of systemic chemotherapy approximately six months prior to venesection. Results from ELISpots to test peptide recognition by the two participants are shown in Figure S1.

Both O629 and T107 responded to PHA, FEC and CMV-NLVPMATV. T107 also responded to HIV-SLYNTVATL (289.2 SFC per million PBMCs). O629 did not respond to any of the KSHV-derived peptides. T107, however, responded to both K8.1-LVLILYLCV (91.7 SFC per million PBMCs) and one of the novel late lytic KSHV epitopes derived from ORF28 (ORF28-P29; 40.0 SFC per million PBMCs). Thus the frequency of recognition of peptide ORF28-P29 was 7.1% in the whole cohort of 20 HIV-positive, KSHV-positive, HLA-A*0201-positive individuals tested (O629, T107 and the 18 participants discussed in section 5.2). Interestingly, although ORF28-P29 was identified as a candidate epitope in the HLA-A*0201-peptide binding and off-rate assays (section 5.1), it did not score particularly well compared to the other candidate

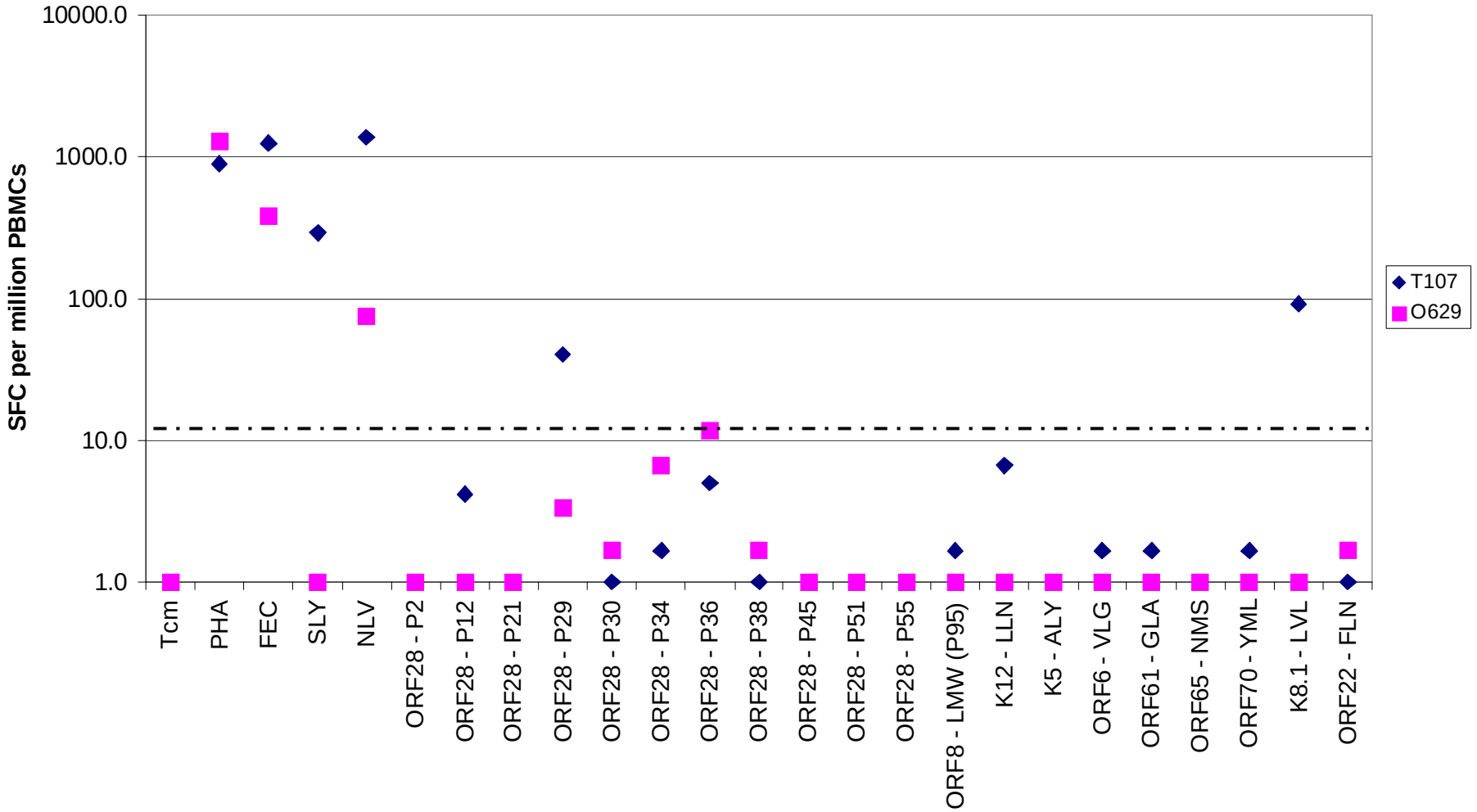


FIGURE S1: CD8 T-cell responses to A*0201-restricted peptides by two HIV-positive, KSHV-positive, HLA-A*0201-positive study participants. PHA and control peptides (FEC, SLY, NLV) were used at 5µg/ml, KSHV peptides were used at 20µg/ml. Responses shown as SFC per million PBMCs with background response to T-cell media alone (Tcm) subtracted. Responses below 1.0 were set to 1.0 to enable plotting of data on a logarithmic scale. Dashed line indicates positive response cut-off value of 20SFC per million PBMCs.

epitopes identified, ranking 9th out of 13 in the binding assay and 13th out of 13 in the off-rate assay (Table 5.5).

Despite its low frequency of recognition, this epitope represents a novel KSHV epitope derived from the late lytic glycoprotein ORF28, aa29-37 (IGLITVLFL). Furthermore, as eight of the nine previously identified KSHV epitopes were not recognised by any of the 20 KSHV-positive individuals, the new ORF28-P29 epitope may represent an epitope of equal, if not greater, biological importance than many of the current known epitopes.

A pentamer containing the KSHV peptide IGLITVLFL from ORF28 aa29-37 (ORF28-P29) was synthesised and used in an experiment to determine the memory phenotypes of CD8 T cells specific for this KSHV epitope from participant T107. Results are shown in Figure S2.

In this individual, the proportion of NLVPMVATV-specific T cells was unusually high (4.8% of total CD8 T cells). KSHV-specific T cells against ORF28-P29 were much lower in frequency (0.01%), in line with the data from the ELISpots (Section 5.2.c and Figure 5.5.b). The KSHV-specific T cells were skewed towards a T_{EM} phenotype (85.2% T_{EM} cells and 9.3% T_{EMRA} cells). Unfortunately, it would not be rational to attempt to draw conclusions from this single experiment, and it is worth noting that in this individual, CMV-NLVPMVATV-specific T cells were unusually highly skewed towards a T_{EM} phenotype (95.5%). Nonetheless, the pentamer containing the ORF28-P29 peptide may prove to be a useful tool for future investigations of this nature, if

more responders to the peptide can be identified. This would enable a more thorough exploration of the memory phenotypes of KSHV-specific CD8 T-cells during chronic infection and disease, as was originally proposed as an aim of this thesis.

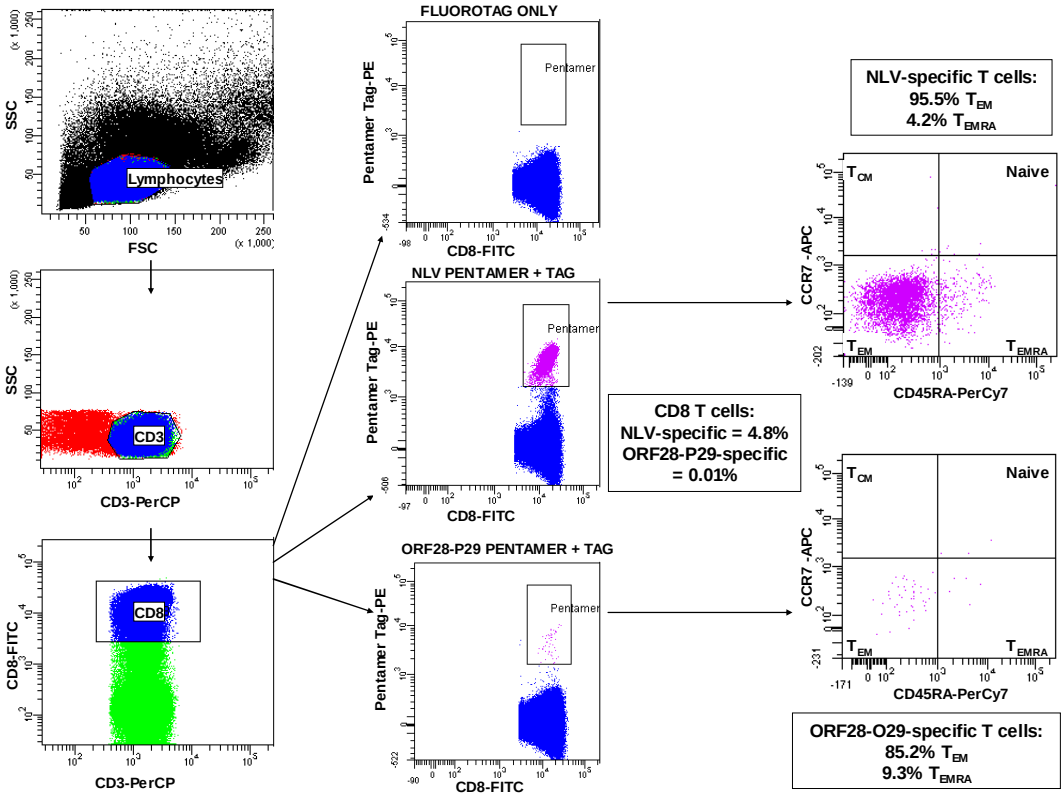


FIGURE S2: Comparison of memory phenotypes of CD8 T cells specific for CMV or for KSHV. Results shown are from one individual, T107.