Investigating the role of Epstein-Barr virus in the pathogenesis of NK and T cell lymphoproliferations

by

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Abstract

Epstein-Barr virus (EBV) is strongly associated with rare but aggressive lymphoproliferative diseases of NK and T cell origin. The finding of clonal and episomal forms of the virus in tumour cells from these clinically diverse diseases indicates involvement of EBV at an early stage of lymphomagenesis. However, many fundamental questions about EBV's contribution to pathogenesis remain unanswered. *In vivo* analyses herein found that infection of tonsillar (but not peripheral blood) T cells occasionally occurred in primary and persistent infection, whilst infection of NK cells was a rare event. By contrast, a high EBV load was found in peripheral blood NK cells from 3 adult patients with EBV-associated haemophagocytic-lymphohistiocytosis, a disease previously associated with $CD8^+$ T cell infection in children.

Complementary studies examined EBV latent gene expression in EBV^+ T/NK malignancies. Notwithstanding an apparent absence of latent membrane protein 2A and 2B (LMP2A/B), these tumour cells were recognised and killed by LMP2-specific cytotoxic T lymphocytes. This paradox was resolved by identifying a novel LMP2 mRNA, initiated from within the terminal repeat (TR) region of the viral genome and containing downstream epitope-encoding exons. Expression of LMP2-TR in T/NK cell lines and primary tissue implicates this truncated viral protein in T/NK lymphomagenesis.

Dedication

For my wife Ashini and our daughter Olivia Raaya, whose timely arrival coincided with the writing of this thesis.

For parents Fox and Jayasuriya for their enduring support, particularly Padmini’s valiant efforts in allowing some sleep during Olivia’s early weeks - and thus the production of this thesis!

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ABBREVIATIONS

aa	amino acid
ANKL	aggressive NK leukaemia
APC	antigen-presenting cell
β2m	beta 2 microglobulin
BART	*Bam*HI-A rightward transcript
BCR	B cell receptor
BL	Burkitt Lymphoma
bp	base-pair
CAEBV	chronic active EBV
C-terminal	carboxyl-terminal
cDNA	complementary deoxyribonucleic acid
CD	cluster differentiation (antigen)
CGH	comparative genomic hybridisation
CMV	cytomegalovirus
CR	complete remission
CSA	Cyclosporin A
CTAR	C-terminal activating region
CTL	cytotoxic T lymphocytes
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
EA	early antigen
EBER	Epstein-Barr virus-encoded small RNA
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr virus
EDTA	diaminoethanetetraacetic acid
EFS	event-free survival
ENKTL	extra-nodal NK/T lymphoma
FACS	fluorescence activated cell sorting

FAM	6-carboxy-fluorescein
Fc	fragment crystallisable (region)
FCS	foetal calf serum
FISH	fluorescent in situ hybridisation
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
gp	glycoprotein
GVHD	graft-versus-host disease
HDT	high-dose therapy
HINGS	heat inactivated goat serum
HIV	human immunodeficiency virus
HL	Hodgkin's Lymphoma
HLA	human leukocyte antigen
HLH	haemophagocytic lymphohistiocytosis
HSCT	haematopoietic stem-cell transplantation
IE	immediate early
Ig	Immunoglobulin
IHC	immunohistochemistry
IL	interleukin
IM	infectious mononucleosis
IPI	international prognostic index
ITAM	immunoreceptor tyrosine-based activation motif
IFN	interferon
IRF	interferon regulatory factor
JAK	janus kinase
kbp	kilobase pair
kDa	kiloDalton
KIR	killer immunoglobulin-like
KO	knock-out
LCL	lymphoblastoid cell line

LCV	lymphocryptovirus
LGL	large granular lymphocyte
LMP	latent membrane protein
LP	leader protein
MA	myeloablative
MAb	monoclonal antibody
MACS	magnetic activated sorting
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
miR	microRNA
MOI	multiplicity of infection
MNC	mononuclear cell
NCAM	neural cell adhesion molecule
N-terminal	amino-terminal
NFκB	nuclear factor kappa B
NGFR	nerve growth factor receptor
NK	natural killer
NPC	Nasopharyngeal carcinoma
ORF	open reading frames
oriP	origin of plasmid replication
OS	overall survival
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PNA	peptide nucleic acid
PTLD	post-transplant lymphoproliferative disease
PVDF	polyvinylidine difluoride
RACE	rapid amplification of cDNA ends
REAL	Revised European and American Lymphoma

RIC	reduced-intensity conditioning
RNA	ribonucleic acid
RS	Reed Sternberg
RT	reverse transcription
SAP	SLAM-associated protein
SCID	severe combined immunodeficient
SLAM	signals for lymphocyte activation molecule
SLT	secondary lymphoid tissue
SDS	sodium dodecyl sulphate
STAT	signalling transducer and activator of transcription
TAP	transporter associated with antigen processing
TCR	T cell receptor
TM	transmembrane
TNF	tumour necrosis factor
TR	terminal repeats
Tween	polyoxyethylenesorbitan monolaurate
VCA	viral capsid antigen
vIL-10	viral IL-10
WHO	World Health Organisation
WT	wild type
XLP	X-linked lymphoproliferative

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Introduction

Discovery of EBV

In 1957 whilst working at Mulago Hospital, Kampala, Uganda, a British surgeon named Denis Burkitt became intrigued by the unusual clinical presentation of jaw tumours in children, often associated with intra-abdominal tumours[1]. The curiously high prevalence of such extranodal tumours in areas of holoendemic malaria prompted his initial hypothesis that this may be caused by transmission of an infectious agent by mosquitos[2-3]. Subsequent collaboration between Denis Burkitt and Anthony Epstein led to the first lymphoid cell line grown in suspension *in vitro* – established from disaggregated fresh tissue of a Burkitt lymphoma biopsy, flown to the UK from Kampala (and subsequently named EB1[4]). A pellet of cells from EB1 was examined by electron microscopy by Dr Anthony Epstein, unequivocally demonstrating virus particles whose morphology suggested membership of the herpes family[5]. Subsequent virological, immunological and biochemical analyses confirmed that this was indeed a newly identified herpes virus[6-9], although no biological activity was initially demonstrable.

Within BL cell lines, a minority of cells hosted viral replication allowing development of an immunofluorescent assay that reacted with the viral capsid antigen (VCA)[9]. The detection of high viral antibody titres in the serum of BL patients and subsequently in carcinomas of the post-nasal space (nasopharyngeal carcinoma, NPC)[10] prompted seroepidemiological surveys of healthy adults. Unexpectedly, approximately 90% of the adult human population[11] appeared to have been exposed to the virus, now termed Epstein-Barr virus (EBV). It was later established that EBV was able to persist in serologically immune hosts; latent virus was found in B lymphocytes and active lytic replication was observed in the oropharynx[12]. A seminal study of 42 children and young adults with the clinical syndrome of infectious mononucleosis (IM) showed that they developed VCA antibodies in the acute stages of this illness, whereas their baseline sera were negative[13]. This and other evidence directly implicated EBV as the causative agent of IM[13-14].

Critically, it was also elucidated that EBV from irradiated BL cell lines or cell free virus could infect resting B lymphocytes *in vitro* resulting in transformed, continuously proliferating lymphoblastoid cell lines (LCLs). Together with its association with BL, this key demonstration of EBV's B-lymphotrophic transforming ability implicated it as a ubiquitous and potentially oncogenic virus[15-16].

Classification of EBV

Epstein-Barr virus (EBV), or Human Herpes virus 4 (HHV4), is one of eight known human herpesviruses, genetically stable infectious agents with large double-stranded DNA genomes that have evolved over millions of years to co-exist with our species and its antecedents.

This family is divided into Alpha (α), Beta (β) and Gamma (γ) sub-groups based on genome homology. The γ-viruses are further separable into two genera, γ-1 and γ-2, with different sets of latent cycle genes and molecular strategies that have evolved to induce latent cell proliferation. Kaposi's sarcoma herpesvirus (KSHV) and its distant relative, mouse herpesvirus 68 (MHV68), are classical γ-2 viruses (or rhadinoviruses), that can establish latency in B cells but have no autonomous capacity to drive B cell growth[17]. This contrasts with the ability of γ-1 viruses (or lymphocryptoviruses, LCV), a more recently evolved genus whose members are found only in primates, that can independently actuate B cell growth transformation[18]. EBV is considered the prototype LCV and has been found exclusively in humans. Each primate species is infected by a closely related member of the LCV family, similar in both genome structure and gene organisation to EBV[19].

EBV structure

EBV's nucleocapsid is composed of 162 capsomers, a protein tegument and an outer envelope with external glycoprotein spikes. The icosahedral viral capsid comprises 3 major tubular proteins and a number of minor virion proteins. The tegument comprises a mixture of cellular and virally encoded proteins, whereas the envelope is dominated by the virally encoded 350 or 220kDa glycoprotein (gp350/220)[20].

The EBV genome comprises a linear double-stranded DNA, approximately 184kb long[21-22], wrapped around a toroid-shaped protein core[23]. The genome is divided into long unique sequence domains by 3kbp internal direct reiterations[24].The prototype B95-8 strain, the first virus to be fully cloned and sequenced[21], is unusual as it has a 11.8kb deletion[22]. The complete sequences of two more strains of EBV have recently been determined: GD1[25], derived from a NPC tumour in a Chinese patient; and AG876[26], derived from a Ghanian BL patient. The ends of the genome are flanked by 4-12 tandem copies of the 538bp terminal repeats[27] (TR) which mediate episomal circularisation. The precise number of TRs is determined during viral replication which serves as a invaluable marker of clonality, as the progeny of latently infected cells retain an identical number of TRs to the parental cell[28]. Since the prototype sequencing was achieved by a BamHI fragment cloned library, genes and genomic locations are often referred to by specific BamHI fragments - alphabetically in order of descending fragment size (Figure 1). EBV can potentially encode approximately 80 proteins, some of which remain uncharacterised[29].

There are 2 major strain subtypes of EBV, referred to as type 1 and type 2, which can be further subdivided into different EBV strains[30]. All EBV isolates are highly homologous and share a common genome organisation[31]. The predominant sequence differences between type 1 and type 2 viruses relates to genes encoding the Epstein-Barr nuclear antigen (EBNA) 2, 3A, 3B and 3C[32]. EBV strains within a designated type can be distinguished on the basis of single nucleotide changes, sequence repeats and, notably, the presence or absence of a 30bp region within the latent membrane protein 1 (LMP1) gene[33-34].

Although it had been proposed that certain EBV gene polymorphisms are specifically associated with some malignancies and confer a more aggressive phenotype[35], this has not borne out; no definitive evidence exists that a given strain is more ‘tumourogenic’ than another. Rather, it appears that the majority of EBV gene polymorphisms discernible in healthy virus carriers occur at a similar frequency in EBV^+ tumours from the same geographical region[33].

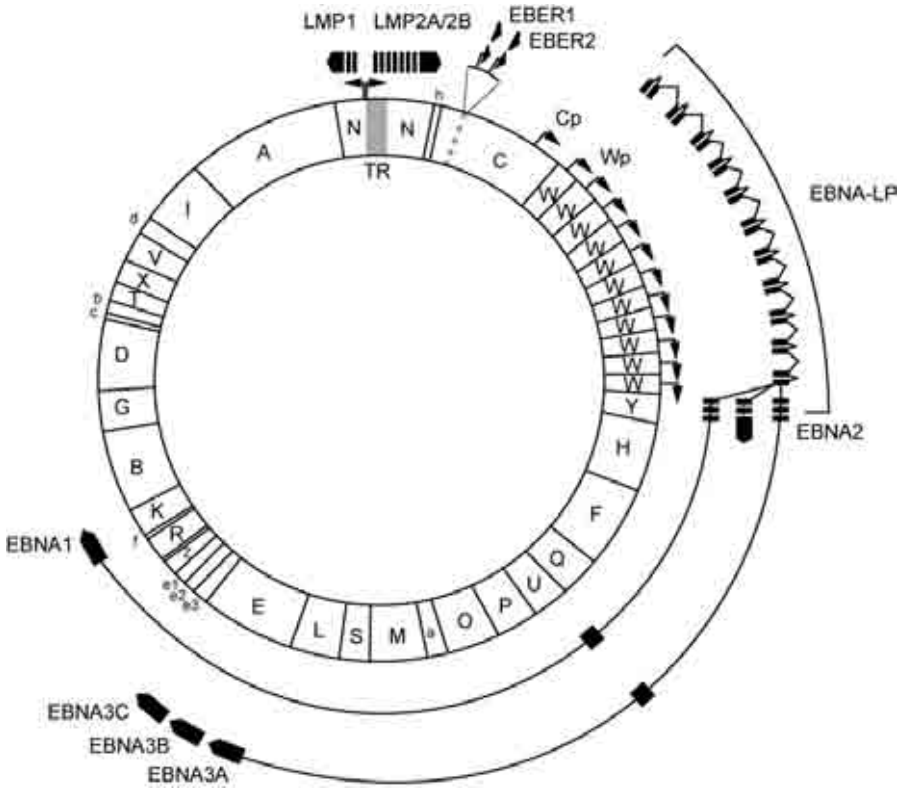


Figure 1 Map of EBV genome

Schematic map of the EBV genome divided into specific BamHI digest fragments, referred to alphabetically in order of descending fragment size. Arrows represent latent promoters, black filled segments represent genes encoding the latent proteins whilst the grey shaded region represents the terminal repeat region (TR).

Latent infection in transformed primary B cells

The Latency I, II and III nomenclature was originally established following key observations on the pattern of EBNA and LMP protein expression in BL and LCLs [36]. Exclusive expression of EBNA1, the viral protein essential for replication and maintenance of its own episome, was considered the most restricted form of latency: i.e. Latency I observed in BL. However, in non-dividing cells such as memory B lymphocytes in healthy carriers[37] EBNA1 appears not to be critical, thus the term Latency 0 is applied to such cells where no viral antigen expression is seen. These terms will be used for discussion of viral gene expression within this thesis, although caveats relating to such nomenclature, particular when applied to an *in vivo* context, do exist. For example, considerable variation is seen to exist in B cell lymphomas of the immunocompromised, both between

tumours[38] and - at the single cell level[39] - within a malignant population. Nonetheless, an accurate picture of latent gene expression within a tumour can usually be established by combining an assessment of viral proteins at the single cell level alongside quantitative RT-PCR to analyse the range of latent transcripts expressed.

Following *in vitro* infection of primary B lymphocytes, circularised viral genomes are appreciable within the nuclei 12 to 16 hours after virus binding. At around the same time, the W promoter (Wp), present in the BamHI-W long internal repeat element, initiates rightward transcription[40-41] . The B cell specificity of this promoter is facilitated by B cell lineage specific activator protein (BSAP, or Pax5) binding sites upstream of Wp[42-43]. Wp promotes the transcription of the initial viral RNAs, which are differentially spliced to generate mRNAs coding for the EBNA-LP and EBNA2 proteins. Transcription is undertaken by host cell RNA polymerase II.

Twenty-four to 36 hours following B cell infection, levels of EBNA-LP and EBNA2 protein are approximately equivalent to those seen in established LCLs[41]. These early latent proteins cooperate to initiate transcription from the upstream BamHI C promoter (Cp)[44-45]. The mutual exclusivity of Wp and Cp activity within a single cell results in Cp replacing Wp as the dominant promoter for transcription of the EBNAs[46]. The switch from Wp to Cp results in expression of the remaining EBNA transcripts[47]. All EBNA mRNAs have exons in common from the BamHI W viral genomic fragment but alternative splicing into one of 4 alternative acceptor sites determines whether EBNA3A, 3B, 3C or EBNA1 is translated[48], each of which encodes its own polyadenylation signal. Expression of these EBNAs occurs at approximately 24 hours following infection and reaches a plateau after 48-72 hours. A positive feedback loop enhancing Cp activity, initially mediated by EBNA2 and EBNA-LP and thereafter by EBNA1, is countered by the repressive action of the EBNA3 proteins[49]. In LCLs, transcription of EBV's latent membrane proteins is activated by EBNA2. The expression and regulation of LMP1 and LMP2 is discussed later under the individual latent gene headings (pages 9-17). The non-coding, non-polyadenylated EBER1 and EBER2 transcripts are the last of the latent transcripts to be expressed, reaching substantial expression levels 3 days after infection. Transcription is

predominantly mediated by cellular RNA polymerase III and EBERs are the most abundantly expressed EBV RNAs in established LCLs[50].

More recently a relatively large number of EBV-encoded microRNAs (miRs) have been identified[51-52], encoded within two primary transcripts: the BHRF1 transcript which also contains the BHRF1 open-reading frame (ORF); the miR-BARTS, processed from the introns of the long, alternatively spliced BARTS which contain two clusters of miRs, designated cluster 1 and cluster 2; and an isolated mirBART2 at the 3′ end of the BARTs. The BARTs are transcribed across a region of the viral genome that is deleted in the prototype B95-8 and thus removes the coding sequence for part of BART Cluster 1 and all of the Cluster 2 miRs. The miR-BARTs are expressed in all forms of latency and in lytic cycle, differing to the more restricted expression of the BHRF1-derived miRs. BHRF1 transcripts in latent infection are almost exclusively initiated from the Cp/Wp promoters[51-52].

Alternate patterns of latent gene expression

Figure 2 is adapted from reference[29] and represents a schematic of the four alternate patterns of viral gene expression observed in latent EBV infection.

Latency III

Following infection of primary B lymphocytes *in vitro,* almost all nuclear genome-positive B cells promptly express EBNA2 which initiates and drives the virus' growth transforming programme, inducing cell cycle transit (usually within 48-72 hours); ultimately leading to an established LCL[53]. Latently infected B cells express 6 EBV nuclear antigens (EBNAs), EBNA 1, 2, 3A, 3B, 3C and LP and 3 latent membrane proteins (LMPs), 1, 2A and 2B[54]. More recently, a tenth latent antigen expressed from Wp-initiated transcripts - the BHRF1 protein (a Bcl-2 homologue with anti-apoptotic function) - has also been associated with Latency III type infection[55]. In addition, small, non-polyadenylated EBER1 and EBER2 transcripts, the highly spliced *Bam*HI-A rightward transcripts (BARTs) and a number of EBV-encoded microRNAs (miRs)[52] are expressed. Such pattern of latent gene expression is referred to as Latency III[36], or the 'growth programme'[56].

The latency III pattern of gene expression seen in LCLs is inextricably accompanied by an equally characteristic cellular phenotype; markers of B cell activation and adhesion, usually absent (or weakly expressed) on resting B cells, are readily identified[57]; LCLs resemble lymphocytes proliferating in response to antigen, mitogen, or stimulation with CD40 and IL4[58-62]. Such a transformative impact of Latency III gene products on B cell phenotype implicated these viral proteins as key effectors of cell 'immortalisation' and, importantly, is analogous to EBV-driven B cell lymphoproliferations occurring in the context of impaired T cell immunity[39,63-64] (see PTLD, pages 47-50).

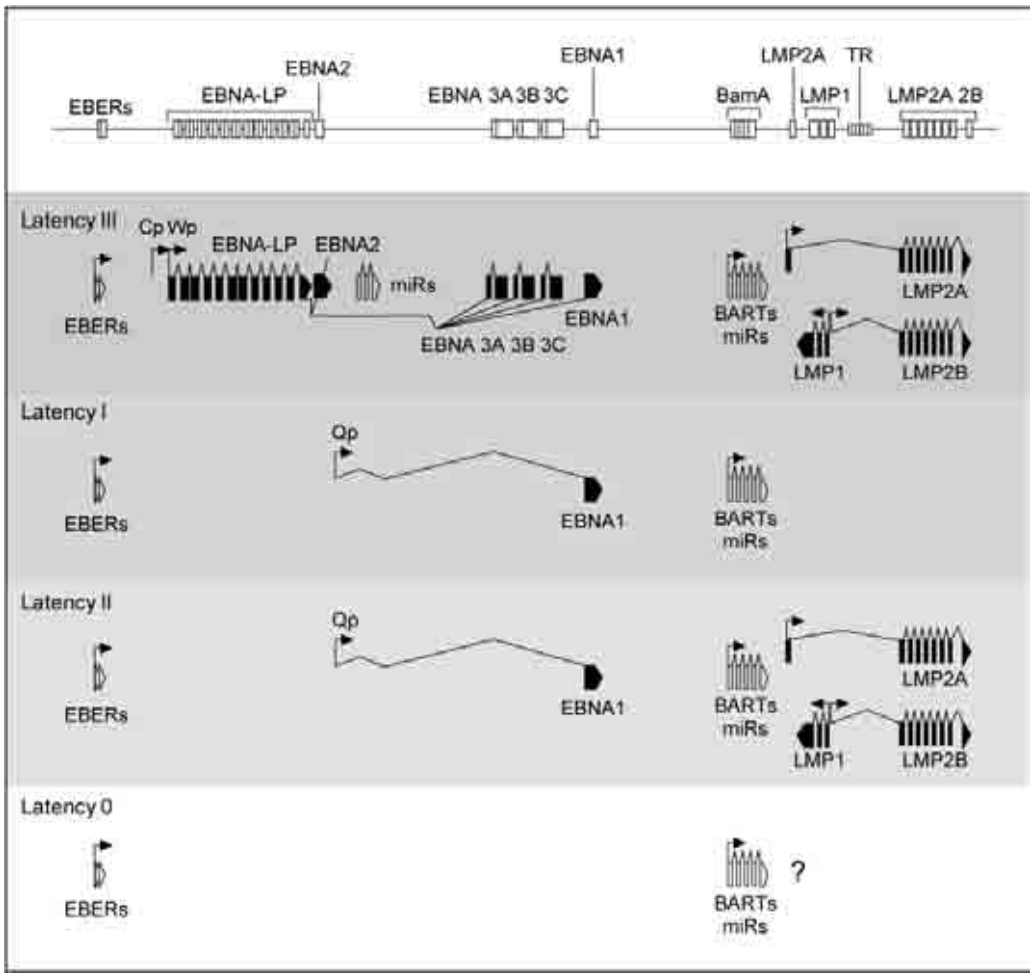


Figure 2 Patterns of latent gene expression

This figure is adapted from reference[29]. Boxes represent open-reading frames for latent genes; shaded boxes indicating those that are translated into viral protein, whereas open boxes represent non-coding viral RNAs. A linear EBV genome is shown at the top of the schematic.

Latency I

The characterisation of latent gene expression in EBV-associated tumours and cell lines established from BL biopsies highlighted two alternate patterns of Latency. EBNA-1 is the only protein consistently expressed in BL[57], alongside the non-coding EBERs, BARTs[65] and miRNAs[51]. This restricted pattern is referred to as Latency I and is characterised by activity of the Q promoter (Qp), for EBNA1 transcription, and silence of Cp, Wp, and the LMP promoters[66]. It should be noted, however, that isolated cells within a Burkitt tumour biopsy can express LMP1 and EBNA2[67] similar to the in vitro phenomenon of BL cell lines that are seen to 'drift' towards a Latency III phenotype.

Latency II

An intermediate pattern of EBV gene expression was originally identified in undifferentiated nasopharyngeal carcinoma (NPC) [68-70], and thereafter in EBV-associated gastric carcinomas[71], Hodgkin's lymphomas[72], and extra-nodal NK/T cell lymphomas (ENKTL)[73].

Akin to latency I, EBNA1 is transcribed from a highly spliced Q-U-K transcript driven by Qp, a TATA-less promoter whose configuration resembles the promoters of housekeeping genes - suggesting that Qp may act as a default promoter for EBNA1 in the absence of a 'growth program'[74]. This Q-U-K-spliced structure of the EBNA-1 transcript has been demonstrated in HL[75], NPC[70,76] and ENKTL[73]. As in BL, EBNA 1 expression is accompanied by the EBER and BART transcripts, but in Latency II additional latent membrane proteins (LMP1, LMP2A and LMP2B) are expressed (Figure 2). Acknowledging that these tumours arise from different cell lineages, and that cellular factors regulating LMP1 and LMP2 expression are yet to be delineated, it should be noted that heterogeneity of viral gene expression within the Latency II pattern exists. For example, LMP2 expression in the malignant cells of gastric carcinoma is at a low level compared to very high levels of both LMP1 and LMP2A in the Reed-Sternberg cells of HL, whilst expression of the LMP2 proteins in ENKTL has not been elucidated[54].

EBV Latent gene products

LMP 1

In LCLs, LMP1 is translated from an RNA transcribed from the BNLF1 gene at the right end of the virus genome[77-78] , initiating from a site (ED-L1) within the *Bam*HI N-*Eco*RI D fragment of the EBV genome[77,79]. The unprocessed transcript - which lies entirely within the antisense strand of the first intron of LMP2A - is spliced to a 2.4kb mRNA, with an ORF of 1.3kb which crosses two introns[77] (Figures 2 and 3). Later analyses of a nude mouse-passaged NPC detected two 3' co-terminal LMP1 transcripts of 2.8kb and 3.5kb, at apparently equal levels as assessed by Northern blotting[68]. An alternative (TATA-less[80]) LMP1 promoter (pL1-TR) was subsequently mapped within 20 bp of the start of the first TR - approximately 600 bp upstream of ED-L1 - and gives rise to the larger mRNA. Both transcripts utilise the same ATG initiator, hence the encoded LMP1 protein is identical. The 3.5kb mRNA was also found to be expressed in LCLs (IB4) and the BL Akata line, although levels of the 2.8kb mRNA were substantially higher in these B lymphoid cells[81].

By 36 hours following *in vitro* infection of primary B lymphocytes, LMP1 mRNA is measurable and, within the first few days, approaches a level similar to an established LCL[41,82]. The nucleotide sequence of the ORF predicted that LMP1 would comprise six hydrophobic transmembrane domains and a 200 amino acid acidic carboxy terminus. A monoclonal antibody, S12, confirmed that LMP 1 is distributed throughout the cytoplasmic and plasma membrane where it forms distinct aggregates[83-84].

Further characterisation of the structure confirmed that LMP1 is an integral membrane protein of 63 kDa and delineated three distinct domains. The amino-terminal cytoplasmic tail (aa 1–23) functions to tether LMP1 to the plasma membrane and orientates the protein; six hydrophobic transmembrane loops (aa 24–186) are involved in homo-oligomerisation and self-aggregation; an elongated carboxyterminal cytoplasmic region (amino acids 187–386) mediates most of the signalling activity of the molecule[85].

Interest in LMP1 as a crucial growth transforming protein of EBV was initially stimulated by a study demonstrating that LMP1 functions as a classic oncogene in a rodent fibroblast-transformation assay[86]. Subsequent studies corroborated its transformative properties by showing a requirement for LMP1 in EBV-induced B cell transformation *in vitro*[87-88]. Mutational analyses indicated that LMP1's cytoplasmic C-terminus and transmembrane domain 1 (TM1) were essential for B cell transformation[89-90]. Deletion of the cytoplasmic N-terminus adversely impacted, but did not prevent transformation[91]. *In vivo,* transgenic mice expressing LMP1 under the Ig heavy chain promoter developed monoclonal/oligoclonal splenic B cell lymphomas expressing LMP1 – confirming the potent oncogenic potential of this viral protein[92].

LMP1 has pleiotropic effects on cellular phenotype, resulting in the induction of cell-surface adhesion molecules and activation antigens[93], and upregulation of anti-apoptotic proteins (eg BCL2)[94]. LMP1 functions as a constitutively activated member of the tumour necrosis factor receptor (TNFR) superfamily and activates a range of signalling pathways in a ligand-independent manner[95-97]. Functionally, LMP1 resembles CD40 – a *bona fide* member of the TNFR superfamily - and can partially substitute for CD40, as shown in LMP1 transgenic mice, providing both growth and differentiation signals to B cells[98].

The activation of several downstream signalling pathways contributes to the multiple phenotypic consequences of LMP1 expression. Two distinct functional domains referred to as C-terminal activation regions 1 and 2 (CTAR1 and CTAR2) were identified by virtue of their ability to activate the nuclear factor B (NF-κB) transcription-factor pathway[99]. The signalling abilities of LMP1 result from the ability of tumour necrosis factor receptor (TNFR)-associated factors (TRAFs) to engage either directly with CTAR1 or indirectly by interacting with the death-domain-containing protein TRADD, which binds to CTAR2 [85]. This results in the recruitment of a multiprotein catalytic complex containing the NF-κB-inducing kinase (NIK), the IκB kinases (IKKs) with consequent activation of both the classic IκB-dependent[100] and independent[101-102] NF-κB pathways. Other kinases are also recruited to LMP1 through interactions with TRAF molecules, including mitogen-activated protein kinase kinase kinases (MAPKKKs), which in turn contribute to the activation of

the NF-κB, MAPK and phosphatidylinositol 3-kinase (PI3K) pathways[85]. Taken together, it is clear that LMP1 effectively utilises its position within the plasma membrane to influence a large number of cellular genes and signalling pathways to promote the transformation of B cells and epithelial cells.

Factors regulating LMP1 expression

The dominant (2.8kb) LMP1 mRNA found in LCLs is expressed from the ED-L1 site upon transcriptional activation by the EBNA-2 protein, which interacts with cellular transcription factors such as the repressor RBP-J kappa (CBF1 - involved in the Notch signalling pathway) and PU-1(Spi1). Both cellular proteins bind to distinct sites in the LMP-1 promoter [103-107]. By contrast, regulation of expression of LMP1 from the alternate, EBNA2-independent, L1-TR promoter is less well understood. pL1-TR is transcriptionally active in NPC cell lines[68,81], NPC tumour tissue[108] and Hodgkin lymphoma tissue[108] and appears to be the dominant LMP1 promoter in these tumours, although the PCR methods in these studies were not quantitative[124,108]. Sadler *et al* found that transcription was initiated from within the first TR but not from within distal TR elements and suggested that unique sequences adjacent to the first TR may contribute to promoter activity[109].

Chen *et al* found that both LMP1 promoters responded to activation of the JAK/STAT pathway, with evidence of functional STAT-binding motifs at both sites[108,110]. This feature is in common with STAT-mediated positive regulation of Qp-driven transcription of EBNA1[111] and, specifically, STAT3 is implicated as a biologically relevant candidate for activation of the Qp and L1-TR promoters[108]. Other cellular factors such as Sp1[112] and the unfolded protein response (UPR) protein XBP-1[113] are also thought to contribute to promoter activity.

It seems likely that the EBNA2-independent pL1-TR promoter is transcriptionally dominant in LMP1-expressing cells of EBV^+ NK and T cell lymphoproliferations, but this remains to be formally shown. LMP1 expression in ENKTL cell lines can be regulated by cytokines such as IL10[114] (a potent activator of STAT3), IL15 and IFNγ[115], although such cytokines were not sufficient for their

proliferation in the absence of IL-2. Recent work on Latency I BL lines found that IL-21 could potently induce LMP1 expression (in the absence of EBNA2), with the majority of the LMP-1 mRNA originating from the ED-L1 promoter[116].

Thus, it is feasible that rather than LMP-1 being a stable characteristic of a 'Latency II' EBV-infected cell, expression may be dynamic and influenced by extracellular signals.

LMP1 expression and function in T cells

Studies examining the role of LMP1 expression in T and NK cells are restricted to in vitro models expressing LMP1 in EBV-negative T cell lymphoma lines[117,118-121], whereas NK cells have not been studied in this context. Following from an initial observation that in vitro infection of a T cell lymphoma line with EBV resulted in up-regulation of TNF-α expression[120], it was subsequently shown that the candidate protein was LMP1[121]. Further work from the same group, investigating the pathogenesis of EBV-HLH, indicated that LMP-1-expressing T cells were relatively resistant to TNF-α-induced apoptosis likely due, in part, to downregulation of TNFR1. Moreover, TRADD was constitutively recruited by LMP-1 in T cells with consequent suppression of activity of apoptotic caspases 3, 8, and 9. These data suggested a potential mechanism whereby LMP1-expressing T cells in EBV-HLH could escape from cytokine-induced injury, although it must be noted that the pattern of EBV gene expression in EBV-HLH has not been clearly elucidated[122].

Interestingly, LMP1 also functions to suppress signals for lymphocyte activation molecule (SLAM)-associated protein (SAP)/*SH2D1A* gene expression in T cells, thereby inducing SLAM-mediated enhancement of ERK/IFN-γ signalling[118]. This LMP1-induced transcriptional suppression of SAP is mediated via activating transcription factor-5 (ATF5)[119]. Such deregulation of SAP/SLAM/ERK by LMP1 and enhanced IFNγ-secretion by T cells prompts comparison with the pathogenesis of X-linked lymphoproliferative disease, where mutations in the SAP gene similarly results in Th1 cytokine production[123-124].

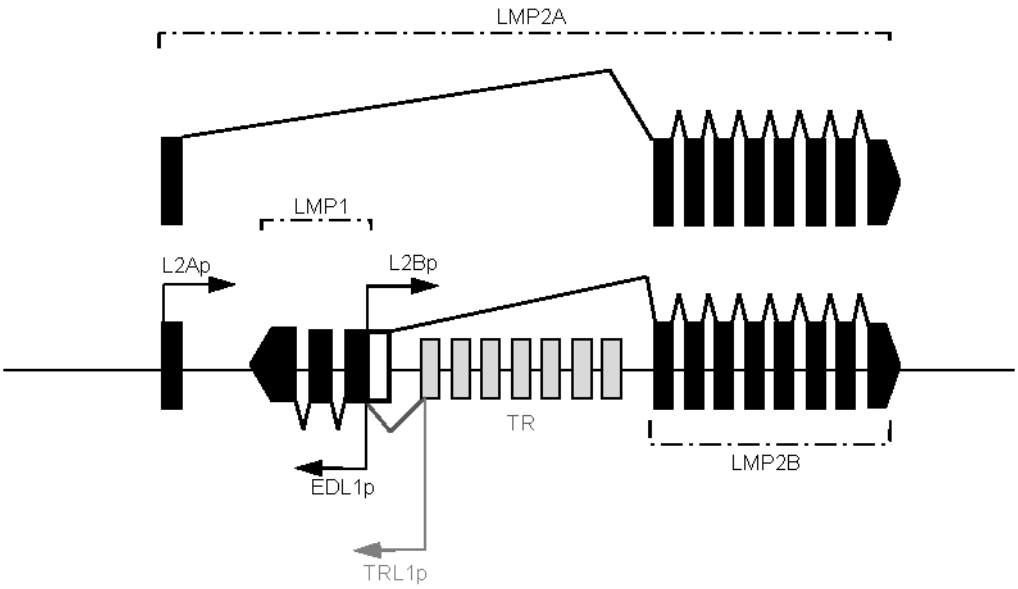


Figure 3 Schematic of LMP1 and LMP2 expression

A schematic of the region of the viral genome encompassing the LMP1, LMP2 genes and the terminal repeats. The arrows represent LMP promoters (L2Ap for LMP2A, L2Bp for LMP2B and EDL1p/TRL1p for LMP1). Shaded black segments represent coding exons and unshaded are non-coding. Splicing patterns are indicated by diagonal lines. Grey rectangles are the terminal repeats (TR)

LMP2A and LMP2B

Discovery

Two polyadenylated RNAs of 1.7 and 2.0kb were first identified by Northern blot analysis in 1985 and found to be constitutively expressed from the unique short region in latently infected B lymphocytes [79]. Subsequent analysis of the cDNA sequence of the larger mRNA showed that the gene consisted of nine exons and that the transcription unit crossed the terminal repeats[125]. The latter observation suggested that creation of a functional transcription unit required circularization of the linear viral DNA molecule at the terminal repeat region. These mRNAs were initially termed TP (terminal protein) 1 (LMP2A) and TP2 (LMP2B) transcripts. Characterisation of the smaller, 1.7 kb TP2 mRNA revealed its close relation to TP1 mRNA: the TP2 transcript was found to be expressed from a different promoter with a distinct 5' exon from TP1, but was also found to splice across the terminal repeats[126-127]. The remaining eight exons are common to the TP1 and TP2 RNAs (Figure 3). The TP2 promoter was identified 3.3 kb downstream of the TP1 promoter and found to form part of a bidirectional latent EBV promoter region transcribing the TP2 and the LMP1 RNAs in rightward and leftward directions respectively[126]. One principal open reading frame was identified in each of the TP1 and TP2 mRNAs. The initial observation that these mRNAs were predicted to encode 54kDa and 40kDa integral membrane proteins in latently infected cells, led to the now established terminology of latent membrane 2A and 2B[127].

Expression and regulation

The LMP2A first exon (here termed exon 1A) contains an initiator of translation resulting in a 119 amino-acid (aa) terminal hydrophilic sequence. The LMP2B first exon (exon 1B) is non-coding and its translation initiates at a methionine codon (corresponding to aa 120 of LMP2A) near the beginning of exon 2, downstream of the TR region. This methionine codon immediately precedes the first of the 12 transmembrane (TM) domains, encoded by eight exons common to both LMP2 species [127]. The highly hydrophobic TM domains are separated by short reverse turns and followed by a 27 aa C-terminal cytosolic tail.

Initial attempts to immunoprecipitate LMP2A and LMP2B *in vitro* translation products with EBV-immune human serum were unsuccessful and no antibody response could be elicited following early attempts of immunization of rats[127]. The relatively weak immunogenicity of the recombinant protein is likely attributable to the high degree of hydrophobicity[128]. Experimental confirmation of LMP2 protein expression in almost all cells in latently infected lymphoblastoid cell lines was achieved using antibodies purified from immunised rabbits[129]. Importantly, this study also provided evidence of the association of LMP2 expression with latent infection.

Antibodies to LMP2A were subsequently detected in sera from 16 of 42 nasopharyngeal carcinoma patients[128]. Later studies generated rat monoclonal antibodies specific for the N-terminal domain of LMP2A, by immunization with bacterial trpE-LMP2A fusion protein[130], allowing its detection in tissue sections and cell lines[130-132]. To-date, there are no available reagents to detect the LMP2B protein which lacks the N-terminus. However, PCR-based methods to distinguish between the LMP2 species are well-established[76,133], capitalising on the unique first exons for design of 5' PCR primers.

Initial observations on EBV^+ BL cell lines and LCLs showed that LMP2A was not expressed in the absence of the viral protein EBNA2[134]. It then became clear that regulation of LMP2 expression in B lymphocytes is dependent upon EBNA2, expressed early in the full growth transforming programme of the virus. This is achieved via transactivation of the LMP2A and 2B promoters[134] via an EBNA2 responsive cis-element[135]. However EBNA2 is not expressed in 'latency II' tumours and alternate factors initiating LMP2 expression in this context have been less clear.

Function

LMP2A and LMP2B form aggregates in the plasma membrane of B lymphocytes[129] and, whilst understood to be non-essential for EBV-induced B-cell transformation in vitro[136], some evidence exists that LMP2A confers a proliferative and survival advantage to B cells in the absence of signalling through the B-cell receptor (BCR)[137]. The N-terminal domain of LMP2A has been shown to have important signalling function; altering B cell receptor signalling by interacting with

cellular tyrosine kinases, such as Lyn and Syk, via its ITAM motifs. Similarly to LMP1, LMP2A has been shown to have a pro-survival function in B cells[138-141]. LMP2A can activate the Ras/PI3K/Akt pathway[138,141-142], the MAP kinase pathway[143], NFκB[144-145] and Bcl-2 and Bcl-x[141]. In addition to providing a developmental signal that bypasses the requirement for Ig heavy chain gene expression in bone marrow, LMP2A appears to provide a pro-survival signal to progenitor and peripheral B cells as evidenced by the increase in $CD19^{+}IgM^{-}$ cells in the splenic tissue of Eμ-LMP2A transgenic mice. Moreover, the Eμ-LMP2A transgene allows IgM^{-} B cells to mature into $CD43^{-}$ cells in the bone marrow and thereafter colonise the spleen in a RAG-$1^{-/-}$ mouse model[137]. These data indicate that LMP2A can drive the proliferation and survival of B cells in the absence of signalling through the B-cell receptor.

LMP2A contributes to transformation of epithelial cells *in vitro* and is thought to promote their adhesion and motility[146], the latter effect mediated by the tyrosine kinase Syk[147]. A role for LMP2A in the pathogenesis of NPC is further suggested by its effect on epithelial cell migration[148] and invasion in primary epithelial cell models[149].

The function of LMP2B, in both B cells and epithelial cells, remains much less clear. LMP2B has been shown to co-localise with LMP2A in B cells although the exact nature of their interaction remains to be clarified[150-151] and such co-localisation may not occur in epithelial cells[152]. Co-expression of LMP2B with LMP2A in a B cell model did not affect LMP2A localisation, but LMP2B did appear to block tyrosine phosphorylation of the amino-terminus of LMP2A. By impairing phosphorylation, LMP2B is understood to restore normal BCR signal transduction and levels of Lyn protein[151]. The implication of this finding is that LMP2B expression negatively regulates LMP2A activity, at least in B cells. Over-expression of LMP2B in EBV^{+} BL cells augmented switching from latent to lytic cycle following BCR cross-linking[153-154], providing further supportive evidence for the 'negative regulation' of LMP2A by LMP2B. However, recent work in epithelial cells suggests a 'positive' function of LMP2B showing that both LMP2A and 2B are able to attenuate cellular IFN responses by targeting the IFNα and IFNγ receptors for degradation[155].

No data exists on the function of the LMP2 proteins in EBV-infected NK and T cells, indeed the level and pattern of expression in these cell types has not yet been clarified.

Functional interactions of LMP1 and LMP2

LMP1 and LMP2A/B, are frequently co-expressed in EBV-associated tumours such as NPC, HL, and PTLD [75-76,107,156] and share common features such as an integral membrane topology and signalling function mediated by their cytoplasmic domains. As discussed above, individually, these viral proteins have pleiotropic effects on cell phenotype. However, studies evaluating their functional relationship are limited, in contrast to the detailed analyses of CD40/BCR interactions in B cells[157-158].

Dawson *et al* [152] found that LMP2A augmented the signalling capacity of LMP1 by stabilisation and half-life prolongation of the LMP1 protein in epithelial cell lines. This indirect effect of LMP2A on signalling activation was found to be dose-dependent and postulated to be mediated by the N-terminus, since LMP2B appeared not to augment LMP1 signalling. Although such an effect was not apparent in EBV^+ immunoblastic lymphoma lines, knockdown of respective mRNAs demonstrated that the constitutive NF-κB activity requires both LMP1 and LMP2A; both proteins play a role in the activation of this pathway [159]. Individual silencing of LMP1 and LMP2A in this system had comparable effects on increased sensitivity to apoptosis, although no synergistic effect was apparent.

EBNAs

EBNA1

EBNA1 is expressed in all virus-infected cells, with the exception of Latency 0 memory B lymphocytes[160], where it functions to maintain and replicate the episomal viral genome - achieved through sequence-specific binding to the plasmid origin of viral replication, OriP[29]. The interaction of cellular DNA, EBNA1 and oriP results in a single replication of the EBV genome during S phase, followed by equal segregation of replicated EBV genomes to progeny cells during cell division[161].

The amino- and carboxy-terminal domains of EBNA 1 are separated by a variably-sized Gly-Ala repeat sequence, the chief function of which appears to be stabilisation of the mature protein, preventing its proteasomal breakdown degradation[162] and/or suppressing EBNA1 mRNA translation initiation[163]. EBNA1 is not critical for B-cell transformation *in vitro*, but seems to substantially promote the efficiency of this process[164]. A more direct effect on lymphomagenesis is inferred by the ability of B-cell directed EBNA1 expression to produce B-cell lymphomas in transgenic mice[165] and by evidence of pro-survival effects in BL cells in vitro[166]

Expression of EBNA1 is augmented by a number of cellular factors, including the interferon regulatory factors, IRF1 and 2 and the cell cycle E2F transcription factors; all of which bind to distinct regions close to the (Fp/Qp) EBNA1 transcription initiation site[66,167]. In restricted forms of latency, EBNA1 restrains its own transcription by binding downstream of the transcriptional start site.

The function of EBNA1 in EBV-associated NK and T lymphoproliferations has been largely uninvestigated. An isolated report showed that partial silencing of EBNA1 expression in an ENKTL line retarded cell proliferation, associated with an accumulation of cells in G_0/G_1 phase[168], although the mechanism was not clearly delineated.

EBNA 2

An early clue to the essential role of the EBNA2 protein in B cell transformation came from the inability of the EBNA2 deletion mutant virus, the P3HR1 strain, to establish transformed primary B cells[169-171]. Reinstatement of the deleted DNA region restored the virus' transformative properties, delineated its functional elements and confirmed EBNA2's critical role in B cell immortalisation in vitro[172].

EBNA2 functions as a potent transcriptional activator of both viral and cellular genes and upregulates specific B cell antigens such as CD21 and CD23, in addition to LMP1 and LMP2. The protein contains a number of distinct structural elements, most notably the RBP-Jκ binding

domain, the acidic activation domain and the homotypic association domains - all considered essential to the transformation and transcriptional properties of EBNA2[29].

EBNA2 is not expressed in EBV^+ NK and T cell lymphoproliferations or other tumours with restricted patterns of latency, where the Wp/Cp promoters are dormant, and will therefore not be discussed further. Similarly, other Latency III-associated proteins (LP, EBNA3A,B,C, BHRF1) are also not expressed in T/NK lymphoproliferations and will only be discussed briefly below.

EBNA LP

EBNA-LP, encoded by the leader of each of the EBNA mRNAs, encodes a protein of variable size according to the number of BamHW repeats within a given viral isolate[173]. The major role of EBNA-LP is to specifically potentiate the effect of EBNA2-mediated transcriptional regulation[174] of both viral (eg LMP1[175]) and cellular genes – the latter exemplified by cyclin D2-mediated induction of G0 to G1 cell cycle transit[176].

EBNA 3 family

Each of these large, related, multifunctional proteins has a highly charged N terminus, dimerisation domains and a nuclear localisation region. Despite expression of relatively few EBNA3 mRNA transcripts per infected cell, the stability of all 3 proteins results in intranuclear accumulation[177-179]. Degradation of the EBNA3s leads to the presentation of highly immunogenic peptides by surface MHC class I, serving as attractive targets for $CD8^+$ CTLs[180].

The EBNA3 proteins impart significant influence on EBV-mediated viral and cellular transcription. A common conserved region allows binding of RBP-Jκ, resulting in competition with EBNA2 and Notch for RBP-Jκ and thus negatively regulating EBNA-2 activity[181]. EBNA3A and EBNA3C are essential for B-cell transformation *in vitro*[182] ,whilst EBNA2B appears not be critical in this system. In addition to their key role as transcriptional regulators, accumulating evidence implicates the EBNA3 family in EBV-mediated lymphomagenesis[54]. For example EBNA3C is seen to cooperate with RAS in rodent-fibroblast transformation assays, deregulating cell-cycle checkpoints[183].

Other proteins expressed in latent infection

BHRF1

Although primarily a lytic antigen, BHRF1 (a homologue of the cellular BCL-2 protein[94]) has recently been identified in the context of latent infection in LCLs and a subset of BL cells. The 17kDa protein is thought to be non-essential for virus replication but may contribute to cellular transformation *in vitro*[184]. Notably, it is consistently expressed in BL cells where the Wp promoter (rather than Qp) is activated – termed Wp-restricted latency[55]. Such expression is thought to confer a survival advantage to the tumour cells.

BARF1

The BARF1 ORF encodes a soluble molecule of 33kDa that is quickly secreted into the medium of cultured cells[185-186]. Initially thought to be exclusively expressed during early lytic cycle, its presence in the context of NPC and BL – seemingly in the absence of replicative infection – prompted consideration of a putative oncogenic function[185,187]. Of interest, BARF1 transcripts were identified in cell lines derived from patients with extra-nodal NK/T lymphoma (ENKTL) and chronic active EBV (CAEBV), without evidence of full lytic cycle, at levels comparable to a LCL[188]. By contrast, levels of BHRF1 mRNA in these T and NK lines were considerably lower than the LCL control.

Non-coding transcripts

EBERs

In addition to protein-coding transcripts, all types of latent EBV-infected cells contain abundant non-polyadenylated, non-coding transcripts generated by viral hijack of cellular RNA polymerase III. EBER1 (166 nucleotides) and EBER2 (172 nucleotides) form stem-loop structures by intermolecular base-pairing, resulting in double-stranded RNA (dsRNA)-like molecules which are the most highly expressed viral transcripts in latently infected cells[189-191]; estimated at 10^7 RNA copies per B cell. That the EBERs are so widely and abundantly expressed in latently-infected cells, provides a highly sensitive and specific diagnostic indicator of EBV infection *ex-vivo*: both at

the single cell level via *in-situ* hybridisation[192] and by RT-Q-PCR of RNA derived from tissue biopsies and cell lines[82]. Although seemingly ubiquitous in latent infection, irrespective of cell type, an isolated report has shown that cells undergoing replicative infection may not express EBERs – at least in the context of oral hairy leucoplakia[193].

Some controversy exists as to whether EBERs are required for transformation of B cells *in vitro*. Initial work indicated that they were dispensable[194] whilst more recent studies found that loss of EBER2 but not EBER1 considerably impaired the B lymphocyte transformation ability of EBV[195]. With some difficulty, B cells could be transformed with the EBER KO recombinants but growth of the resultant EBER KO LCLs was sluggish although, interestingly, could be enhanced by IL-6 supplementation. EBERs have also been shown to induce anti-inflammatory cytokines, such as IL10[196] and type I interferon[197], mediated through RIG-1 (retinoic acid-inducible gene-1). Recently, a paracrine effect of EBERs was demonstrated, inducing signalling through toll-like receptors (TLR) and the subsequent release of type I IFN and proinflammatory cytokines such as TNFα[198]. This 'immune activating' effect may be relevant to the immunopathology of diseases such as IM, CAEBV and HLH, in which high levels of EBER1 were found in *ex vivo* serum samples from most cases examined[198].

Whilst the EBERs seem to have some influence upon cellular phenotype and play a role in immunomodulation, the mechanistic details are not well understood and their role in disease pathogenesis remains unclear. In particular, their impact on the phenotype of EBV-associated NK and T cell lymphoproliferations has not been investigated.

BARTs

Originally identified in NPC tissue, an intricate cluster of non-polyadenylated RNAs are transcribed from the BamHI A region[68,199] and are collectively termed BamHI A rightward transcripts (BARTs). The primary transcripts contain a number of potential ORFs but uncertainty still exists as to whether or not these rightward transcripts encode protein[200-201], but they do yield a number of EBV miRs (discussed below). Similarly to the EBERs they are found to be widely

expressed across the spectrum of EBV-associated malignancies[73,75,202] and in peripheral blood B lymphocytes[203]. The biological function of the BARTs is not clear, but it has been proposed that they may potentially regulate gene transcription or modulate kinase activity[200].

EBV-encoded micro-RNAs

Fully processed miRNAs (miRs) are short (21–24 nucleotides), single-stranded RNAs derived from double-stranded RNA precursors that post-transcriptionally regulate gene expression. This is most commonly achieved by binding to sequences within the 3' untranslated region (with partial or complete complementarity) of target mRNAs and thus inhibiting their translation or directing their degradation[204] via RISC (RNA-induced silencing complex).

Eukaryotic cells express a large number of miRs and, since the initial discovery of virus-encoded miRs in a BL cell line[52], a number (>23) of EBV-encoded miRs have also been identified. The EBV miRs are derived from two separate regions of the viral genome: the BamHI H region (containing the BHRF1s miR sequences either side of the BHRF1 mRNA open reading frame); and the BamHI A region (encoding the BART miRs, within the intronic region of the BARTs). To-date, aside from the prototype B95.8 EBV strain[22] (which contains a 11.8 kbp deletion in the BART region), 25 EBV-encoded pre-miRNAs - resulting in 4 mature BHRF1 miRNAs and 40 BART miRNAs - have been identified[51-52,205].

Analysis of NPC biopsies has shown high-level expression of most EBV miRNAs derived from the BART miRNA cluster, whilst the BHRF1 miRNAs are not expressed in primary NPC tissue[51,206]. As the Qp promoter is located between the BHRF1 and EBNA1 ORFs, viral RNA initiating from Qp cannot yield any of the BHRF1 miRNAs[52]. Current data is consistent with the notion that expression levels of BART miRNAs mirror that of the BART mRNA transcripts (Amoroso *et al*, Journal of Virology 2010 *in press*). Studied in the context of NPC, EBV miRs can evidently target other EBV transcripts including LMP1 and LMP2A[207-209]as well as cellular genes[210]. It should be noted, however, that high expression of a given miR may not consistently equate to the expected target effect in all tumours[210].

In the context of NK/T lymphoproliferations, although certain cellular miRs have been proposed to confer a pro-survival advantage to ENKTL cells *in vitro*[211], the expression and function of EBV miRs in this spectrum of disease has not yet been investigated.

Lytic cycle genes

For all herpesviruses, including EBV, the virus lytic cycle involves the sequential expression of: immediate early (IE) proteins, typically transactivators of viral early gene expression; early (E) proteins, including requisite components of the viral DNA replication complex; and late (L) proteins comprising many of the virion's structural proteins.

In vivo, activation of the EBV lytic cycle is thought to be instigated by differentiation of infected B cells into plasma cells following antigen stimulation of the BCR[212] or by differentiation of infected squamous epithelial cells [213]. Various methods have been developed to induce lytic cycle *in vitro* but the most extensively studied is directed engagement of the B cell receptor[214] in the Akata-BL cell line which results in synchronous and rapid induction of as many as 50% of cells into lytic replication[215].

Immediate early lytic gene products

The IE genes products BZLF1 and BRLF1 are transcription factors that serve as master regulators of EBV's lytic cycle. They synergistically activate one another's promoters resulting in considerable amplification of the primary lytic stimulus[29]. Together they initiate a cascade of viral gene transcription, starting with several early genes after about 2 hours. The BZLF1 protein is a viral homolog of c-Jun and c-Fos, containing 3 separate functional domains to facilitate replication, transcriptional regulation and homodimerisation[216]. BRLF1 is also a DNA sequence specific acidic transactivator; it has some homology to c-myb, which can interact synergistically with BZLF1 in transactivation of the early lytic BMLF1 promoter[217].

Early lytic cycle

Genome-wide studies have aided the identification of 38 early lytic mRNAs [218] including the EBV DNA polymerase (BALF5) and the major DNA binding protein (BALF2). Expression of these E

proteins ultimately leads to the production of a concatenated linear EBV DNA molecule; the replicative form of viral DNA[219].

Late lytic cycle

Late lytic proteins are produced following DNA replication and, of the 40 known genes, 28 are transcribed into virion proteins including the EBV glycoproteins, capsid proteins, tegument proteins and the virion kinase. The viral protease BVRF2 thereon provides a scaffold for the assembly of viral particles in readiness for the encapsulation of viral DNA[29].

Non-structural late lytic cycle proteins include BCRF1, a viral gene highly homologous to human IL-10[220]. Similarly to human IL-10, viral IL-10 (vIL-10) is a growth factor with autocrine and paracrine effects including promotion of B cell survival and proliferation. [221]. vIL-10 also mediates multiple and complex effects on the immune system, including downregulation of surface HLA-1 through transcriptional inhibition of TAP-1[222] and, conversely can promote the reactivation of anti-EBV T and NK cell responses[223]. Maximum expression of late proteins, including structural viral capsid antigens (VCA) and envelope glycoproteins, is seen approximately 12 hours after expression of BZLF1 and BRLF1 and release of virions can be detected by approximately 24 hours, although lytically infected cells can survive beyond this[29].

The relative deficiency of significant $CD8^+$ T cell responses to late lytic proteins[224] is now understood to reflect the temporal expression of viral immune-evasion genes. Four such genes (*BNLF2a, BGLF5, BILF1* and *vIL10*) can demonstrably interfere with the HLA-I antigen presentation pathway and thus modulate the $CD8^+$ immune responses to EBV[225].

EBV infection *in vivo*

B cells and epithelial cells

EBV transmission between humans usually occurs by contact with oral secretions[226]. The virus replicates within the oropharynx resulting in active shedding of virus in the vast majority of seropositive individuals[54,227]. It remains somewhat unclear whether oropharyngeal epithelial cells

or B lymphocytes are the primary site of replication[228-230], although infected B cells identified in IM tonsils were seen to be predominantly latent, with no clear evidence of full viral replication[230]. Evidence of virus replication has been observed in squamous epithelial cells of the tongue in patients with acquired immunodeficiency syndrome (AIDS)[231] and occasionally in post-mortem tissue from immunocompetent virus-carriers[232]. Viral shedding from the oropharynx is abrogated in acyclovir-treated patients, whilst the number of EBV-infected circulating B cells is unchanged by such treatment[233]. Taken together with data from *ex vivo* cultures of tonsillar epithelium[234], this suggests that infection of oropharyngeal epithelium is an integral part of the in vivo EBV–host interaction.

The process whereby oropharyngeal replicative infection results in latently infected B lymphocytes is not well understood, but tonsil sections from IM patients readily demonstrate EBER-expressing B lymphoblasts with a proportion of these cells co-expressing EBNA2 and LMP1[229-230,235]. Expression of these viral proteins is indicative of a growth-transforming infection akin to EBV-transformed lymphoblastoid cell lines *in vitro*, where the full spectrum of latent cycle genes (EBERs, BamHI A-encoded RNAs, EBNAs 1, 2, 3A, 3B, 3C, -LP, and LMPs 1 and 2) are expressed[54]. In vivo, however, these EBV-transformed lymphoblasts appear to down-regulate viral antigen expression, resulting in a reservoir of EBER-positive antigen-negative memory B cells, considered to be in a resting state[160,236] that persist within blood and oropharyngeal lymphoid tissues[54].

In normal adults, approximately 1 to 50 circulating B cells per million are infected with EBV, and the number of latently infected cells within a person remains stable over years[237-238]. Interestingly, the observation that EBV can be eradicated in bone marrow–transplant recipients undergoing myeloablative therapy[239] (thus eliminating their hematopoietic cells but not their oropharyngeal epithelium) provided further evidence that B cells are the site of EBV persistence.

T cells

Although distinctly B lymphotropic both *in vivo* and *in vitro,* the characterisation of a spectrum of EBV^+ T and NK cell lymphoproliferative diseases[240-242] inevitably signifies an *in vivo* infection event of their T and NK counterparts, although conflicting data exists regarding the frequency and nature of these occurrences.

In the context of primary EBV infection, Anagnostopoulos *et al* examined tissue sections from tonsils excised during the acute phase of IM[229]. EBV-harbouring T cells were consistently identified in 9/9 cases using *in-situ* hybridisation for EBERs combined with antibodies directed against CD45RO and, more specifically, the TCR β-chain. The number of infected T cells was stated to be considerably lower than $EBER^+$ B lymphocytes within the same tonsil, though this observation was not formally quantified. The $EBER^+$ T cells were located predominantly within the inter-follicular areas and morphological differences were evident within and between tonsils; some T cells exhibited blastoid features whilst many appeared as small/medium-sized lymphocytes. Similar findings of appreciable T cell infection in the context of primary infection were reported by a Japanese group who examined the lymph nodes of IM patients[243]. By contrast, other studies of tonsils and lymph nodes from patients with IM - applying either CD3 or CD4/CD8 antibodies - could not identify any $EBER^+$T cells[244-246]. In keeping with the latter data, a further study of 14 IM tonsils found no evidence of T cell infection, although it should be noted that 30-50% of $EBER^+$ tonsillar lymphocytes in this study could not be clearly assigned a B or T lineage, using CD20, CD79a, CD3 and CD45RO as markers[247].

In the case of EBV persistence, studies of primary and secondary lymphoid tissue have suggested rare instances of $EBER^+$ T cells in vivo[248], more often (at least in tonsillar tissue) of $CD4^+$ phenoype[249]. Similarly, a study of reactive lymph node tissue from Peruvian patients, a region where the incidence of EBV-associated extra-nodal NK/T lymphoma (ENKTL) is thought to be relatively high[250-252], found in a minority of lymph nodes ascertained to contain $EBER^+$ lymphocytes, that as many as 24% of $EBER^+$ cells per section expressed CD3[253]. This contrasted

with a separate investigation of intramucosal lymphocytes in inflamed tissues where no definite evidence of T cell infection was seen[254].

Noting the characteristic anatomical presentation of ENKTL in the nasopharynx, small studies from China examined both normal nasopharyngeal tissue (obtained post-mortem[255,256]) and excised nasal polyps[257]. Very rare intraepithelial lymphocytes co-expressing $EBER^+CD3^+$ [255] and $LMP1^+CD3^+$ [257,256] were identified in a minority of cases and at a lower frequency than their $EBER^+$B cell counterparts.

NK cells

EBV infection of NK cells *in vivo* has also been investigated using an IHC/ISH approach in IM and reactive tonsillar tissue. Trempat *et al*[246] used an antibody directed to an NK-restricted epitope PEN5[258-259] (see NK cell phenotype: page 34 and Figure 4) to identify $EBER^+$ NK cells in the context of primary infection. Of the 6 cases of IM lymph nodes and tonsils examined, small $EBER^+PEN5^+$ cells were appreciable, albeit very infrequent; corresponding to 10^{-2} to 10^{-3} of all $EBER^+$ lymphocytes. The vast majority of $PEN5^+$ cells however were EBER negative, and none expressed EBNA2. Applying similar methodology, Hudnall *et al* undertook a comprehensive, semi-quantitative analysis of 20 EBV^+ tonsils obtained from donors undergoing routine tonsillectomy[249]. The majority (82%) of >1000 $EBER^+$ cells weakly expressed CD20, while dual-positive PEN5/EBER NK cells were very rare (1.7%); numerous NK cells identified beneath the crypt epithelium were EBER negative. Notably, and similarly to an IM study[247], a population of cells (comprising 11% of $EBER^+$ lymphocytes) could not be assigned a lineage using B, T, NK and epithelial markers.

Thus, within the limitations of dual IHC/ISH labelling on fixed tissue sections, the occurrence of EBV-infected T and NK cells *in vivo*, during primary infection and persistence, appears to be a relatively rare event.

EBV infection *in vitro*

B cells

The observation that EBV possessed the ability to infect and transform human B cells *in vitro*[15,260] was a critical discovery in the pursuit of understanding the pathogenesis of B lymphomagenesis. Continuously proliferating lymphoblastoid cell lines (LCLs) could be generated by infecting resting primary B lymphocytes, where the majority population do not replicate virus. B lymphocytes derived from peripheral blood, tonsils or foetal cord blood could all be readily infected *in vitro* [15,260-263].

EBV's tropism for B lymphocytes is accomplished through engagement of the major viral envelope glycoprotein gp350 with the 145-kDa transmembrane, C3d complement receptor CD21[264] on the B cell surface[265-267]. Thereafter, a second envelope glycoprotein gp42[268-269] binds to cell surface HLA class II molecules[270], initiating virus–cell fusion - a process requiring gp85/gp25 and the gp110 glycoprotein[271] - and permitting viral entry. Expression of CD21 during B-lymphocyte development correlates with the efficiency of viral binding and EBV infection can be substantially reduced by blocking antibodies to CD21, purified CD21 or by saturation of B-lymphocyte receptors with gp350/220[272-273]. However, although gp350 represents EBV's primary mechanism for B cell binding, a gp350 knock out (KO) recombinant EBV retains some ability to infect and transform B cells, albeit with considerably reduced efficiency[274].

In vitro, although virus binding to the B-cell surface is highly efficient, only 10–15 % of bound virus genomes reach the cell nucleus[275]. Depending on the multiplicity of infection applied, 10-90% of cells will become latently infected[262,275]. Although the precise mechanisms of EBV capsid dissolution and genome transit to the nucleus have not been delineated, it is likely from analogy to other DNA viruses that EBV capsid transport to nuclear pores is mediated by the cytoskeleton[276]. Subsequent covalent closure of the linear viral DNA can then form the circular EBV genomes observed in virally infected cells[277-278]. The consequent growth-transforming effect of EBV, mediated by the co-ordinated expression of nine latent proteins results in the

establishment of a long-lived lymphoblastoid cell line (LCL), as discussed under latent gene expression (pages 4-7).

Epithelial cells

Epithelial cells do not express CD21 and infection by cell-free virus is much less well understood, but is thought to involve gp85/gp25 acting both as a ligand for binding (to a hitherto unidentified receptor) and, in concert with gp110, as a fusion complex[268,279]. Some uncertainty exists regarding the role of gp42 in epithelial cell infection, which may be a consequence of which experimental system is used[279-280]. Virions produced in HLA class II-positive B cells tend to be gp42-low, as a result of sequestering into endosomes by nascent HLA class II molecules, thus limiting its representation in virus particles. As a result, gp42-low viruses (produced in B cells) preferentially target epithelium, whereas gp42-high viruses (produced in epithelial cells) are equipped to efficiently infect B cells[279-280].

Efficient infection of epithelial cells *in vitro* has proved more challenging. Exposing squamous epithelial cells[281] or the apical surface of columnar epithelial cells[282] to cell-free virus *in vitro* results in very low rates of infection. When epithelial monolayers were co-cultured with EBV-positive B cell lines containing lytic cells, infection of epithelial cells was somewhat augmented [281]. More strikingly, virus bound to the surface of recently exposed primary B cells is highly infectious for epithelium, increasing the efficiency of infection by 10^3-10^4fold compared to cell-free virus preparations[280]. The function of gp350 in such 'transfer infection' appears to act purely as a ligand for B cell binding. Interestingly, although apparently inactive in transfer infection, the gp350 KO virus can directly infect epithelial cells[280]. This suggests that gp350, whilst of central importance for infection of B cells, may actually impair direct epithelial infection. A number of other studies have provided support for the existence of CD21-independent infection mechanisms

T cells

Following the discovery that B cells express a binding receptor for EBV[283-284], but prior to the molecular characterisation of CD21 (CR2)[264], it was found that such a virus receptor was also

present on a T cell leukaemia line[272,285]. This cell line, named 'MOLT 4', was established from the peripheral blood of a patient with T cell acute lymphoblastic leukaemia[286]. Initial attempts by Menezes *et al* to infect MOLT 4 showed that although EBV could bind to the cell surface, there was no discernible virus penetration or EBV gene expression[285]. Separate studies appeared to corroborate the finding that the MOLT 4 CD21 is unable to facilitate viral entry, although it can bind and efficiently transfer virus to infect epithelial cells *in vitro*[280].

Subsequently, variable expression of CD21 was documented on a proportion of T cell lymphoma/leukaemia lines; HSB-2, Hut-78, CEM and T-ALL-1 were all CD21 negative whilst Jurkat expressed low surface levels and HPB-ALL expressed receptors at a higher density than several B-lymphoblastoid cells[287]. Limited N-terminus sequencing of the HPB-ALL CD21 molecule confirmed its identity[287]. It is noteworthy that the CD21-positive T cell lines were established from lymphoblastic leukaemias, rather than mature T cell tumours.

Importantly, CD21 was shown to be variably but consistently expressed on a proportion (8-18%) of primary human thymocytes, obtained from paediatric patients undergoing elective cardiac surgery[288-289]. An inverse correlation of CD21 intensity with CD3 expression was also apparent in these primary T cells - consistent with the view that these T lineage cells were relatively immature[288]. Moreover, convincing *in vitro* infection of primary thymocytes by co-culture with EBV was seen, with EBNA1 expression appreciable by immunoblotting after 12 days[289] – although the efficiency of infection of $CD21^+$ T cells was not clear. Thymocyte proliferation was augmented by EBV infection and a synergistic effect with IL2 treatment was also apparent. These data, alongside emerging reports of EBV^+ T cell diseases[241-242], challenged the belief that EBV's tropism was restricted to B cells and epithelial cells and suggested a restricted pattern of viral gene expression. Further data concerning the pattern of EBV gene expression in T cells infected *in vitro* is limited, but the serendipitous establishment of EBV^+ T cell lines from peripheral blood T lymphocytes suggested a Latency II pattern of gene expression[290].

In the case of expression of CR2/CD21 on mature peripheral blood T lymphocytes, the available data is somewhat conflicting. Fischer *et al* found low intensity CR2 expression on 30-40% of T cells (both $CD4^+$ and $CD8^+$) using antibodies recognising 3 non-overlapping epitopes of CR2. However, the intensity of staining was approximately 10-fold lower than that of peripheral blood B cells[291], consistent with recent quantitative analyses of virus binding[53]. A separate study could find no expression of CD21 on peripheral $CD4^+$ and $CD8^+$ cells but were able to infect a small proportion of these cells *in vitro*[292]. Applying different methodology, via dual staining with T cell-specific MAbs and fluoresceinated viral preparations, Sauvageau *et al* found that 50% of $CD8^+$ (but not $CD4^+$) T cells bound EBV specifically, albeit at an extremely low level[293]. This low-level interaction was apparently not impeded with an anti-gp350 antibody and is consistent with more recent data[53]. This may imply either a CR2-independent mechanism of infection – akin to epithelial cell infection[274,294] – or possibly the presence of an alternate molecule with some homology to CR2[295]. *S*tudies with murine T cells have demonstrated transcriptional upregulation of CD21 by deacetylase inhibition[296], although the significance of this finding for EBV binding and entry was not investigated.

Taken together, the published *in vitro* data is consistent with the idea that during T cell development in the thymus, transient expression of CD21 is sufficient to allow binding of EBV and, in certain circumstances, viral entry.

NK cells

HLA class II molecules are expressed on the cell surface of NK cells, but CD21 is absent and the process of virus binding and entry into NK cells remains wholly unclear.

One biological mechanism to account for EBV infection *in vivo* is entry of the virus via an immunological synapse. In terms of a latently infected B cell, LCLs are generally resistant to NK cell lysis, although lines with aberrant loss of surface HLA class I expression are sensitive to NK cell killing[297]. NK cells have been shown however to kill EBV^+ B cells in lytic cycle, coincident with downregulation of HLA class I and inhibitory KIRs[298]. Within the tonsil, conjugate formation

between NK cells and autologous EBER$^+$ cells have been documented in the context of primary infection[246] and healthy EBV persistence[249]. Confirming this latter observation in IM lymph nodes, Tabiasco *et al*[299] undertook *in vitro* experiments to demonstrate that NK cells acquire CD21 on their surface following co-culture with CD21$^+$ B cell targets. Such a phenomenon had been previously described and is not specific for CD21[300]. Nonetheless, target-to-effector transfer of the CD21 receptor occurred quickly following transient contact such that, within 1 h at 37°C, activated NK cells acquired up to 1% of the surface CD21 available on conjugated targets[299]. Moreover, the quantity of transferred CD21 was orientated and functionally sufficient to permit EBV binding to the NK cell surface, although viral entry was not pursued in this study.

The only existing report of EBV infection of NK cells *in vitro*, published in 2004, unexpectedly described efficient infection of both EBV-negative NK leukaemia cell lines and primary human NK cells. Co-incubation of these NK cells with a recombinant, eGFP-expressing EBV at an extremely high m.o.i of cell-free virus, resulted in efficient infection rates.[301]. Highly pure, freshly-isolated NK cells from 3 different donors were shown to be infectable at efficiencies from 45-92% as determined by GFP expression at 48 hours. Aside from noting marked morphological changes in the infected population, this striking result was not further characterised and despite attempts at drug-selection, a stable cell line could not be established.

Isobe *et al* instead focused on the NKL[302] and NK-92[303] cell lines that were infectable with reported efficiencies of 25-30%. Considering viral gene expression in these cell lines, the findings were most consistent with an abortive lytic infection (expression of BALF2 and BZLF1 mRNAs), and possibly an underlying latency I (expression of EBER and EBNA1 mRNA, in the absence of LMP1 and EBNA2) restricted pattern of latent gene expression. Although the majority of infected NKL cells became apoptotic after 72 hours, 2 EBV$^+$NKL clones were eventually established using drug-selection.

A subsequent study from the same group[304] analysed sub-clones of EBV$^+$NKL which had developed a Latency II pattern of gene expression, following very long periods in culture,

expressing a lower MW form of LMP1. The authors showed that although these EBV-containing NK cells had no proliferative advantage compared to the parent line, the presence of EBV appeared to confer resistance to apoptosis induction by the anthracycline doxorubicin[304]. This apoptotic protection was thought to be mediated, at least in part, through NFκB activation. LMP1 expression in other cell types is known to activate the NFκB pathway[100] as well as its ability to induce expression of other anti-apoptotic proteins such as BCL2[94].

Such *in vitro* infection of NK cells has not yet been reproduced by others, but if this efficiency of infection is feasible then opportunities exist – at least in the first 48-72 hours – to carefully examine the consequences upon viral and cellular transcription and cell phenotype.

Natural Killer Cells

Discovery

Immune effectors with cytolytic potential, resembling NK cells, apparently constituted a component of innate immunity long before the development of T and B cells of the adaptive immune system approximately 500 million years ago[305]. However, orthologs of recently characterised NK cell receptor families are not found beyond mammals[306-307], suggesting that the functional effector in humans that we now recognise as the NK cell emerged approximately 400 million years after evolution of the vertebrate adaptive immune system[308].

Natural killer (NK) cells were first identified 35 years ago, in mice, as 'small lymphocytes of undefined nature exerting spontaneous selective cytotoxic activity against Moloney leukemia cells[309]. This phenomenon was functionally defined as 'natural cytotoxicity' but the contribution of other cell lineages to this cytolytic function could not be excluded. Further work defined a specific cell population as the predominant and most abundant effectors of non-MHC-restricted cytotoxicity. Importantly, NK cells were recognised to constitute a population distinct from T cells in both lineage and antigen recognition[310]. They are functionally thymic-independent and do not undergo germline rearrangements of their receptors nor express T-cell receptor heterodimers.

Phenotype

The phenotypic definition of NK cells (differing between human, mouse and rat NK cells[306]) is determined by their expression of a unique combination of non–NK-restricted surface antigens. In humans, NK cells are classically defined as $CD56^{+}CD3(\alpha\beta TCR)$- lymphocytes[311-312] and comprise approximately 10% of peripheral blood lymphocytes[313]. NK cells do not possess a complete TCR-complex, but activated NK cells express the ε and ξ chains of CD3 in the cytoplasm. In NK cells, CD56 corresponds to the 140 kDa isoform of the neural cell adhesion molecule (N-CAM)[314]. However, a minority of T-cells[314-315], muscle cells and neurons also express CD56, but it is not expressed by mouse NK cells. An apparently NK-restricted marker, PEN5[258], is a post-translationally-modified epitope on P-selectin glycoprotein ligand-1 (PSGL-1) where it serves as a unique binding site for L-selectin[316]. On the surface of NK cells, the expression of PEN5 is coordinated with the disappearance of L-selectin and the up-regulation of Killer Cell Ig-like Receptors (KIRa). Such a finding, an NK cell-restricted epitope, was encouraging but its expression is restricted to the $CD56^{DIM}$ subset and it is down-regulated in response to proliferative stimuli, limiting its usefulness as an NK marker.

Walzer *et al* have recently proposed that NKp46, a member of the highly conserved natural cytotoxicity receptor family of NK-activating receptors[317] best defines NK cells across species[306] However, NKp46 is not an absolutely specific or sensitive NK cell marker as it is also expressed on a subset of human cytolytic T lymphocytes[306,318] and a minor population of $CD56^{+}CD3$- cells have low-density or absent expression.

Thus, at present NK cells are determined by their expression of a combination of non–NK-restricted surface antigens[308].

NK subsets

More than 20 years ago it was recognised that two distinct subsets of human NK cells exist, based on their cell surface density of CD56[313]. It is now clear that these subsets differ

considerably in their proliferative response to IL-2, intrinsic cytotoxic capability, NK receptor repertoire and adhesion molecule expression[312] (see Figure 4, adapted from reference[312]).

The majority (approximately 90%) of peripheral blood NK cells are $CD56^{DIM}$ and express high levels of FcγRIII (CD16). These cells express homing markers for inflamed peripheral sites and express perforin to rapidly mediate cytotoxicity[319-320], consistent with early observations[313]. The remaining, approximately 10%, of peripheral blood NK cells are $CD56^{BRIGHT}$ and $CD16^{DIM/NEG}$. These NK cells have an important immunoregulatory role, rapidly secreting an abundance of cytokines[319-320] (such as IFN-γ and TNF-α) when activated, yet have little or no ability to spontaneously kill tumour cell targets[321]. The production of IFNγ, considered the prototypic NK-cell cytokine, has pleiotropic effects including: induction of the Th1 immune response[322]; up-regulation of HLA class I on antigen-presenting cells (APC)[323]; augmentation of macrophage killing[324] and antiproliferative activity against viral- and malignant-transformed cells[325]

In addition, they display homing markers for secondary lymphoid organs, namely CCR7 and CD62L[319]. In fact, a substantial number of human NK cells home to secondary lymphoid tissue (SLT), constituting approximately 5% of mononuclear cells in un-inflamed lymph nodes and 0.4–1% in inflamed tonsils and lymph nodes[326-327]. Although, proportionally, $CD56^{BRIGHT}$ NK cells are a minority population, because lymph nodes harbour 40% of all lymphocytes, (compared to peripheral blood which contains only 2% of all lymphocytes), NK cells in SLT are numerically substantially more abundant than peripheral blood NK cells. As may be anticipated from some of its functions, the $CD56^{BRIGHT}$ NK subset resides primarily in the parafollicular T cell– and APC-rich region of SLT[326].

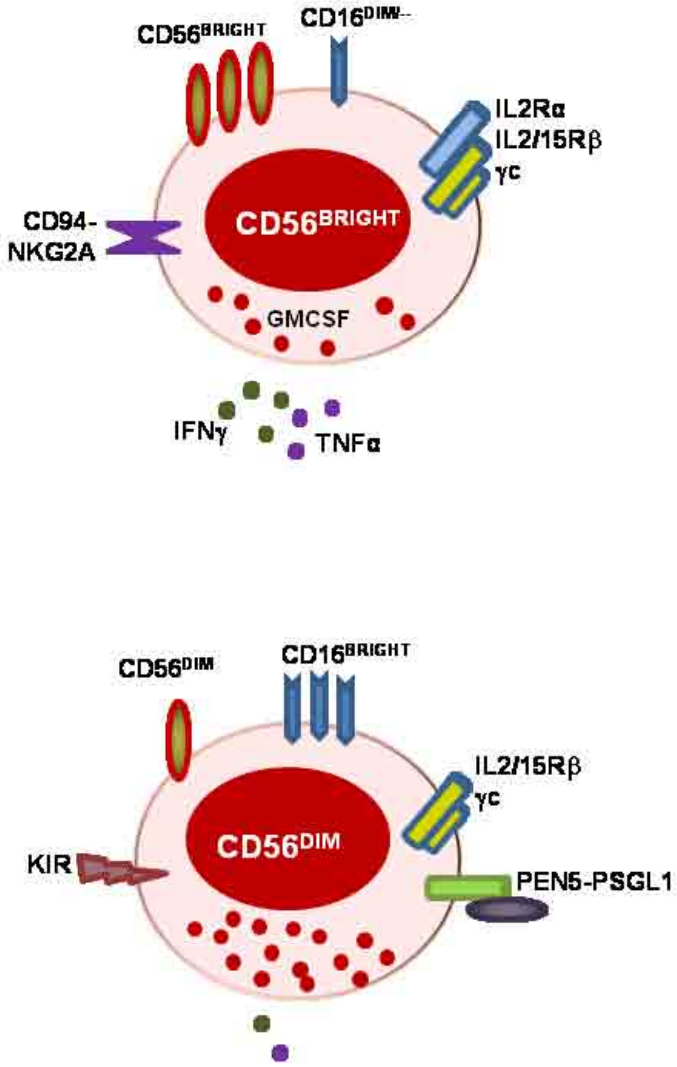


Figure 4 Major NK cell subsets.

The top cartoon represents the $CD56^{BRIGHT}$ NK cell that produces high levels of cytokines when stimluated. Expression of CD16 is dim/absent with accordingly low antibody-dependent cytotoxicity and low natural cytotoxicity. CD94 expression is high but KIRs are low density. The high-affinity IL2 receptor (IL2αβγ) is also highly expressed.

The lower cartoon represents the cytotoxic $CD56^{DIM}$ subset with more granular morphology and high expression of CD16, PEN-5 and KIRs. They lack the high-affinity IL2R subunit α and have less cytokine-producing ability. Their predominant function is to execute natural and antibody-dependent cytotoxicity.

$CD56^{BRIGHT}$ NK cells constitutively express the high- and intermediate- affinity IL-2 receptors (respectively heterotrimeric IL-2R$\alpha\beta\gamma$, and heterodimeric IL-2R$\beta\gamma$) and expand *in vitro* and *in vivo* in response to picomolar concentrations of IL-2[328-329]. Co-expression of the c-*kit* receptor tyrosine kinase can enhance IL-2-induced proliferation[330-331]. By contrast, resting $CD56^{dim}$ NK cells lack the high-affinity IL-2 receptor, are c-*kit* negative, and proliferate weakly in response to high doses of IL-2 (1 to 10 nM) *in vitro*.

$CD56^{BRIGHT}$ NK cells also express L-selectin (CD62L), an adhesion molecule which mediates initial tethering to vascular endothelium[332]. $CD56^{DIM}$ NK cells lack this receptor but, accompanying NK cell differentiation, express PEN5 that partially mediates the binding of L-selectin. This could function to help KIR^+ NK cells home to specific tissues[316], a concept supported by patterns of chemokine expression suggesting differential trafficking of human NK cell subsets *in vivo*[321]. Potential functional differences were also highlighted by a recent study that demonstrated a relative resistance of $CD56^{HIGH}$ NK cells to treatment with the calcineurin inhibitor cyclosporin A, known to suppress T Cell function[333]. Whereas a dose-dependent selective inhibition of $CD56^{DIM}$ NK cell growth and cytolytic function was evident, $CD56^{BRIGHT}$ NK cells were resistant to cyclosporin A treatment and indeed enhanced IFN-γ production was seen[334].

Natural killer receptor (NKR) repertoires also differ between the two NK subsets. All resting $CD56^{BRIGHT}$ NK cells highly express CD94/NKG2 C-type lectin receptors[335] whilst less than 10% expresses KIRs[316]. The converse is true of resting $CD56^{DIM}$ NK cells; the majority are KIR^+ with low levels of CD94/NKG2. The cognate ligands for KIRs are classical MHC class Ia ligands (HLA-A, B, and C), whilst CD94-NKG2A heterodimeric receptors bind the non-classical MHC class Ib (HLA-E)[336]. Every NK cell with killing capacity appears to possess an inhibitory receptor that recognises at least one of the MHC class I molecules on the opposing cell's surface[337]. Engagement of the inhibitory KIRs with classical MHC class I results in a dominant signal (overriding any activation signals) that prevents the NK cell from lysing the candidate target cell[338].

NK cell development

Several studies have confirmed that NK cells are derived from $CD34^+$ haematopoietic progenitor cells (HPCs)[339-341], and thereafter a common T and NK lymphoid progenitor[342], but a detailed characterisation of the site(s) and pathway of NK development is not yet fully understood.

Taking Lanier's initial hypothesis, that $CD56^{BRIGHT}$ cells were less mature than their $CD56^{DIM}$ counterparts[313], together with the finding that $CD56^{BRIGHT}$ cells dominated in SLT[326-327] suggested that the BM may not be the exclusive site of NK development. Selective enrichment of a unique population of $CD34^+$ pre-NK cells ($CD34^+$ $CD45RA^+$ CD10- $CD117^+$ $CD161^{+/-}$) in SLT[343] (and subsequently in mucosa-associated lymphoid tissue in the gut[344], at high levels compared to BM and blood, suggested that SLT may be a site for NK-cell development *in vivo*. The closely proximate and abundant APCs in these tissues, which express surface-bound IL-15[345] required for NK development[346-348], supported this possibility. Moreover, the orderly acquisition of NK cell receptors, with CD161 and NKp46 being among the first NK cell receptors to be evident, followed by CD94 and finally CD16 and KIRs[348-350] suggested differentiation through distinct maturational stages. Detailed *in vivo* and *ex vivo* studies subsequently identified four populations that appeared to represent discrete stages of a human NK cell developmental continuum within SLT. Each population appeared capable of downstream NK cell differentiation *ex vivo* and manifest unique functional and phenotypic attributes. Progression through the stages was characterized with a progressive reduction in non-NK cell lineage differentiation potential and a concomitant increase in CD56 expression with functional features of $CD56^{BRIGHT}$ NK cells[351-352]. Subsequent studies supported the derivation of $CD56^{DIM}$ NK cells from their $CD56^{BRIGHT}$ counterparts [353-356]. Interestingly, a role for the CD56 antigen *per se* in terminal NK differentiation has recently been suggested[353].

Role of NK cells in control of EBV

NK cells appear to play an important role in the early control of cytomegalovirus (CMV) infections in both mouse and man[357-359] but seem to be less crucial for control of the γ-2 herpesvirus MHV68 in mice[360].

NK cell numbers (but not CD1-restricted NKT cells) are expanded in the blood of acute IM patients and, in a small study of patients with primary EBV infection, a relative increase in the $CD56^{HIGH}$ subset was seen. Furthermore, there was an inverse correlation of NK numbers with EBV genome load in the peripheral blood of IM patients[361].

In vitro, NK cells can inhibit the EBV-induced transformation of resting B cells if introduced within a few days of infection, mediated at least in part, by the release of IFN-γ[362]. Consistent with this, tonsillar NK cells can effectively inhibit *in vitro* transformation of EBV-infected B lymphocytes in the presence of mature dendritic cells[363]. Collectively, this data suggests that NK cells may be able to limit primary EBV infection in SLT until the adaptive immune system establishes specific control of the virus.

However, indirect clinical evidence goes against a major role for NK cells in the control of transformation events *in vivo* since, in recipients of T-depleted stem cell transplants, EBV-driven lymphoproliferative disease occurs most commonly in the first 3-6 months post-transplant by which time NK cell numbers have recovered but the patients remain profoundly T cell-deficient[364].

NK cells do seem to have a role in the control of lytic infection; a reduction in surface HLA class I expression seen in lytically-infected cells *in vitro* is accompanied by increased sensitivity to NK cell recognition [298]. Notably, an NK immune evasion strategy by EBV has not yet been identified, in contrast to numerous evasion mechanisms seen in both murine and human CMV infection[357-358], scenarios in which the NK response appears to be critical.

T cell development, subsets and function

The T cells of the adaptive immune system are heterogeneous and functionally complex and include naïve, effector (regulatory and cytotoxic), and memory T cells. T Cells are derived from common lymphoid progenitor in the bone marrow and migrate to the thymus where they differentiate into mature T cells during passage from the cortex to the medulla. It should be noted that although a common progenitor for T and NK cells exists, once committed to the NK cell lineage these cells lose the capacity to progress along the T cell developmental pathway[342].

During thymic maturation, self-reactive T cells are culled whereas T Cells with appropriate HLA specificity are positively selected.[365]

The definitive T cell lineage marker is the T-cell antigen receptor (TCR). There are two defined types of TCR, αβ or γδ heterodimers, which are both disulphide-linked polypeptides with structural similarity. Both receptors associate with the 5 polypeptides that form CD3, and together constitute the TCR complex. The vast majority (>90%) of peripheral blood T cells are αβ T cells whilst 5-10% are γδ T cells. The α,β,γ and δ genes of that encode the TCR polypeptides each include V, D, J and C regions. During T cell ontogeny, rearrangement of these gene segments occurs – similarly to immunoglobulin gene rearrangements in the B lineage. This potentially results in $>10^8$ specific TCR structures, with additional diversity facilitated by the enzyme TdT (terminal deoxynucleotidyl transferase) at the junction sites. The same recombinase enzymes utilised by B cells mediate re-joining of the TCR gene segments[366].

Such uniqueness of receptor genes affords diagnostic opportunity to establish clonality amongst T cell populations in blood or tissue, usually achieved by PCR-based techniques. Naive T lymphocytes that leave the thymus do not actively divide, unless they encounter an antigen-presenting cell (APC) with cognate antigen and are thereon stimulated to clonally expand. Antigen-specific responses are predominantly generated in SLT[367].

αβ T cells are subdivided into two discrete populations. The CD4-expressing subset (the dominant population in peripheral blood and SLT) whose function is principally regulatory, acting via cytokine production (further divided into T_H1 and T_H2) and a $CD8^+$ subset that are chiefly cytotoxic. $CD4^+$ antigen recognition is restricted by HLA class II molecules whilst $CD8^+$ T cells recognise antigen in association with HLA class I. A minority of αβ T cells are termed ‘double negative’ as they express neither CD4 nor CD8. Most peripheral blood γδ T cells are also ‘double negative’ although a proportion express CD8[367].

A significant minority of T cells express non-lineage-specific surface markers, including some in common with NK cells. For example: CD2, expressed by all T cells is also present on a majority

of NK cells; CD16 (FcγRIII), expressed by a minority of T cells and most peripheral blood NK cells; CD56, expressed on a minority of cytotoxic T cells and on all NK cells with variable density.

Virally-infected cells highlight themselves as a target for cytotoxic T cells via HLA class I surface display of peptides derived from processed intracellular viral proteins. The cytotoxic T cell TCR engages with the peptide–MHC complex and then kills the infected cell by two major mechanisms. Perforin insertion through the target-cell membrane creates pores through which granzymes can pass and thereon activate target cell caspases - inducing apoptosis. Alternatively, expression of Fas ligand on cytotoxic T cells binds to target cell Fas, which also results in caspase activation within the target cell and ultimately apoptosis[368].

Specific T cell responses to latent and lytic EBV-infected cells are critically important in preventing EBV-driven disease and maintaining the virus: host balance that exists in healthy persistence.

Primary infection and infectious mononucleosis

Clinical and epidemiological features

Primary EBV infections are rare in the first year of life, presumably as a result of passively transferred EBV-specific maternal antibodies. In developing countries and lower socioeconomic groups, most individuals are exposed to EBV early in childhood. Primary infections in young children are either asymptomatic or manifest as nonspecific viral illnesses, in whom the characteristic symptoms of infectious mononucleosis (IM) are uncommon. However, IM in early childhood is thought to occur, albeit rarely[369].

The clinicoimmunological syndrome of infectious mononucleosis is more likely to occur if primary EBV infection is encountered during or after the second decade of life. Attributed to improved economic and sanitary conditions over recent decades, EBV infection in early childhood has become less frequent[370], leading to more individuals potentially susceptible to ‘late’ primary infection and thus IM. In a recent UK study of university students[371], 25% of young adults were

EBV seronegative at the time of university entry (median 19 years) of whom 46% had seroconverted after 3 years. Of these 'late' seroconversions, one-quarter of individuals had been diagnosed with IM.

A majority of patients with infectious mononucleosis manifest fatigue, lymphadenopathy and pharyngitis, whilst hepatosplenomegaly is clinically evident in a third of individuals but fever is relatively uncommon[372]. Less common complications include haemolytic anaemia, thrombocytopenia, aplastic anaemia, myocarditis, hepatitis, genital ulcers, splenic rupture and neurologic complications such as Guillain–Barré syndrome, encephalitis, and meningitis[373]. So-called 'fulminant IM' cases, due to the development of haemophagocytic lymphohistiocytosis or clonal lymphoproliferation of infected T cells (discussed in detail later), are very rare but are associated with a high mortality. A Japanese survey[374] estimated that the incidence of EBV-HLH was less than 1 case per million population each year, though diagnostic challenges and the nature of the study means that this is likely to be an underestimate.

Most patients with infectious mononucleosis have an absolute leucocytosis in the peripheral blood, predominantly accounted for by increased T and NK cells ($CD8^+$, γδ and $CD56^+$), but mild elevations of monocyte numbers also occur[372]. Mild neutropenia and thrombocytopenia are common and deranged liver biochemistry (most commonly elevated serum alanine transaminase) can be detected in the majority. Heterophile antibodies, detected via the monospot test, are present in all cases whilst anti-VCA IgM is almost as sensitive and affords more diagnostic specificity for IM[372,375].

The clinical manifestations of IM are thought to be, at least partly, attributable to the brisk and substantial T cell response and the associated cytokine release – particularly IFNγ and TNFα[376]. Moreover, a correlation between the intensity of T cell activation and the severity of symptoms has been described [377]. Susceptibility to IM is bestowed by polymorphisms in genes encoding the regulatory cytokine IL-10[378] and, interestingly, HLA class I[379] possibly impacting on efficiency of viral peptide presentation.

In the majority of immune-competent individuals, the illness is self-limiting and recovery can be anticipated within weeks, with resumption of normal activities after 2-3 months[372].

Cellular immune responses

Most of the published data characterising cellular immune responses to primary EBV infection are derived from studies of IM patients, on the assumption that this immuno-pathological syndrome amplifies, but does not distort, the events of sub-clinical primary infection. (The role of NK cells in control of EBV has been discussed on page 38).

$CD8^+$ T cell responses

The markedly expanded populations of $CD8^+$ T cells in the peripheral blood during acute infection were shown to be distinctly oligoclonal in T cell receptor (TCR) usage[380]. Moreover, *ex-vivo* assays demonstrated that these cytotoxic, perforin-expressing $CD8^+$ cells were functionally EBV epitope-specific and were numerically dominant, as shown by HLA class I tetramer staining [381]. A comprehensive body of work has subsequently characterised and quantitated numerous EBV specificities in IM blood[381-385]. Responses to individual epitopes can account for 1-40% of the total $CD8^+$ T cell population, with dominant reactivities to immediate early (IE) and some early (E) lytic antigens usually seen. Responses to latent viral proteins are numerically smaller; individual specificities accounting for 0.1-5% of the $CD8^+$ T cell population.

The EBV-specific $CD8^+$ population express the activation marker CD38, the cell cycling protein Ki-67 and CD45RO, all consistent with recent antigen stimulation[381-382,386-387] and die rapidly by apoptosis *in vitro* unless persistently stimulated. Consistent with this, low levels of Bcl-2 and Bcl-x proteins and high levels of the pro-apoptotic Bax protein are evident, accounting for the rapid culling of T cells in the immediate aftermath of acute IM when access to antigen becomes limiting[383].

In healthy EBV persistence, virus shedding in the throat is often very low[388] and, of the total peripheral B lymphocytes approximately 1-50 cells/million are latently infected with EBV[238]

(>1000-fold less than seen in acute IM). Nonetheless, $CD8^+$ memory responses are maintained by low-level antigen stimulation and display a resting phenotype although a proportion do express perforin and are demonstrably cytotoxic *ex vivo*[386,389]. In healthy carriers, a significant fraction of the circulating $CD8^+$ T cell pool is devoted to control of EBV and remains relatively stable over time (typically 0.2-2% of the $CD8^+$ T cells specific for lytic epitopes and 0.05%-1% for latent epitopes)[383].

In the vast majority of individuals, both in the context of IM[385] and healthy persistence[180], $CD8^+$ T cell responses to latent cycle antigens are highly focused on immunodominant epitopes from the EBNA3A, 3B, 3C family of proteins. Co-existing sub-dominant responses often map to epitopes either from the same EBNA 3 family or from LMP2, whilst responses to EBNA2, EBNA-LP, LMP1 and EBNA1 are substantially less frequent. Such a hierarchy of immunodominance, however, may be influenced by the HLA repertoire of the geographical population studied[383].

LMP2 $CD8^+$ responses

More than 20 $CD8^+$ epitopes from LMP2 have been described, the vast majority of which are derived from the transmembrane domains of the protein and thus common to both LMP2A and LMP2B[390-392]. A comprehensive study examined T cell responses in 32 healthy individuals with persistent EBV infection and 8 subjects with IM including 13 LMP2 epitopes, restricted through 8 HLA class I elements, amongst a panel of 113 EBV-derived epitopes[392]. LMP2-specific responses were common in the setting of healthy persistence and, when seen, occurred in 25-60% of individuals. By contrast, LMP2-specific responses were relatively uncommon in the small number of IM patients tested; where EBNA3 and lytic responses dominated. Low frequency LMP2 responses can also be detected in immunocompetent patients with EBV-associated malignancies[393-394] and have recently been capitalised upon for adoptive cellular immunotherapeutic approaches[391,395-396](discussed under HL, pages 51 and 52).

$CD4^+$ T cell responses

By contrast to their $CD8^+$ counterparts, $CD4^+$ T cell numbers are not expanded in IM and do not demonstrate the same oligoclonal skewing[397]. $CD4^+$ epitope specificities have been more challenging to characterise *ex-vivo*, but $CD4^+$ T cell responses to a lysate comprising both lytic and latent EBV proteins can be detected in IM blood at much lower frequencies than $CD8^+$ reactivities[398]. As for the $CD8^+$ population, latent and lytic epitope-specific $CD4^+$ T cell numbers are observed to peak during acute IM and promptly decline thereafter[383].

In terms of $CD4^+$ T cell memory, multiple EBNA- and LMP- epitope specificities have been identified but are estimated to be at least 10-fold lower than typically seen for the equivalent $CD8^+$ T cell memory[399]. $CD4^+$ memory responses to IE, E and late lytic cycle antigens have also been isolated in healthy carriers[383].

In terms of an immunodominance hierarchy, there appear to be no particular CD4 epitopes that consistently dominate the latent antigen-induced response[399]. The C-terminal domain of EBNA1 provides an abundance of epitopes for a range of HLA class II alleles, to which $CD4^+$ memory responses are frequent[400-401]. EBNA2 and EBNA3C are also relatively frequent epitope sources for $CD4^+$ recognition. More recently $CD4^+$ T cell clones specific for a range of LMP1- and LMP2-derived epitopes were shown to efficiently recognise latently infected targets and prevent their outgrowth through perforin-mediated cytolysis[402].

Interestingly, a component of the LCL-stimulated $CD4^+$ T cell response of EBV-immune and –naive donors, despite recognising the autologous LCL in a classically HLA-class II-dependent manner, is not EBV antigen-specific[403]. These cytotoxic 'LCL-specific' (Figure 5) cells appear to recognise cellular targets, whose expression is specifically upregulated as an early event in EBV-induced transformation. Such T cells recognise a broad range of human B-lymphoma-derived cell lines *in vitro* including BL and HL lines irrespective of EBV genome status, providing those lines express the relevant HLA class II restricting allele.

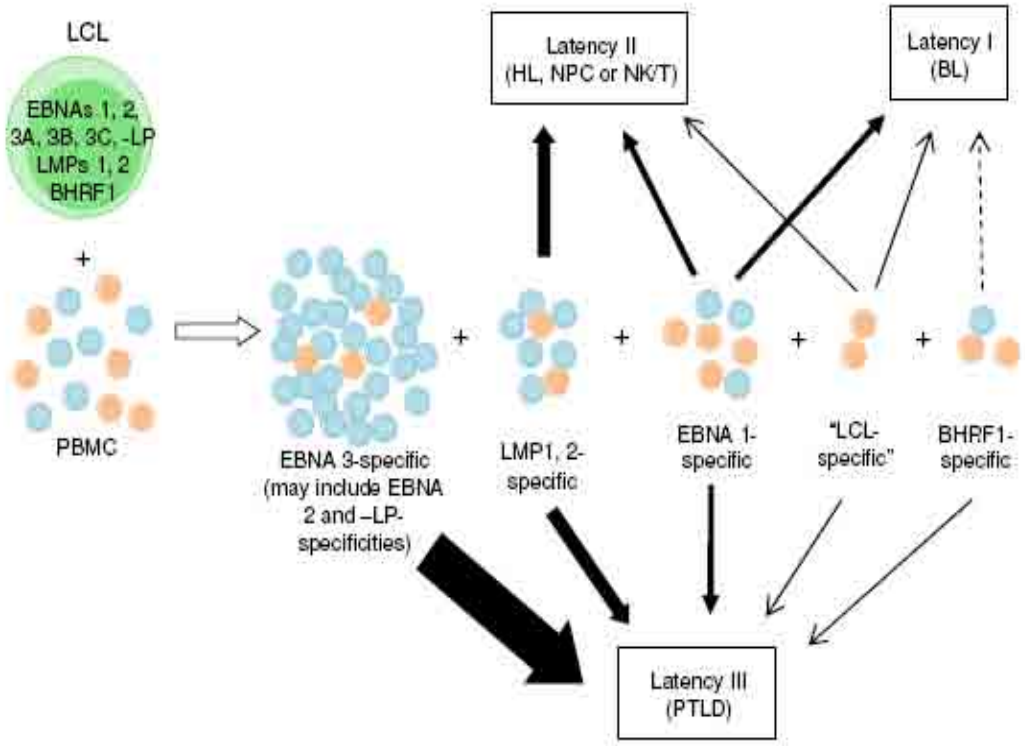


Figure 5 Latent antigen specificities of EBV-specific CTLs

A schematic representing the specificities and frequencies of effector T cells generated using conventional LCL stimulation protocols (adapted from reference[404]). $CD8^+$ T cells of indicated specificity are shaded blue, whilst $CD4^+$ effectors are orange; the number of cells shown indicates their relative frequency within the polyclonal populations. The width of each arrow represents the potential contribution of the respective T cell sub-population to target malignancies displaying alternate patterns of latency.

Post-transplant lymphoproliferative disease

Association with EBV

The importance of intact T cell immunity in controlling EBV's transforming and replicative capabilities is exemplified by EBV-positive lymphoproliferative diseases opportunistically arising in the context of inherited or acquired compromise of T cell number and/or function. Histologically diverse lymphoproliferations arising as a result of iatrogenic immunosuppression following solid-organ or haematopoietic stem-cell transplantation (SOT or HSCT) are the best studied of these diseases and are termed post-transplant lymphoproliferative diseases (PTLD).

The risk of PTLD increases with the degree of T-cell impairment; thus, T-cell depletion of the graft or using incompletely human leukocyte antigen (HLA)–matched grafts that require greater immunosuppressive therapy will increase PTLD incidence. EBV-positive tumours occur earlier following allografting (3-6 months for HSCT, 12-24 months for SOT) when immunosuppression is most severe[405-406]. In most such cases of PTLD the proliferating cells express the full growth-transforming programme of EBV-latent proteins[39,63], akin to EBV-transformed LCLs *in vitro,* although EBNA2/LMP1-negative cases have been described[407].

Pathology

PTLD represents a pathological spectrum that can resemble reactive and neoplastic lymphoid and plasmacytic proliferations, in terms of their histological appearance and cell of origin. A significant minority of cases (20-30%) arising following SOT are EBV negative, whereas almost all post-HSCT are EBV-associated. Most SOT PTLD are of host origin and the converse is true for HSCT PTLD; where most arise from a donor lymphocyte[408].

The WHO[409] classifies PTLD into: 'early lesions' akin to reactive proliferations resembling IM; 'polymorphic PTLD' comprising heterogeneous populations of lymphocytes and plasma cells with architectural destruction of the host tissue, of which there is no immunocompetent counterpart;

'monomorphic PTLD' that most commonly resemble Diffuse Large B cell lymphoma (DLBCL), plasma cell neoplasms, occasionally classical HL, or rarely T or NK cell lymphoma.

B cell PTLD not only arises from the pool of antigen-selected memory cells in which EBV resides in healthy persistence, but from a range of B cells at all stages of the normal differentiation process. Tumour development seems often to be associated with the EBV-facilitated rescue and expansion of B cells that have failed the physiological process of germinal centre selection into memory[407]. Malignant cells displaying germinal centre (GC) markers, post-GC characteristics or plasmacytic/plasmacytoid features can be seen[410].

PTLD of T or NK cell origin account for approximately 7–15% of PTLD in Western countries and appear to be more common in Japan, though incidence data is limited[411]. Compared to their B cell counterparts, T or NK PTLD tend to occur later in the post-transplant period (median 4-5 years) and are less commonly EBV-associated; EBV is demonstrable in 30-40% of cases. Peripheral T cell lymphoma unspecified and $\gamma\delta$-hepatosplenic lymphomas are the most frequently identified subtypes but very rare cases of extranodal NK/T lymphoma have been reported; the latter usually harbour clonal EBV [411-412].

EBV-specific T cell therapy

Although the advent of Rituximab, a therapeutic monoclonal antibody that targets the pan-B cell antigen CD20, has changed the treatment paradigm of PTLD, a significant proportion of patients will not respond to, or relapse following such treatment[405-406]. In this setting, EBV-specific adoptive T cell therapies have a potentially important therapeutic role.

The latent viral proteins that drive B cell PTLD also constitute the target antigens through which, in healthy EBV carriers, virus-transformed cells are recognised and destroyed by virus-specific T-cell surveillance. Accordingly, PTLD provides an ideal opportunity for the application of adoptive cell therapy using EBV-specific T cells. In the context of HSCT, early attempts to treat PTLD using total peripheral blood mononuclear cells from the EBV-immune stem cell donor were marred by collateral graft-versus-host disease (GVHD) [413]. To negotiate this problem, existing

laboratory methods for *in vitro* generation of EBV specific cytotoxic T-cell (EBV-CTL) preparations (see figure 5) were adapted for use *in vivo*, and, by 1995 (a year before others showed proof of principle in a SCID mouse model[414]) clinical efficacy against PTLD was demonstrable[415]. The long-term safety and efficacy of EBV-CTL infusions in a post-HSCT setting is now patent. Ten year outcome data now exists on the largest cohort treated with CTLs post-HSCT[416]. One hundred and one high-risk patients have been treated prophylactically resulting in a fall in EBV genome load in the circulating B-cell pool; more importantly, none of these patients developed PTLD, compared with an observed incidence of 11% in a control cohort receiving similarly T cell–depleted allografts. A further 13 patients received EBV-CTLs therapeutically for either biopsy-proven EBV-positive PTLD or probable disease diagnosed on radiologic and clinical grounds. Complete responses were confirmed in 11 of 13 patients, some with extensive extra-nodal disease including 1 patient with biopsy-proven monoclonal lesions in the central nervous system. Dramatic inflammatory reactions at the site of disease (shown to be mediated by infiltrating EBV-CTLs) occurred in 2 patients, both of whom fully recovered, but no other toxicity or GVHD attributable to EBV-CTLs was observed. Gene-marking of the CTLs enabled *in vivo* tracking. The CTLs persisted in the circulating memory pool for as long as 105 months after infusion and remained capable of expanding *in vitro* in response to EBV antigen challenge.

The situation is somewhat different in the setting of SOT, where the challenge lies in generating autologous EBV-specific CTLs that can function and persist in the face of ongoing immunosuppression. Although EBV-specific CTLs can indeed be expanded *in vitro* from such patients and significant short-term clinical responses[417]and/or reductions in EBV DNA load[418]following CTL infusion have been demonstrated, questions remain over the long-term persistence of the transferred CTLs. Two recent studies have attempted to address this issue by generating EBV-CTLs resistant to Calcineurin inhibitors (e.g. Tacrolimus (FK506) and Cyclosporin A), a commonly used class of immunosuppressive drugs in this context. Employing different mechanisms to render the EBV-CTL resistant to these agents, the authors demonstrated that the modified T cells were shown to expand in the presence of drug without impairment of

cytotoxicity or antigen specificity[419-420]. These data suggest that such genetically modified EBV-CTLs may be of clinical value where ongoing immunosuppression is required.

Two considerable disadvantages of the above strategies however, are the time required for CTL production (6-12 weeks) and the necessity to generate a separate line for each patient. To address this, Haque *et al* generated a bank of 100 polyclonal CTL lines from EBV-seropositive blood donors (i.e. third parties), potentially available to treat patients with PTLD following SOT [421]. CTLs for infusion were selected on the basis of the best HLA match and specific *in vitro* cytotoxicity. Overall response rates were encouraging (17/33 patients at 6 months), with five of the responders having previously failed to achieve a sustained response to Rituximab and/or chemotherapy. Patients receiving more closely HLA-matched CTLs and those receiving higher numbers of $CD4^+$ cells achieved significantly better responses when assessed at six months.

Hodgkin Lymphoma

Association with EBV and pathogenesis

This is an unusual malignancy in that the malignant clone of Reed-Sternberg (RS) cells is a minority population within the tumour tissue; vastly outnumbered by pleomorphic reactive lymphocytes and granulocytes. Approximately 40% of cases of classic Hodgkin lymphoma in the developed world are associated with EBV and tend to co-segregate with the mixed-cellularity histological subtype. In HIV-infected individuals, instances of HL are almost all EBV associated[422-423]. Risk of EBV^+ HL is thought to be increased in individuals who experienced IM as a result of primary EBV infection[424].

In EBV-positive tumours, the clonal viral genome is present in every RS cell and expresses the EBNA1, LMP1 and LMP2 latent proteins in addition to the non-coding EBER and BART mRNAs – a latency II pattern[75]. The identification of RS cells as failed products of germinal-centre reactions by Ig genotyping[425] indicated a conceivable pathogenic role for the virus, based on rescuing RS progenitors from apoptosis. EBNA1 may directly contribute to the pathophysiology of Hodgkin

lymphoma by upregulating CCL20, enticing regulatory T cells (T_{Reg}), and/or by downregulating potential tumour suppressor genes[426]. LMP1 aggregates in the RS cell membrane and mimics an active CD40 receptor, thereby inducing constitutive NF-κB activation[96]in addition to multiple downstream effects, as discussed earlier. LMP2A expression serves as a surrogate BCR[137,140] and can provide a variety of pro-survival signals[139,141-142], but the expression and role of LMP2B in HL has not been established. Given that signalling though the BCR and CD40 are two major survival signals for germinal centre B cells[427], the principal hypothesis is that EBV has hijacked these pathways to facilitate survival of GC cells[428]. It has also been proposed that LMP1 and LMP2A contribute to the loss of B-cell phenotype in EBV^+ HRS cells[429-430].

EBV-specific T cell therapy

Although a large majority of HL patients are cured with dose- and time-intense, multi-agent chemotherapy regimens with or without adjuvant radiotherapy[431], a significant minority fail to respond to conventional treatment or relapse following an initial remission. A proportion of patients with relapsed disease will respond to high-dose chemotherapy with autologous stem cell support[432] and evidence has also emerged of a graft-versus-HL effect in the context of allogeneic stem cell transplantation[433]. However, these approaches are associated with considerable morbidity and mortality, prompting investigators to ask whether the success of EBV-specific CTL therapy in PTLD[416] can be applied to EBV^+ HL.

Early clinical studies using CTLs that had been expanded *in vitro* by stimulation with LCLs, expressing the full range of latent viral proteins, produced limited tumour responses. A major hindrance to this approach was that the CTL lines contained a preponderance of specificities directed towards the immunodominant EBNA 3A, 3B, 3C antigens (see figure 5) or against early lytic cycle proteins – all of which are absent in HL. However, the small numbers of LMP2 CTLs present within the administered CTL preparation were seen to expand and persist *in-vivo*[434].

The consistent expression of LMP2A in Reed-Sternberg cells and the apparent conservation of LMP2 epitope sequences between viral strains and within HL biopsies[156,390,435] renders LMP2 an

attractive target, if an appropriate biased CTL product could be generated from HL patients. Thus, recent research has used LMP2-expressing dendritic cells (DCs) to reactivate LMP2 reactivities and expand this CTL population using autologous LCLs over-expressing LMP2 from a chimeric adenovirus vector (Ad5F35-LMP2)[391,396]. Such an approach increased the frequency of LMP2-specific T cells by up to 100-fold in the resultant polyclonal line, which contained both $CD4^+$ T-helper cells and $CD8^+$ CTLs, targeting multiple LMP-2 epitopes. These LMP2-containing CTL preparations have produced encouraging clinical results in patients with relapsed/refractory HL, some of whom achieved sustained complete remissions following the infusion[395].

Recognising that EBV^+ NK cell lymphoproliferative diseases also display a latency II pattern of viral antigen expression, a small number of such patients were also treated with LMP2-containing CTLs within the same study. Notwithstanding the uncertainties relating to LMP2 expression in these malignancies, a particularly striking clinical response was experienced by one patient with multiply relapsed ENKTL - who achieved a complete response lasting nine months following CTL therapy[395].

Nasopharyngeal carcinoma

Association with EBV

EBV infection of epithelial cells has been associated with various tumours including nasopharyngeal carcinoma (NPC), a subset of gastric adenocarcinomas and certain salivary gland carcinomas[436]. The undifferentiated form of NPC (WHO type III) is most consistently associated with EBV, with particular geographical predilection for areas of China and south-east Asia[437]. Genetic and dietary cofactors are well described[54]. NPC tumours characteristically contain a prominent lymphocytic infiltrate alongside the undifferentiated carcinoma cells. EBV latent gene expression in NPC (latency II) is predominantly restricted to EBNA1, LMP2A and the BamHIA transcripts, whilst approximately 20% of tumours also express the LMP1 protein[436] and a minority also express relatively low levels of LMP2B[76]. Southern-blot hybridization of DNA from NPC confirms viral monoclonality, indicating the presence of EBV prior to expansion of the

malignant clone[436]. Seroepidemiological studies in high-incidence regions have shown that elevated EBV-specific antibody titres, particularly IgA directed to the EBV capsid antigen and early antigens have proved useful in diagnosis and in monitoring the effectiveness of therapy[438]. Carcinomas with similar features to undifferentiated NPC, occurring at various anatomical sites including the thymus, tonsils, lungs, and skin, have been referred to as lymphoepitheliomas and are variably associated with EBV[439].

EBV-specific T cell therapy

Several small studies have treated patients with relapsed and refractory NPC with autologous[440-443] and allogeneic[444] (from an HLA-identical sibling) EBV-specific CTLs, generated by *in vitro* stimulation with LCLs. Although some studies have reported increases in circulating LMP2-specific CTLs, reductions in blood viral load[440] and evidence of $CD8^+$ tumour-infiltrating cells[444] following CTL infusion, significant clinical responses are uncommon[440,442-443]. Recently, lymphodepleting strategies, designed to augment expansion of infused CTLs using anti-CD45 MAbs prior to CTL infusion, have not improved tumour responses substantially although one patient did achieve a sustained CR[441].

Burkitt lymphoma

EBV and pathogenesis

EBV is present in all cases of 'endemic' (that is, holoendemic malarial regions of Africa and New Guinea) Burkitt lymphoma (BL) and in up to 85% of cases in areas of intermediate incidence such as Brazil and North Africa, but in only 15% of the low-incidence 'sporadic' tumours seen in the West. The exception to the latter situation is EBV's more frequent association in those cases of BL arising in CD4 deficient HIV-infected persons[439]. The unifying feature of all BL cases is the translocation of *MYC* to one of the, constitutively active, Ig heavy or light chain promoters – resulting in a recurrent t(8,14), t(2,8) or t(8,22)[445-447]. Although deregulation of the *C-MYC* proto-oncogene is of fundamental importance in BL pathogenesis[448], commonly occurring in

association with defects in the p53 protein[449], EBV is thought to play a key and complementary role in pathogenesis.

The typical phenotype of BL cells ($CD10^+$, $CD77^+$, $BCL6^+$) is commensurate with germinal centroblasts being the 'normal cell counterpart' for these tumours[450], and the detection of ongoing Ig-gene mutation in tumour cells[451] is indeed consistent with a GC origin.

Most cases of EBV^+BL are regarded as Latency I: expression of EBNA1 initiated from the Qp-promoter, in addition to the non-coding EBERs, BARTs and viral miRs. However, two other types of latent gene expression have been described in association with BL. The first involves expression of all six EBNAs in the absence of LMP1 and LMP2. This is considered to be very unusual and may occur as a result of viral genome integration into a cellular chromosome[452]. The second is a latent infection that lacks EBNA2 and LMP1, LMP2 but unusually expresses the EBNA3 proteins. This is thought to occur in approximately 15% of endemic BL cases[453-454] and is due to a deletion including the EBNA2 gene. In this scenario, the Wp promoter rather than the Qp promoter initiates EBNA transcription; hence the term 'Wp-restriction'. Such Wp-restricted latency BL cells express BHRF1, a viral protein previously thought to be exclusively lytic[55].

There are two hypotheses as to how EBV might contribute to BL pathogenesis. One is that the virus might increase the likelihood of the characteristic C-MYC translocations and the second is that EBV might complement the activity of c-myc, perhaps by suppressing the apoptotic activity of the oncoprotein – a feature of MYC that co-exists with its potent proliferative effect[455]. The viral mediator(s) of the putative MYC complementation are unclear: EBNA1's role in this setting remains controversial; BHRF1 has anti-apoptotic activity but is only expressed in the Wp-restricted subtype; and the functions of EBERs, BARTs and the EBV miRs remain under investigation in this context[439,455].

Therapy

The development and application of non-cross resistant, dose- and time-intense, multi-agent chemotherapy regimens that, importantly, contain central nervous system-directed drugs[456-457]

have revolutionised the therapy of BL (where access to such therapies exists). Where this (or similar) therapeutic protocol(s) can be successfully delivered, a large majority of patients expect to be cured of BL; this highly proliferative disease is inherently chemo-sensitive.

Thus, together with the low frequency of EBV^+ BL in the developed world, the case for development of EBV-directed therapies for BL is weaker than for the other EBV-associated tumours. Furthermore, BL presents the greatest challenge for immunotherapy due to the limited repertoire of available viral antigens in association with global deficiency in HLA class I antigen processing, impairing $CD8^+$ T cell recognition[458-459]. According to some reports, BL tumours retain class II processing function and some EBNA1-specific $CD4^+$ T cells can recognize and kill HLA-matched EBNA1-positive BL cells – although this is somewhat controversial.[404]

Association of T and NK lymphoproliferations with Epstein Barr Virus

EBV is so markedly B-lymphotropic when exposed to human lymphocytes *in vitro* that the association of EBV with rare but distinct types of T and NK cell lymphoproliferations was quite unexpected. A spectrum of such lymphoproliferations have only been relatively recently characterised but are now recognised to comprise a heterogeneous spectrum of diseases, affecting humans through all stages of life and conferring considerable morbidity and mortality.

In substantiating the association with EBV, studies throughout the 1980s and 1990s examined both biopsy tissue and circulating lymphocytes for the presence of EBV in patients with a range of unusual clinical syndromes. In some cases of systemic, apparently infectious, illness there followed the development of EBV-positive malignant T cell lymphomas[241]. Similarly to the findings in lymphoma tissue[460-462], clonal forms of the virus were identified in circulating NK cells, $CD8^+$ and $CD4^+$ lymphocytes in patients across a clinical spectrum of lymphoproliferations[242-243,245,463-471].

The fundamental unifying feature of such illnesses appears to be the clonal expansion of EBV-infected T and/or NK cells, although the specific viral and host factors initiating and potentiating the disease processes remain largely unresolved. Furthermore it remains quite unclear why

Table i Spectrum of EBV-associated NK and T cell disease: comparison of clinicopathological features

	Systemic EBV⁺ T-cell lymphoproliferative disorders of childhood (W.H.O 2008)		T and NK malignancies, typically presenting in adulthood (W.H.O 1999)	
	HAEMOPHAGOCYTIC LYMPHOHISTIOCYTOSIS	CHRONIC ACTIVE EBV	EXTRA-NODAL NK/T LYMPHOMA	AGGRESSIVE NK LEUKAEMIA
Age of presentation (years)	2-15[i]	11 (range 0-53)[ii]	45-50[iii]	40[iv]
Major clinical features	Fever Splenomegaly Hepatomegaly Cytopenias	Fever Hepatitis Lymphadenopathy Hepatosplenomegaly Cytopenias	Nasal mass Systemic, extra-nodal sites	Fever/Sweats Weight loss Leukaemia Cytopenias
Typical duration of symptoms	Days to weeks	Months to years	Weeks to months	Days to weeks
Typical cell phenotype	sCD3⁺CD8⁺	sCD3⁺CD4⁺	sCD3⁻CD56⁺⁺ *or* sCD3⁺CD8⁺(cytotoxic)	sCD3⁻CD56⁺⁺

[i] References 374, 495, 500. Variation in survival rates reflect retrospective studies in adults versus children; a more favourable survival seen in *de novo* EBV-HLH cases in children.

[ii] Refs 556, 561. Based on retrospective data from non-uniformly treated children

[iii] Refs 473, 616, 620-22, 660

[iv] Ref 660

Latent EBV[*] gene expression[*]	Unclear	EBERs EBNA1 (Qp) LMP1 (variable)	EBERs EBNA1 (Qp) LMP1 (variable)	EBERs LMP1 negative
Typical treatment approach	Immunosuppression and chemotherapy (i.e. steroids, cyclosporin A and etoposide[vi])	Intensive immunochemotherapy with/without allogeneic stem cell transplant[vii]	High-dose radiotherapy with/without concurrent chemotherapy[viii]	Systemic chemotherapy with/without allogeneic stem cell transplant
Outcome following treatment	32%-76% 4-year survival[ix]	58% 10-year survival[x]	10-71% 5-yr survival[xi]	Median survival <2 months[xii]

[*] See introduction pages 59-60, 67-68, 74-75, 82 for discussion and references

[vi] Ref 476

[vii] Ref 567

[viii] Refs 633, 636-7

[ix] Survival improved following development of HLH 2004 protocol and more favourable in children cf adults. Ref 500.

[x] Based on historical data. Survival may be improved by modern immunochemotherapy and allogeneic stem cell transplant. Ref 567

[xi] Marked difference in outcome according to stage of disease. Refs 473, 623, 625

[xii] Refs 657,663-666.

infection of similar or identical cell types is associated with such a diverse spectrum of clinical illnesses, occurring both in previously EBV-naive and ostensibly EBV-immune individuals. A relationship between the phenotype of the infected cell and the nature of the clinical presentation has been suggested, at least for the systemic childhood illnesses[472]. Thus, in the context of chronic active EBV (CAEBV – discussed later), the predominantly infected cells appear to be either $CD4^+$ T lymphocytes or NK cells, whereas the dominant infection in HLH is found within the $CD8^+$ lymphocyte fraction[471]. However, this is not evidently the case for ENKTL in adults, where the normal cell counterpart is an NK cell or cytotoxic T cell – with apparently indistinguishable clinical presentations[473-474].

The clinical, pathological and biological features of the individual diseases will be detailed separately. However it is interesting to note that their characterisation and association with EBV began with clinical observations on unusual illnesses, in small numbers of patients, with corresponding analysis of clinical material with emerging molecular biological techniques, rather than routine clinical diagnostic tools. Such progress in understanding the pathobiology of this group of diseases would not have been achieved without close clinical-scientific collaboration.

EBV-positive T-cell lymphoproliferative disorders of childhood

WHO definition

"'Systemic EBV^+ T-cell lymphoproliferative disorders of childhood' are life-threatening illness of children and young adults characterised by a clonal proliferation of EBV-infected T cells with an activated cytotoxic phenotype. It can occur shortly after primary infection or in the setting of chronic active EBV (CAEBV)."

Haemophagocytic Lymphohistiocytosis

History

The earliest description of a distinct clinical syndrome associated with histological evidence of erythrophagocytosis was detailed in the Lancet in 1939 by two Oxford Pathologists[475]. A detailed

clinical and pathological description of four fatal cases, termed 'histiocytic medullary histiocytosis' was summarised as follows. *"These cases illustrate what we have come to regard as the typical clinical course of the disease: fever, wasting and generalised lymphadenopathy are associated with splenic and hepatic enlargement and in the final stages jaundice, purpura and anaemia with profound leukopenia may occur. Post mortem examination shows a systematised hyperplasia of histiocytes actively engaged in phagocytosis of erythrocytes"*. These prescient observations remain central features of internationally agreed diagnostic criteria of the present-day clinicopathological syndrome now termed Haemophagocytic Lymphohistiocytosis (HLH)[476] (Table 1, page 63).

Reports of similar clinicopathological entities accumulated in the literature under various terminology, including familial haemophagocytic reticulosis[477], familial erythrophagocytic lymphohisticytosis[478], histiocytic medullary reticulosis[479] and malignant histiocytosis[480]. These reports included seemingly inherited syndromes and some attributable to, or associated with, malignancy although many cases remained of uncertain aetiology. Although a possible link with viral infection had been suggested, it was not until a study of HLH in a group including immunocompromised patients that a causative link with viruses was formally considered likely[481]. Fourteen of 19 patients studied (median age 28 years) were immunosuppressed with Azathioprine and Prednisolone, following *in vivo* T cell depletion (with anti-lymphocyte globulin) and splenectomy at the time of renal transplantation. The remaining five patients were previously healthy. All 19 patients presented with high fever and constitutional symptoms consistent with a viral illness in conjunction with evidence of histiocyte proliferation and haemophagocytosis in the bone marrow (Figure 6). Consistent with subsequent characterisation of HLH[476], abnormalities of liver enzymes, coagulation dysfunction including hypofibrinogenaemia were noted in some cases. Serological or culture evidence of active viral infection was obtained in the majority of cases, of which the members of the herpes virus family dominated. This entity was termed virus-associated haemophagocytic syndrome[481] (VAHS) and two cases with high IgM titres to EBV, suggested a role for this virus in disease pathogenesis - at least in some cases of HLH.

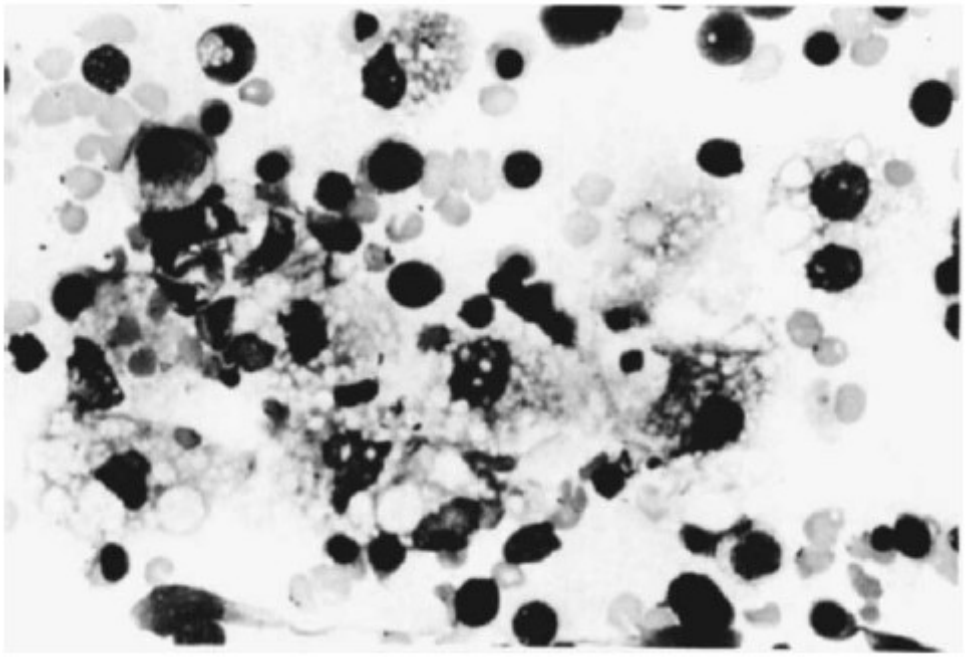


Figure 6 Bone marrow haemophagocytosis in virus-associated haemophagocytic syndrome
From Risdall *et al*[481], the first description of VAHS in 1975. A photomicrograph of a bone marrow smear which shows mature histiocytes with abundant vacuolated cytoplasm. Many histiocytes contain haematopoietic cells.

Association with EBV

Stronger evidence of an association of EBV infection with HLH accumulated. These diagnoses were initially founded on typical clinicopathological features of the HLH syndrome, in association with high serum titres of anti-EBV antibodies[482-484] followed by the finding of viral particles or DNA in affected tissues[485]. The increasing recognition of EBV-HLH and the apparent association with primary EBV infection prompted a detailed analysis of 52 cases of fatal IM that, following a characteristic clinical presentation of IM, severe pancytopenia developed, with bone marrow evidence of infiltration by lymphoid cells, cellular necrosis and marked histiocytic haemophagocytosis. The median survival time of these patients was six weeks, in whom an Epstein-Barr virus-associated haemophagocytic syndrome was strongly implicated in the cause

of death[486]. It is now clear that EBV is the most frequent cause of acquired HLH in the immunocompetent[374,487-488].

A major breakthrough in implicating EBV as a key aetiological agent in HLH was the identification of viral genomes within T lymphocytes in tissue biopsies of affected individuals[240,489-490]. Molecular findings in the majority of these cases confirmed, by southern blot analysis of viral terminal repeats, that EBV was present in monoclonal form. Moreover, clonal rearrangements of germline TCR gene sequences provided supportive evidence of clonal proliferation of a T cell infected with EBV. This notion was supported by studies employing lineage-specific markers with EBER in-situ hybridisation. Kawaguchi *et al* detected EBER-specific signals exclusively within a $CD45RO^+$ $TCR\beta^+$ population; importantly, EBERs were not detectable in B cells or macrophages[465]. Notably, the findings of some reports indicated that EBV^+T cell lymphoma could arise from, or co-exist with, HLH[489] although clear delineation between the two entities is not always straightforward[245,491-492].

The majority of published data has confirmed that $CD3^+$ T cells, most often the $CD8^+$ subset, both in tissue biopsies[240,245,464-465,467,469,493] and circulating lymphocytes[471,494], are the dominant infected population in EBV-HLH. By contrast to CAEBV [472], definitive evidence for EBV infection of NK cells in HLH (in the absence of co-existing NK leukaemia[495]) has not been available.

The pattern of EBV gene expression in EBV-HLH remains unclear. EBERs are frequently expressed by infected T cells and are a useful diagnostic target for in-situ hybridisation on tissue biopsies[465]. However, whether such EBV^+ T cell lymphoproliferations display a Latency II pattern of viral gene expression - in keeping with the related T or NK diseases CAEBV[496] and ENKTL[73] - has not been adequately studied. A limited study analysed mRNA from splenic and peripheral blood mononuclear cells in 3 patients with EBV-HLH and found expression of EBERs, Wp/Cp- and Qp-initiated EBNA1, EBNA2, LMP1 and LMP2A transcripts[122]. This suggested alternate promoter usage in different cell populations and suggests the possibility of B cell derived transcripts, limiting interpretation of the data. An animal model of EBV-HLH, using the baboon

lymphocryptovirus herpesvirus papio (HVP), found mRNA transcripts using end-point PCR to detect HVP-EBNA1, HVP-EBNA2 and HVP-LMP1 in affected tissues[497]. However, interpretation of this study is again limited by lack of analysis at the single-cell level, compounded by lack of MAbs to HVP-encoded latent proteins.

Epidemiology and risk factors

The majority of cases of EBV-HLH occur in the context of primary infection[240,485-486,490] in children and adolescents[498-500]. Adult cases of HLH are rare and usually have an underlying cause other than EBV infection, usually attributable to malignancy and most commonly lymphoma[374,501]. In common with other entities within the spectrum of EBV^+ T and NK lymphoproliferations, the literature is dominated by a majority of EBV-HLH cases arising from study groups in East Asia[499]. However, reports in patients of European[240,494], Middle Eastern[492], North American and Hispanic[245] ethnicities have been described.

Although rare, inheritance of specific immune defects is recognised to be an important and strong predisposition for the development of HLH. The majority of such inherited abnormalities are associated with impaired NK and/or cytotoxic T cell function[502-503]. Familial HLH (FHLH) is an autosomal recessive condition originally described in 1952[477] with an estimated incidence of 1 in 50,000 births. HLH is the primary clinical feature and usually occurs within the first year of life. Several independent genetic loci related to cytolytic granule release by the effector T or NK cell have been implicated in the pathophysiology of genetic HLH. In 1999 mutations in the perforin gene at locus 10q24 were described in 8 patients with FHLH.[504] and a number of further defects have been subsequently characterised[502,505]. Other autosomal recessive immunodeficiencies such as Chédiak-Higashi syndrome[506] and Griscelli syndrome[507] are also recognised to predispose to HLH, which occurs frequently albeit sporadically and often later during the natural history of their disease[502].

XLP (X-linked Lymphoproliferative Disease), initially described in 1975[508] and molecularly characterised in 1998[509-510] is a condition associated with mutations in the signalling lymphocytic

activation molecule (SLAM) associated protein (SAP/SH2D1A). SAP functions as an adaptor, binding to and recruiting signalling molecules to SLAM family receptors expressed on T and NK cells. XLP is associated with marked sensitivity to primary Epstein-Barr virus (EBV) infection, often leading to a lethal infectious mononucleosis and/or HLH[510].

Notwithstanding the original report of VAHS in a largely immunocompromised patient group[481] – in which EBV was implicated in at least two cases, as discussed earlier - most patients presenting with EBV-HLH do not have apparent immunodeficiency. In the context of iatrogenic immune dysfunction CMV appears to be a more frequently implicated agent, although EBV-HLH has been reported and can be associated with a poor outcome[511].

Clinical and laboratory features

Awareness of the clinical presentation and established diagnostic criteria[476] for HLH (Table 1, page 63) is of fundamental importance to allow the prompt initiation of appropriate therapy.

In keeping with Scott and Robb-Smith's original description of a HLH syndrome[475], the cardinal clinical features of EBV-HLH are prolonged high fever, hepatosplenomegaly and cytopenias. Lymphadenopathy, jaundice or neurological manifestations[512-513] such as cranial nerve palsies or symptoms of encephalitis may also be present. Characteristic laboratory findings include elevated serum triglycerides, ferritin (often extremely high and a sensitive marker of disease activity)[514], LDH[494], transaminases, bilirubin and hypofibrinogenaemia[515]. Although not routinely assayed in UK clinical laboratories, serum levels of soluble IL-2 receptor-α (CD25) are frequently elevated in HLH[516], form one component of the diagnostic criteria[476] and appear to be a sensitive marker of disease activity with some prognostic value[517].

Involvement of the central nervous system is common and an elevated cerebrospinal fluid protein with a moderate pleocytosis can be seen[512,518]. Cytopathological evidence of haemophagocytosis is often not evident on initial bone marrow biopsy, particularly if performed early, but is usually manifest as the disease progresses[502]. The apparent absence of haemophagocytosis on initial biopsy, can compound the diagnostic uncertainty related to the non-specific clinical features

which can mimic a wide range of infective and inflammatory conditions[487], including IM[486]. However, evidence of progressive hepatosplenomegaly, cytopenias and biochemical parameters should alert the physician to the possibility of HLH.

Elevated VCA-IgM or -IgG antibodies in the serum, in the absence of EBNA antibodies, may reveal a primary EBV infection, whilst elevated EADR-IgG or -IgA antibodies are thought to represent a reactivation phenomenon[519]. However, serological assays may not always be definitive[492,500] and have been complemented by a substantially more powerful diagnostic tool: quantitative PCR for EBV genomes. Although the biological significance of plasma or serum EBV DNA levels are not clear, cell free viral load is frequently elevated across a range of EBV-associated diseases[520-521] and has also been the assay of choice for some studies on HLH. At the time of active disease, EBV copy numbers ranging from 10^2 to 10^7/ml of plasma[522-523] or serum[524] have been documented. Such levels usually exceed those seen in cases of uncomplicated IM assayed in parallel[494,524]. More limited data is available on the viral load in circulating lymphocytes, although a recent European study found copy numbers of 10^3 to 10^5 (per million cell equivalents) in B lymphocytes and 10^2 to 10^6 in total T cells[494].

Pathophysiology

The clinical manifestations of HLH are, at least in part, a result of a dramatically dysregulated inflammatory response due to release of pro-inflammatory cytokines including interferon (IFN)-γ, tumour necrosis factor (TNF)-α, IL-6, IL-10 and macrophage-colony-stimulating factor (M-CSF)[525]. These mediators are secreted by activated T-lymphocytes and infiltrating histiocytes, which can instigate tissue necrosis and organ dysfunction. In particular, $CD8^+$ T lymphocytes and IFNγ have been shown to be necessary mediators of the disease process in an *in vivo* model[526]. Inflammatory cytokines are also responsible for the haematological and biochemical manifestations such as cytopenias, coagulopathy and elevated triglycerides[502]. Serum levels of Fas ligand (a membrane protein expressed on cytotoxic T and NK cells) has also been noted to be elevated in patients with HLH[527] and may explain features such as liver dysfunction, as is the case in NK leukaemia[528].

Table 1 Diagnostic criteria for EBV-HLH

Clinical and Laboratory features*	Ranges/Parameters
Original criteria[529]	
Fever	
Splenomegaly	
Cytopenias (≥2 lineages)	Hb ≤9g/dl Plt ≤100x10^9/l Neuts ≤1 x10^9/l
Hypertriglyceridaemia	≤3mmol/l
Hypofibrinogenaemia	≤1.5g/l
Haemophagocytosis†	(BM, spleen, liver, LN)
New criteria[476]	
Low or absent NK activity	(local lab. reference range)
Elevated serum ferritin	≥500µg/l
Elevated soluble serum CD25 (soluble IL2 receptor)	≥2400U/l
Supportive features	(local laboratory reference ranges)
CSF pleocytosis and/or elevated protein	(may resemble chronic persistent hepatitis)
Histological evidence of hepatitis	
Features consistent with the diagnosis	
Cerebromeningeal symptoms	
Lymphadenopathy	
Jaundice	
Oedema	
Skin rash	
Hepatic enzyme derangement	

* Note that patients with a molecular diagnosis consistent with HLH do not necessarily need to fulfil the criteria here.

† Note that initial biopsy/ies may lack evidence of haemophagocytosis or be inconclusive. Further biopsies are encouraged if a strong clinical suspicion exists.

Hb=Haemoglobin, Plt=platelet count, Neuts=neutrophil count, BM=bone marrow, LN=lymph node, NK=Natural killer cell, CSF=cerebrospinal fluid

Immunocompetent rabbits, infected with HVP, a baboon lymphocryptovirus[530], developed a HVP-driven, fatal monoclonal/oligoclonal T cell lymphoproliferative disease with similar clinicopathological characteristics to EBV-HLH[497]. Another study, employing the same *in vivo* model of EBV-HLH described the emergence of erythrocyte and platelet antibodies at the time of peak HVP genome load. This appearance of antibody-coated erythrocytes anteceded erythrophagocytosis in tissues and heralded the onset of the full clinical syndrome. Such phagocytosis was shown to be specifically mediated by Fc-mediated macrophage activation[531].

Impairment of the cytotoxic function of NK cells and CTLs seems to be a common denominator across both inherited and acquired HLH syndromes[488,532], although the mechanisms leading to cytolytic defects in immunocompetent patients with EBV-HLH are not clear. Elevated levels of cytokines such as IL-12 have been shown to impact on NK function[533]. Lastly, the apparent geographical disparities in the incidence of EBV-HLH may suggest a hitherto unidentified genetic susceptibility resulting in a dysfunctional immune response to infected cells.

Prognosis and Therapy

The aims of HLH therapy are to suppress the augmented inflammatory response with immunosuppressive/immunomodulatory agents, support and restore organ function, and (in the case of EBV-HLH) eliminate EBV-harbouring T cells with cytotoxic drugs.

A key therapeutic advance in HLH was the observation that use of the epipodophyllotoxin derivatives etoposide, and later teniposide, in combination with corticosteroids could induce prolonged symptomatic resolution[534-536]. Moreover, the early application of etoposide (within 4 weeks of diagnosis) has been shown to substantially improve outcome in EBV-HLH[537]. Intravenous (human) immunoglobulin usually results in sub-optimal responses[537]. The immunosuppressive drugs cyclosporin (CSA) [538] and anti-thymocyte globulin (ATG) are also effective in FHL[539]. CSA has shown to be of clinical value in improving cytopenias in the context of an Etoposide-based regimen[540]. In the international HLH-94 protocol (initially designed for familial HLH[541] and later adopted for patients with acquired disease, including EBV-HLH

patients[476,542], etoposide and dexamethasone were combined with CSA[541]. Due to the concern of CNS morbidity and mortality[512-513], dexamethasone is the preferred corticosteroid as it penetrates the blood-brain barrier. Intrathecal Methotrexate has also been used for cases of CNS involvement, particularly if no improvement with systemic therapy is seen[541]. For those patients with refractory disease[500] and those with familial defects[543], allo-HSCT can result in long-term disease-free survival.

Focusing on the prognosis of patients with EBV-HLH, Imashaku *et al* described the outcome of 78 consecutive children. Although treatment was somewhat heterogeneous, the majority received an etoposide-containing regimen – many as part of a therapeutic protocol based on HLH-94 immunochemotherapy[541]. With a median follow-up of 43 months, evidence of disease recrudescence was noted in 13 patients (19.4%) and a total of 12 patients required allo-HSCT, of whom 9 are alive and well. Haemorrhage and infections accounted for the 11 early deaths, whilst late deaths were due to relapse (4 patients), transplant-related mortality (3 patients) and secondary leukaemia (1 patient). Overall, after a median follow-up of almost 4 years, 59 (75.6%) of the cohort are alive and well, indicating the effectiveness of similar immuno-chemotherapy protocols as used for familial disease[476,541]. The prognosis and outcome of adult patients with EBV-HLH has been less well studied. One study analysed the outcome of 20 young adults (aged 17-33 years) treated for EBV-HLH[495]. At a median follow-up of 2.5 years, 32.5% of patients remained alive and well – although a caveat is that some cases in this cohort had co-existing NK leukaemia, conferring a poor prognosis. Patients treated with Etoposide appeared to have a more favourable prognosis. Nonetheless, the available published data[245,492,495,501] suggests a more adverse prognosis for adults with EBV-HLH than in children, although a higher incidence of co-existing lymphoma in adults, and inter-study treatment heterogeneity should be noted.

Chronic Active EBV

History and terminology

In 1948, Isaacs described a cohort of patients with fatigue, fever, splenomegaly and small volume lymphadenopathy persisting for 3 months to over 4 years after an initial episode of clinically-defined infectious mononucleosis [544]. All patients were found to have variable numbers of mononuclear cells termed ‘infectious mononucleosis cells’ in their peripheral blood although the absolute peripheral blood counts were normal. A subsequent study that examined antibody responses to EA following acute IM noted that although many patients had an unremarkable clinical course, a subset of patients experienced protracted symptomatology over periods of 4 to 28 months. These patients tended to have late anti-EA antibody responses and relatively high persistent VCA titres[545].

Reports of patients with clinical syndromes consistent with ‘chronic symptomatic EBV infection’ accumulated [546-549] although terminological inconsistencies caused some confusion in the literature. Reference to 'chronic mononucleosis' was considered misleading, as the chronic illness was not associated with persistent lymphocytosis and in many cases was not preceded by acute IM[550]. On the basis of available clinicopathological data, diagnostic criteria based on clinical and serologic findings were proposed[551-552]. The term chronic active Epstein–Barr virus (CAEBV) disease was applied for patients with a systemic EBV-positive lymphoproliferative disease characterised by fever, lymphadenopathy and splenomegaly developing after primary EBV infection in patients without known immunodeficiency[552]. Proposed diagnostic guidelines required persistence of symptoms for at least 6 months associated with high IgG antibody titres to the viral capsid antigen (VCA) and early antigen (EA). This definition arose from observations on affected children from the Western hemisphere and was proposed to result from progressive EBV infection of B cells, although this was not formally proven.

The evidence for a causal relationship between persistent EBV infection and a chronic IM-like disease strengthened with reports of patients in whom chronic symptoms first appeared following

proven IM, with a serological picture (high anti-EA, very low or absent anti-EBNA titres) that never developed beyond that expected exclusively during acute primary infection. All four patients in this study had reduced EBV-specific cytotoxic T-cell activity, without apparent impairment of other CTL responses[553].

Infection of T and NK cells

The first evidence of an association of CAEBV with infection of non-B cells arose from a detailed clinicopathological study of a young child with clinical and serological evidence of CAEBV[242]. $EBNA^+$ cells were detected in blood, bone marrow and lymph node and, unexpectedly, clonal EBV genomes were identified in peripheral blood $CD4^+$ T lymphocytes. There followed a series of reports, predominantly arising from Japan and East Asia, that contributed to an emerging picture of the clinical and virological features of CAEBV[463,469-470,554-555,523,555-559]. The striking pathological feature evident within these studies was the finding of EBV-infected T or NK cells in the blood or tissue of affected patients[463,469-470,554-555,471,556]. Importantly, Southern blot analysis of viral terminal repeats consistently demonstrated clonal or oligoclonal EBV genomes[463,469-470,554-555,556], implicating the virus in the early stages of disease pathogenesis.

The advent of quantitative PCR for EBV genome load in peripheral blood and tissue biopsies[560] has proved invaluable and is considered a more sensitive diagnostic parameter than EBV serology data, which can be normal in a minority of patients with clear clinicopathological evidence of CAEBV [556]. The published data suggests that patients with clinically apparent CAEBV, assessed prior to therapy, have viral load values in the order of 10^3-10^7 genomes/million PBMC[556,561] and 10^2-10^6 copies per ml of plasma[523,557-558]. One study analysed whether viral loads correlated with disease severity: patients experiencing 2 or more life-threatening complications had significantly higher viral loads than those with fewer complications ($p=0.046$)[556].

On the basis of analyses of EBV-encoded proteins in cell lines established from patients with CAEBV[562], the available data suggests a Latency II pattern of viral gene expression in EBV-

infected T and NK cells, at least *in vitro.* Studies examining viral gene expression in *ex-vivo* lymphocytes from CAEBV patients have been scarce. Iwata *et al*[563] recently described a pattern of EBV latent antigen expression including Qp-initiated EBNA1, LMP1, and LMP2. Although this study used RNA extracted from total PBMC, the viral load in each case was confirmed to be predominantly within the T or NK population. In particular, LMP2 was consistently expressed amongst CAEBV patients although the PCR assay did not distinguish between LMP2A and LMP2B. EBNA2 and lytic transcripts were absent; thus the pattern was representative of 'Latency II'. This was consistent with previous, non-quantitative PCR studies on CAEBV PBMC *ex-vivo*[564-565].

Although monoclonal or oligoclonal EBV has been consistently identified in the infected T/NK populations of patients with CAEBV, further details relating to the genome copy number per cell and whether the virus genome is episomal or integrated (or both) into cellular chromosomes, are limited. An early study examined lymph node tissue from 4 patients with CAEBV and found, by in-situ hybridisation studies, evidence of viral genome integration into the host chromosomes. For two cases in which integration events were more frequently identified, an abnormal karotype was also evident: one with an oligoclonal abnormality of chromosome 6 and one case with a clonal 6th deletion (del(6)(q15q23)). Further data on EBV genome integration in CAEBV has not been forthcoming, but metaphase analysis of a larger number of patients with CAEBV has confirmed the frequent finding of associated chromosomal aberrations[556]. PBMCs from 10 of 20 patients examined exhibited a range of chromosomal abnormalities, some complex, but no evidence of recurrent abnormalities.

Clinical features, prognosis and therapy

A detailed Japanese study of 30 CAEBV cases[556], followed by a nationwide survey that captured data on 82 patients[561], included elevated EBV DNA load in blood or tissue as a diagnostic criterion alongside the increasingly recognised clinical features (including fever, hepatitis, lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme[566], or hypersensitivity to mosquito bites[555]). Patients in these analyses could be

delineated into two groups according to whether T cells or NK cells were the predominant EBV-harbouring cell, as assessed by quantitative PCR or in-situ hybridisation. Interestingly, although all patients fulfilled the stipulated diagnostic criteria, the two respective groups seemed to exhibit different clinical features and prognosis. T cell-type infection was characterised by fever and high titres of EBV-specific antibodies, whereas patients with NK cell–type infection exhibited hypersensitivity to mosquito bites and high titres of IgE as distinguishing features. Importantly, patients with T cell–type infection appeared to have significantly poorer outcomes[556,561,564].

The relatively large study of 82 patients[561] provided a more representative insight into the natural history and prognosis of CAEBV, at least for East Asian cases. The mean age at onset of symptoms was 11.3 years (range 9 months to 53 years). Consistent with previous descriptions, fever, hepatomegaly, splenomegaly and liver dysfunction were manifest in almost all cases whereas lymphadenopathy, thrombocytopenia and anaemia featured in 40-45% of patients. The previously reported phenomenon of hypersensitivity to mosquito bites[555] was seen in one-third of patients and skin rash was noted in 25%. Life-threatening complications were common and included haemophagocytic syndrome (24.4% - no data was available for whether these HLH cases were of T of NK origin), malignant lymphoma (18.3%), disseminated intravascular coagulopathy (15.9%), hepatic failure (14.6%), digestive tract ulcer/perforation (11.0%), coronary artery aneurysms (8.5%), CNS involvement (8.5%), myocarditis (6.1%), interstitial pneumonia (4.8%), and leukaemia (4.8%).

Survival probabilities for the whole cohort were estimated at 68% (range 62-76%) and 58% (51-65%) at 5 and 10 years respectively. Patients with T cell-type infection fared much worse, with 59% probability of survival at 5 years compared with 87% for those with predominantly NK-type infection. Other adverse prognostic features evident on multivariate analysis included the severity of thrombocytopenia at diagnosis and age >8years at disease onset. The impact of therapy in this retrospective study could not be assessed due to the wide range of treatment approaches undertaken in multiple study centres. However, given the generally poor outcomes following immunoregulatory drugs and antiviral agents, one study group has investigated a new therapeutic

algorithm for CAEBV comprising sequential immuno-chemotherapy, combination chemotherapy and allogeneic hematopoietic SCT[567] (allo-HSCT) in an attempt to reduce and/or eliminate EBV-infected T/NK cells. In the experience of the investigators, complete responses are rare with a correspondingly high relapse risk and therefore allogeneic haematopoietic stem cell transplantation (allo-HSCT) is considered for all patients in whom evidence of residual disease exists. Since the first report of allo-HSCT for severe CAEBV in 2000[568], Dr Kawa's group have undertaken allo-HSCT for 29 patients with CAEBV, using either conventional myeloablative (MA) conditioning (11 patients) or reduced-intensity conditioning (RIC) (18 patients). A recent analysis of their clinical outcomes[567] showed a high transplant-related mortality in the MA group (45%), resulting in a 3 year event-free survival (EFS) and overall (OS) of 54.5±15.0% for both outcome measures. By contrast, the 3-year EFS for the RIC group was 85.0±8.0% and the 3-year OS was 95.0±4.9%. Importantly, for both groups, a plateau in the survival curves is evident – indicating the remarkable potential of allo-HSCT to achieve long-term disease-free survival for patients with severe CAEBV.

Extra-nodal NK/T-cell lymphoma, nasal type

World Health Organisation definition

'A predominantly extra-nodal lymphoma characterised by vascular damage and destruction, prominent necrosis, cytotoxic phenotype and association with Epstein-Barr virus (EBV). It is designated "NK/T" (instead of "NK") because while most cases appear to be genuine NK-cell neoplasms, some cases show a cytotoxic T-cell phenotype'[474].

History

Extra-nodal NK/T-cell lymphoma (ENKTL) is a relatively recently characterised clinicopathological entity, being formally incorporated into the WHO classification of haematopoietic and lymphoid tumours in 1999[569]. However, a historical trail of descriptive clinical and pathological literature suggests that this entity was recognised over a century earlier and provides a fascinating insight into early attempts to characterise, classify and understand this initially enigmatic disease.

The first clinical description of a disease resembling what is now recognised as the most common manifestation of ENKTL – a destructive nasal lesion – was recorded in 1897 by McBride who described a patient in whom an ulcer developed on the left lateral surface of the nose that within a year, at the time of death, had extended to both cheeks causing extensive tissue damage to the nose and upper lip[570]. Although syphilis and tuberculosis were not specifically excluded as diagnoses, a review of tissue sections by a pathologist (Dr. Muir) was *"strongly of the opinion that the disease does not correspond to any type with which we are familiar.*"

A perhaps clearer account was subsequently published by Sir Robert Woods in 1921[571] when he described two patients with destructive nasal lesions, in whom syphilis was excluded as a diagnosis and no infectious organism could be identified. He described the lesion as a *"wave of granulation tissue advancing irregularly into healthy parts, breaking down behind as it advanced in front, so that there was never any great depth of pathological growth present."* The term "malignant granuloma" was suggested by his colleague Dr. O'Sullivan. Further clinical and histological accounts of such a disease accumulated over the following decades[572 573-579].

However, in spite of the increasing recognition of this disease as a distinct entity, confusion of the nature of the lesion remained. It had been recognised that patients with 'Stewart's nasal granuloma' may eventually die with disseminated malignant lymphoma[580] but difficulties remained in distinguishing a malignant nasal lymphoma from non-malignant conditions such as Wegner's granulomatosis[581]. Harrison suggested that this was likely to be due to the frequently 'under-representative' biopsy material available for pathological study[581] and went on to review the clinical and pathological cases of 28 patients with non-healing granuloma. He proposed that '*where the lesion remained localized to the nose or pharynx, irrespective of the histological appearances, then this condition should be considered as a neoplasm, probably attenuated to a varying degree by the individual's own immunological defences'*. Harrison also made the prescient observation that patients with localised lymphoma *'may have a surprisingly good prognosis when treated with radiotherapy'*, in fact a documented response to radium therapy was also described in one of Robert Woods' original cases[571].

Phenotype and genotype

Following the development and application of immunological techniques to pathological diagnosis, a study by Ishii *et al*[582] was the first to demonstrate that the malignant cells in cases of 'lethal midline granuloma' or 'rhinitis gangrenosa progressiva' (other terms included polymorphic reticulosis or malignant midline reticulosis) reacted with anti-sera directed to T cell, but not B cell, antigens[582]. Importantly, a separate analysis of DNA derived from such tumours found evidence of rearranged TCR genes indicating a clonal T-cell proliferation[583].

The apparent T-cell origin of this lymphoma was corroborated by further pathological studies in East Asia[584-585] and the United States[586]. However, the development of antibodies against the CD56 antigen questioned the T cell phenotype of the malignant cells[587-590]. A body of evidence then emerged supporting the notion that the majority of these tumours were of natural killer cell origin, with germline T cell receptor gene configurations[461,588,591-595]. The reactivity of polyclonal anti-CD3 antibodies with the cytoplasmic subunit (ε-chain) of the CD3 molecule in formalin-fixed tissues is the likely explanation for the original phenotypic interpretation[596-599].

Emile et al[600] hypothesised that lymphomas expressing some T-cell-associated antigens, but without surface expression of either a αβ or γδ TCR, may have arisen from NK cells. Of 35 'TCR-silent' lymphomas identified, 15 were found to have rearranged TCR γ genes with a clinical presentation predominantly involving lymph nodes (14/15). By contrast, 16 cases were $CD56^{+}$ and of 9 cases analysed for TCR γ gene rearrangements, 8 cases were negative. Of note, all but one of these cases had an extranodal presentation, and 11 of 16 fulfilled the criteria for the entity termed nasal T/NK cell lymphoma[594,601]. This study provided support to the concept that nasal T/NK cell lymphoma is a distinct entity and substantiated the case for a derivation from NK rather than T cells, although did not investigate the EBV status of the tumours.

Consequent upon such difficulties in morphological and phenotypic characterisation was that the evolving lymphoma classification systems - Rappaport[602], Kiel[603] and the Working Formulation[604] - did not include ENKTL as a distinct entity. In the Revised European and American Lymphoma

(REAL) Classification[605], the entity 'angiocentric lymphoma' was used, although it has since been recognised that this pathological description is not unique to ENKTL.

The characteristic phenotype of ENKTL is now understood to comprise $CD2^+$, $CD56^+$, surface CD3- (as demonstrated on fresh/frozen tissue) and cytoplasmic CD3ε (as demonstrated on FFPE tissues). Although CD56 is a very useful marker for NK cells, it can also be expressed on peripheral T cell lymphomas, particularly those that express the γδ T cell receptor[474]. The largest clinicopathological study of ENKTL since its incorporation into the WHO classification[474,569] has provided further information on the phenotype of the tumour cells. The International T cell project studied 136 cases of ENKTL and confirmed the expression of CD56 and cytotoxic markers (TIA-1 and Granzyme) in a majority of cases, but also identified a minority of tumours (14%) with a $CD8^+$ phenotype. Rearranged T cell receptor genes (by Southern Blot analysis) were found in approximately one-third of 52 cases tested[473]. Note that such a proportion of cases with TCR rearrangements is greater than might be expected from the phenotypic studies, indicating that further detailed phenotypic/genotypic correlative studies are required.

Association with EBV

The first compelling evidence that EBV infection could be implicated in the development of T and NK lymphomas arose from a report that described 3 patients with clinical and serological features suggesting a background of chronic active EBV (CAEBV) who subsequently developed fatal T cell lymphoma[241]. These tumours were shown to contain clonal EBV as determined by viral terminal repeat analysis. Subsequent studies identified EBV genomes and/or EBV-encoded RNAs (EBERs) within the tumour cells of both nasal and extra-nasal T and NK lymphomas arising in children and adults[460-462,589,593]. The association with EBV was observed to be most robust in extra-nodal lymphomas and those arising in the nasopharynx[606]. The clonal and episomal form of the virus in the tumour cells[607-608], together with the expression of EBV-encoded transcripts and proteins[73,469,607-609], suggested a causative role for the virus in disease pathogenesis.

Although the incidence of ENKTL is higher in East Asia and South America than in Europe and North America, an invariable association with EBV is apparent irrespective of geographical origin[473]. Indeed, demonstration of the virus in the malignant cells, usually by virtue of EBER *in-situ* hybridisation, is virtually a requisite for diagnosis[474].

Viral gene expression in ENKTL

Initial analyses of six cases of ENKTL, confirming the presence of the virus within the malignant cells[607], also performed immunostaining for LMP1 and found numerous positive cells in four of six cases. A follow-up study from the same group in Hong Kong comprehensively analysed 23 cases of ENKTL, to establish a more comprehensive picture of EBV gene expression at the mRNA and protein level[73]. Immunostaining for LMP1 revealed heterogeneous membrane positivity in a sub-population of $EBER^+$ cells in 15 of 23 cases. Of note, both cases of extra-nasal tumours analysed were LMP1 negative and, importantly, none of the 23 cases expressed EBNA-2 or BZLF1 protein. Further characterisation was performed at the RNA level by conventional end-point RT-PCR. The BARTs were expressed in the majority of cases, whilst EBNA-1 transcripts were present in 15/23 cases and, similarly to BL[74] and NPC[76] confirmed to be Qp-initiated. LMP1 transcripts were readily detected in all cases, whilst LMP2A and LMP2B mRNAs were low or absent in the majority of tumours. A similar study examining Japanese cases of ENKTL found comparable results: LMP1 and Qp-initiated EBNA-1 were detected in all seven cases at the mRNA level. Data from a study of 7 European cases was also in keeping with the East Asian data[610]. QUK spliced EBNA1 was present in the majority of cases, whilst LMP1 was variably expressed at the mRNA level in 5 of 7 biopsies examined. By contrast, a small study could find no LMP1 mRNA or protein in 4 cases[611] although two tumours were not phenotypically characteristic of ENKTL (CD4 positive, CD56 not tested) and, unusually, a leukaemic phase was manifest in all cases. Moreover, the gene expression analyses were performed on the circulating tumour cells, rather than the tissue from the primary tumour, and may therefore not be representative of the original malignant population.

Taken together, the burden of published evidence[73,469,608-610,612-614] is most consistent with a 'Latency II' pattern of gene expression akin to HL[75] and NPC[76], although intra-tumour heterogeneity exists. In particular, LMP1 expression is variable and heterogeneous at the single cell level in ENKTL[73,614], whereas LMP2 mRNA levels appear low or absent in analysed cases[73,610,612].

Epidemiology

Extra-nodal NK/T lymphoma (ENKTL) is an aggressive malignancy with a unique geographical distribution; rare in Western countries and more frequently encountered in East Asia and Central/South America[250-252,473,615-618]. Importantly, only studies applying the WHO diagnostic criteria for ENKTL[474,569] can be expected to provide a meaningful estimate of incidence. A single-centre study documented that ENKTL comprised 6.27% of almost 1000 incident NHL cases studied – equating to over one-third of all T and NK cases in this Chinese NHL population[617]. Similar incidence data was recently published from a large Korean study[616] – in which ENKTL accounted for 4% of all NHL cases and 30% of mature NK and T cell neoplasms. By contrast, the best estimate of incidence in Europe and the United States is that ENKTL represents 4% of all NK and T cell lymphoma subtypes[473] - equating to approximately 0.5% of all NHLs[619].

Patients with ENKTL are usually immunocompetent. The median age of presentation is 45-50 years with a male: female ratio of 2-3:1[473,616,620-622] but it should be emphasised that much of the basic demographic data on this disease relating to incidence, age/sex distribution and patient ethnicity is not well characterised outside East Asia.

Clinical Features

ENKTL commonly affects the upper aero-digestive tract (characteristically the nasal cavity), though extra-nasal disease (for example skin, gastrointestinal tract, testis) can account for one quarter of cases[473]. Primary nodal involvement, in the absence of extra-nodal disease, is rare. Bone marrow involvement at diagnosis, as determined by conventional immunohistochemistry, occurs in a minority (6-14% of cases[473,623]) although this may be an underestimate[624]. Clinical

presentation is typically referable to local symptoms from a nasal mass, including obstructive symptoms and bleeding. Hoarseness of voice, dysphagia, proptosis, ophthalmoplegia and dysphonia can also occur according to the extent of local tumour invasion[625].

In contrast to the more prevalent high-grade lymphoma subtypes, clinical outcome is not accurately predicted by the traditional prognostic index (IPI)[626-627]. This is partly attributable to the younger median age of patients with NK/T lymphoma and the high frequency of stage I/II disease (present in approximately three-quarters of patients[473,623]). Published data suggests that a poorer prognosis is conferred by local invasiveness, elevated serum LDH, advanced stage disease and the presence of B symptoms[623], termed the NKIPI and recently validated in a separate, multi-institutional cohort[473].

Therapy

Conventional chemo-radiation

ENKTL is clinically aggressive, displaying inherent resistance to anthracycline-based chemotherapy regimens such as CHOP[628] thought to be mediated, at least in part, by expression of the drug-efflux pump P-glycoprotein (PGP); encoded by the MDR1 gene[629-630]. The outcome of extra-nasal and advanced stage disease is extremely poor[473,623] with little therapeutic progress achieved in this group of patients[631]. By contrast to their documented chemo-resistance, the tumours are usually sensitive to radiation therapy which, when given at relatively high doses[632], forms an integral and effective part of front-line therapy for limited-stage disease[633]. However, in spite of high rates of initial response following involved-field radiotherapy, up to 50% of those with localised disease will experience relapse, usually within a year of completing first-line therapy[625,633-635]. Recently published data from early phase clinical trials examining the role of concurrent chemo-radiotherapy (using non-cross resistant, non-PGP chemotherapy agents) in localised ENKTL are encouraging[636,637], but remains to be tested in randomised phase III clinical studies. Encouraging data has also emerged, both from retrospective[638-639] and early phase prospective studies[640], on the activity of L-Asparaginase in relapsed/refractory ENKTL when

combined with chemo/radiotherapy. Studies are in progress to further delineate its therapeutic utility for patients with ENKTL.

Nevertheless, despite signs of therapeutic progress, an extremely poor outcome is anticipated for the majority of patients with this disease. This is exemplified by data from the retrospective international T cell project study[473], in which the median overall survival for the whole study cohort was 7.8 months, representing the poorest survival of all T cell lymphoma subtypes examined[641].

High-dose therapy and autologous stem cell transplantation

Attempts to improve outcome in ENKTL have included studies of high-dose therapy (HDT) and autologous stem cell transplantation (ASCT), primarily undertaken in East Asia. The key question is whether HDT-ASCT can overcome the primary chemotherapy resistance frequently associated with ENKTL, reduce rates of recurrence and lead to improved outcomes. The majority of published data is based on retrospective analyses of relatively small cohorts[642-644].

Au et al[642] examined data on 18 patients who received HDT-ASCT for histologically confirmed ENKTL, three of whom were included in an earlier report[645]. The majority of patients (72%) had Stage I disease and seven patients underwent ASCT in first complete remission (CR1). Half of this cohort experienced relapse, all within six months following ASCT. Overall survival (OS) plateaued after six months, at 39% (Confidence intervals [CI] 26.6-51.4%). Interpretation of this outcome data is limited owing to the relatively large proportion of patients with Stage I disease in CR1. However it does suggest that employing ASCT as salvage therapy *beyond* CR1 is associated with a poorer outcome than initially suggested[645]. A comparative study from Korea[646] identified 16 patients, from a large database of NK/T lymphomas[623], who had received HDT/ASCT as post-remission or salvage therapy. This cohort comprised mainly younger patients (median 36 years), although the majority had poor prognostic features by virtue of having stage II-IV disease (63%) and a minority (19%) being in CR1 at the time of ASCT. With a median follow-up of 22 months, progression-free survival (PFS) was 25.8% (CI 11.5%-40.1%) and OS of 71.3% (CI 58.9-83.7%); the latter explained in part by successful salvage of post-ASCT relapses by

allogeneic HSCT or additional chemotherapy. Comparison with a non-ASCT cohort demonstrated no significant survival advantage for patients undergoing ASCT and meaningful interpretation of sub-group analyses is not possible due to small patient numbers.

The data from these two studies and a further cohort from Japan[644] were subsequently pooled and analysed as a multicentre, matched controlled study. Multivariate analyses on this group of 47 patients suggested a survival advantage for those undergoing ASCT, although the survival benefit appeared small. Disease status at ASCT appeared to be the strongest prognostic factor for OS and PFS. The authors tentatively recommend consideration of ASCT for patients with high risk NKIPI in CR1, but acknowledge that longer follow-up of a larger cohort is required. Overall, the published data on the role of ASCT in the management of ENKTL are not definitive and larger collaborative studies are necessary.

Allogeneic haematopoietic stem cell transplantation

The notion of harnessing a graft-versus-lymphoma effect against malignancies inherently resistant to conventional therapies is attractive and clearly has a role for some patients with more common subtypes of T cell lymphomas[647-648]. The invariable presence of EBV in the tumour cells of ENKTL, expressing the viral antigens EBNA1, LMP1 with/without LMP2, provides additional allo-reactive T cell targets. *In vitro* data (based on ENKTL cell lines) suggests that EBNA1[649] and LMP1[650] peptides can serve as targets for $CD4^+$ effector T cells. Moreover, preliminary *in vivo* studies have adoptively transferred autologous, *ex-vivo*-stimulated, LMP2-specific cytotoxic T lymphocytes to patients with ENKTL with encouraging results[395,651].

The role of allogeneic HSCT for patients with ENKTL remains unclear; the two largest reported series comprise six[644] and twenty-two[652] patients. Murashige *et al*[652] studied a total of 28 patients with NK cell malignancies, of which 22 were classified as ENKTL (the remaining cases comprised 3 blastic NK lymphomas and 3 aggressive NK leukaemias), although the outcome data is presented on the whole cohort. The majority of patients received allogeneic stem cells from a sibling donor following a myeloablative (MA) conditioning regimen. Sixteen patients (57%) were

defined as chemo-refractory, 19 patients (68%) had stage IV disease and 8 (29%) were in CR at the time of allo-HSCT. The one-year transplant-related mortality (TRM) in this poor-risk group of patients was 29%, with a further 29% experiencing disease relapse/progression at a median time of 1.8 months post-HSCT. Notably, all patients who remained relapse-free at 10 months post-HSCT continued in CR at a median follow-up of 34 months. Eight patients developed *de novo* chronic graft-versus-host disease; no donor lymphocyte infusions were administered. Suzuki et al [644] analysed six young patients (median 23 years) with ENKTL, as a sub-group of a larger study who received a MA allo-HSCT, all of whom had stage II-IV disease and 5 of whom (83%) were defined as chemo-refractory at the time of allo-HSCT. Two patients died of TRM and one patient experienced relapse. Interpretation of the data is clearly limited owing to the small subgroup, but it is noteworthy that 3 patients experienced long-term disease-free survival (30, 56 and 78 months) following allo-HSCT despite having primary refractory disease (2 patients) or extra-nasal disease beyond CR1 (one patient). A further study from Japan, encompassing a total of 74 cases of EBV-associated T/NK lymphoproliferative disease in children and adults, included a sub-group of 9 patients with ENKTL whose outcome was not separately analysed[653].

The totality of published data on the role of high-dose therapy and autologous or allogeneic HSCT is therefore limited and to-date has been almost exclusively studied by clinical study groups in East Asia.

Aggressive NK leukaemia

WHO definition

'A systemic neoplastic proliferation of NK cells almost always associated with Epstein Barr virus and an aggressive clinical course.'

History

Descriptions of chronic expansions of Large Granular Lymphocytes in the peripheral blood[654] (now understood not be associated with EBV), were initially incorporated into the REAL[605] classification system alongside an emerging, clinically aggressive entity[655]. The first distinct report

of an aggressive NK cell leukaemia in an adult[656] was described in a 71 year old white man from the United States, although the majority of subsequent reports from East Asia have occurred in younger individuals[657]. Establishment of a cell line from this case retained morphological, immunologic, and functional characteristics of NK cells[656]. Further reports of clinically aggressive leukaemias displaying neither a B nor T cell phenotype[588,656,658-659] suggested that such malignancies can arise from non-T cell large granular lymphocytes - or NK cells. The WHO subsequently recognised this aggressive leukaemia as a distinct clinicopathological entity and ANKL was separately incorporated into the lymphoid tumour classification[569].

Clinical features, prognosis and therapy

ANKL is extremely rare, with approximately 100 published cases worldwide[660]. The disease typically affects young to middle age patients (mean age approximately 40 years), with a slight male predominance. Patients with ANKL are almost always systemically unwell at presentation, usually with a high fever and constitutional symptoms such as sweats and weight loss. Hepatosplenomegaly are frequent, but peripheral lymphadenopathy is less common[588,658-659 657,659,661]. Mucosal involvement - such as small intestine, salivary glands and testes - is also frequently observed. A leukaemic picture is invariably found, associated with prominent thrombocytopenia and variable degrees of anaemia and neutropenia[660]. However, an important feature of ANKL, unlike most leukaemias, is the patchy and sometimes minimal involvement of the bone marrow, even with co-existing involvement of the peripheral blood. Consequently, it has been suggested that the bone marrow may not be the primary site of neoplastic transformation[662], although this remains to be clarified.

Circulating leukaemic cells can appear morphologically similar to normal LGLs, or display atypical nuclear features. Prominent azurophilic granules within a pale cytoplasm are usually seen. Bone marrow examination frequently shows reactive histiocytes and haemophagocytosis[660] (Figure 7). Admixed apoptotic cells, stromal degeneration and necrosis are commonly seen on tissues sections and angioinvasion can also be seen[474,660]. Cytogenetic abnormalities are seen in at least two-thirds of cases and are often complex[657,660].

Most cases of ANKL pursue an inexorable clinical course, frequently displaying resistance to cytotoxic therapies. Complications such as coagulopathy, haemophagocytic syndrome and multi-organ failure are not uncommon. In spite of treatment with intensive chemotherapy, mortality from ANKL is virtually inevitable with a median survival of less than 2 months[657,663-666]. Even for the minority of patients who experience an initial remission following an anthracycline-based regimen, relapse occurs invariably and attempts to improve outcome by the use of allogeneic bone marrow transplantation[667] have not proved widely successful[665].

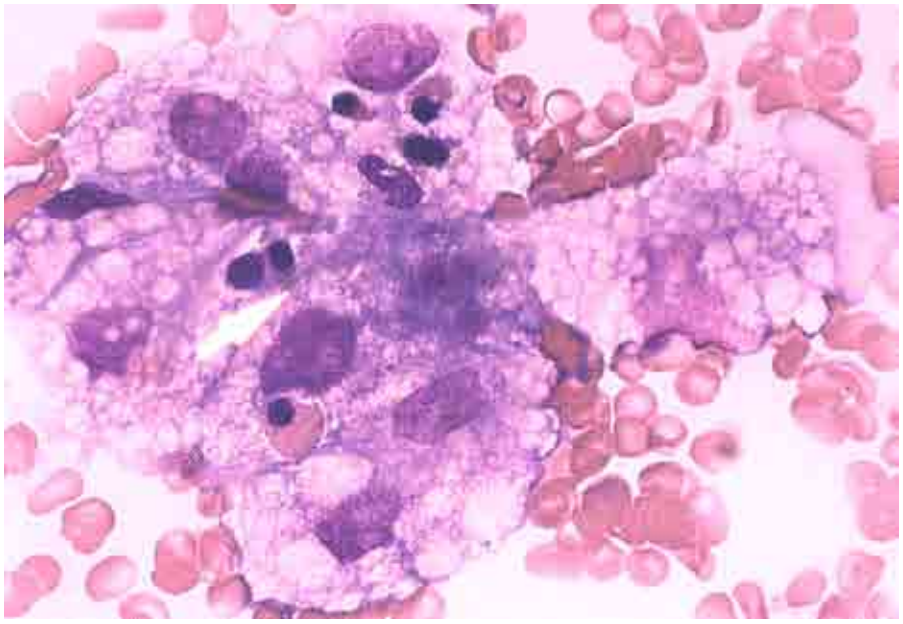


Figure 7 Haemophagocytosis in EBV-associated aggressive NK leukaemia
A photomicrograph of a bone marrow aspirate (original magnification x200) from a UK patient with EBV-associated aggressive NK leukaemia. An area of marked macrophage activity and haemophagocytosis is evident. Lipid-laden macrophages are seen to engulf haemopoeitic precursors.

Association with EBV

In the initial reports of ANKL, studies for EBV were not always performed[656,658-659]. Several small studies documented the presence of clonal episomal EBV in cases of large granular lymphocyte leukaemia [463,668]. Hart et al[669] described a patient who died soon after presenting with an acute hepatitis and rapidly increasing numbers of large granular lymphocytes in the peripheral blood. EBV DNA was found in clonal episomal form within the proliferating cells providing evidence of involvement of the virus in the early stages of leukaemogenesis. It is now recognised that >90% of ANKL cases harbour clonal, episomal EBV[463,666,670].

Due to the rarity of this disease and the often rapidly fatal course, analyses of the pattern of EBV gene expression in ANKL have been scarce. A study from Shanghai found that nine consecutive cases of ($EBER^+$) ANKL were LMP1 negative by immunostaining with CS1-4 MAbs [660].

Differences between ANKL and ENKTL

ANKL shares many features with extra-nodal NK/T lymphoma, including cytological findings of azurophilic cytoplasmic granules in the neoplastic cells, an almost identical immunophenotype[594] ($CD2^+$, $CD3\varepsilon^+$ and $CD56^+$) although CD16 is thought to be more frequently expressed on ANKL than ENKTL [665,671]. Both entities lack T-cell receptor (TCR) gene rearrangements[594,660] and are seen with increased incidence in Asian populations.

However, clinical features of ANKL such as a younger median age at presentation, the absence of a localised mass, invariable involvement of the blood or bone marrow and refractoriness of most cases to therapy allows this entity to be distinguished from ENKTL. In the vast majority of cases these diseases can be clearly delineated although rare patients with NK/T nasal lymphoma may progress to a more aggressive, systemic disease akin to ANKL[601,672].

Cellular genetic changes in ANKL and ENKTL

Initial cytogenetic studies of NK lymphoma and leukaemia demonstrated a common region of genomic loss at 6q21-q25, suggestive of a recurrent chromosomal aberration[673]. Data from subsequent comparative genomic analyses identified further regions of chromosomal loss and

gain[674-675]. Although these early studies provided an indication of the genetic complexity of these malignancies, they did not distinguish between ANKL and ENKTL.

A larger study[676] investigated differences between patterns of genomic abnormalities in 17 cases of ENKTL and 10 cases of ANKL - all tumours were EBV-positive and classified according to the WHO system. In cases of ANKL, loss of 7p and 17p13.1 and gain of 1q occurred frequently but loss of 6q did not. By contrast, loss of 6q21-q22.1, 6q22.33-q23.2, 6q25.3, and 6q26-q27 occurred more frequently in the ENKTL group, implicating tumour suppressor genes within these regions in the development or potentiation of ENKTL. By demonstrating consistent differences in patterns of genomic aberrations between ANKL and ENKTL, this study provides further support to the separation of ENKTL and ANKL as distinct clinicopathological entities

A more recent study employed higher resolution array comparative genomic hybridization (aCGH) technology to analyse NK cell lines and primary tumours and, importantly, correlated genomic abnormalities with transcriptional profiles of genes residing in regions of abnormality[677]. Notably, less than half of such genes showed corresponding increased or decreased expression, compared to normal and activated NK cells. Despite this apparent lack of correlation, many of the upregulated genes in regions of gain are thought to be functionally important for neoplastic proliferation. Those genes downregulated in regions of loss included many transcription factors or repressors, tumour suppressors or negative regulators of the cell cycle. Three candidate genes were identified within the minimal common region of deletion in 6q21 (PRDM1, ATG5 and AIM1) and showed generally low expression. Complementary functional studies in cell lines showed that reversal of methylation by decitabine induced expression of PRDM1 and resulted in cell death, implicating PRDM1 as an important target gene in the context of del6q21.

AIMS OF THESIS

1. **Investigate the frequency and phenotype of EBV-infected T and NK cells *in vivo* in peripheral blood and secondary lymphoid tissue in the context of primary and persistent infection.**

2. **Examine the antigen-processing function of EBV-associated T and NK tumour cell lines and their sensitivity to EBV-specific T cell responses. This aspect of the thesis was subsequently developed to investigate the expression of LMP2 in NK and T cell lymphoproliferations and its utility as an immunotherapeutic target.**

3. **Develop an inducible LMP1-expression vector suitable for transduction and expression in primary NK cells to facilitate analysis of the associated genotypic and phenotypic cellular changes.**

Materials and Methods

Donors and Patients

Ethical approval for the work within this thesis was provided from the West Midlands Research Ethics Committee (REC: 07/H1208/62).

Healthy donors working within the School of Cancer Sciences consented to donate peripheral blood for isolation of PBMC. Patients from University Hospital Birmingham (UHB), Queen Elizabeth and the Birmingham Children's Hospital NHS trust undergoing tonsillectomy for acute or chronic tonsillitis provided consent to donate excess tonsillar tissue. In some instances, matched peripheral blood samples were also donated. Newly diagnosed patients from with acute IM were identified via a positive Monospot test performed at UHB Clinical Haematology diagnostic laboratory, and consent gained thereafter from the patients for peripheral blood donation. For analyses of IM tonsils, viable tonsillar mononuclear cells stored in liquid nitrogen and originally obtained through collaboration between Professor Alan Rickinson and Dr Wolfgang Bergler (University Hospital Mannheim, Germany) were used. All such IM patients had undergone tonsillectomy indicated for acute IM with associated airway compromise. A clinical diagnosis of IM was corroborated by a positive heterophile (Monospot) test.

The patients with EBV-HLH were initially referred to our institution for diagnostic investigations with anonymised clinical data subsequently provided by the referring clinicians, in accordance with the REC approval. The ENKTL biopsy sections used for LMP2A immunohistochemistry fulfilled the W.H.O. diagnostic criteria[409], including EBER-positivity, and were reviewed by two expert haematopathologists in the United Kingdom (Dr Simon O'Connor and Dr David M. Clark, Nottingham University Hospitals, UK). ENKTL frozen tissue for PCR studies had undergone histology review in the United States (Professor W C Chan) for inclusion in a previous study[677].

Isolation and manipulation of cells

Isolation and manipulation of tonsil mononuclear cells

Fresh tonsillar tissue was typically processed within 1 hour of tonsillectomy. The tissue was washed briefly in phosphate-buffered saline (PBS) prior to processing in 'tonsil media' = RPMI-1640, with 10% v/v FCS and Gentamicin (Sigma) at a final concentration of 8µg/ml and 100U/ml penicillin/streptomycin (pen/strep) 100U/ml final concentrations). Tonsil mononuclear cells (T-MNC) were disaggregated to form single-cell suspensions by carefully incising the tissue and fine mincing with sterile scalpels. Isolated mononuclear cells were strained through a 40µm filter (Partec), aliquoted, cryopreserved, and stored in liquid nitrogen or as cell pellets (at -80°C) for extraction of DNA or RNA.

As B cells constitute a considerable proportion (30-70%) of total tonsillar lymphocytes, B cell depletion with CD19 Dynabeads (see below) was performed on *ex-vivo* tonsillar MNC prior to MAb staining and sorting in order to reduce subsequent B cell contamination of sorted T and NK cell fractions.

Separation of PBMCs from whole blood

50-100ml of peripheral blood was diluted 1:1 with PBS and layered onto Lymphoprep™ iso-osmotic medium (Axis-Shield) in a 250ml centrifuge tube (Corning). This was centrifuged for 30 minutes at 1800rpm, without a brake to maintain the density gradient. PBMCs were extracted from the interface using a Pasteur pipette. The PBMCs were then washed twice in sterile PBS by centrifugation at 1600 rpm for 10 minutes and then 1200 rpm for 5 minutes. Viable PBMCs were either processed immediately for NK cell isolation or frozen in 10% dimethyl sulphoxide (DMSO) for future cell sorting.

Isolation of B cells

Expression of the pan-B cell marker CD19 allows the specific isolation/depletion of B lymphocytes from PBMC and tonsil MNC. Magnetic polystyrene beads coated in a CD19 MAb (Dynabeads CD19 Pan B, Invitrogen 111.43D) were used. The equivalent of 4 Dynabeads per B cell were

added to PBMC/T-MNC (assumed 5% B cells in PB and 50% B cells in T-MNC) at a concentration of 10^7 Dynabeads per ml in a 14ml round bottomed tube and incubated at 4°C for 20 minutes (isolation) or 30 minutes (depletion) in RPMI-1640 with 1% v/v FCS (used throughout) on a roller. A magnet was then applied for 2 minutes, allowing removal of non- B cells with a Pasteur pipette followed by washing of the Dynabead-bound B cells; this cycle was repeated 5 times. Dynabead-bound B cells were re-suspended in 1ml with 50µl of Detachabead™ CD19 (InvitrogenCat. no. 125.06D) and incubated at room temperature for 45 minutes on a roller. The bead/cell mix was resuspended in 9ml and the Dynabeads removed from the B cells by exposure to a magnet. This was repeated once, the unattached B cell suspensions combined and used in subsequent experiments.

Isolation of primary NK cells from PBMC

NK cells were isolated from 5-20 x10^7 PBMC using a commercially available kit (Dynabeads® Untouched™ Human NK cell kit – Invitrogen) and following the manufacturers protocol, without methodological modification. The principle of isolation is one of 'negative selection', whereby the application of a mixture of biotinylated monoclonal antibodies ('Antibody mix [human NK cells]', Invitrogen) directed against antigens expressed on T cells, B cells, monocytes, dendritic cells, platelets, macrophages, granulocytes and erythrocytes, allows their specific removal from PBMC using superparamagnetic 1µm polystyrene beads coated with streptavidin. Figure 8 is a schematic of the isolation procedure. The isolated bead- and antibody-free NK cells were subsequently analysed by FACS to assess purity.

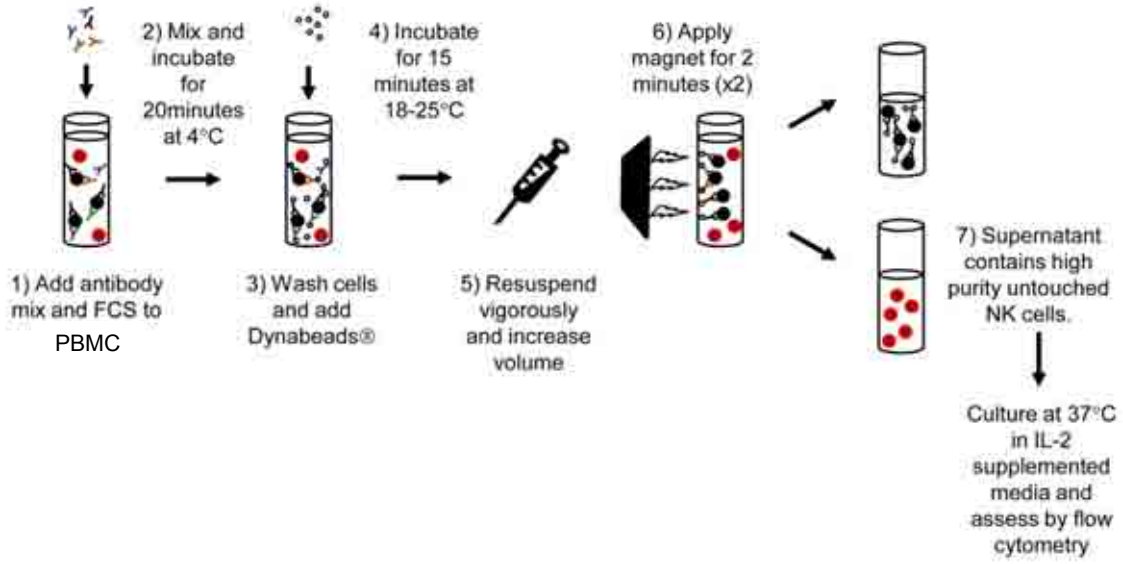


Figure 8 Schematic of NK cell isolation from peripheral blood mononuclear cells

Cell culture

All cells were grown in tissue culture-treated plates (Iwaki) in a humidified incubator at 37°C, 5% CO_2. Primary lymphocytes were cultured at a density of 1-2 x 10^6 cells per cm^2 in 6-, 12-, 24- or 48-well plates. Suspension cell lines were cultured at densities of 0.5-1.5 x 10^6 per ml in 25cm^2 flasks.

Culture of tonsil mononuclear cells

Tonsillar mononuclear cells (T-MNC) were cultured in 'tonsil media' (RPMI-1640, with 10% v/v FCS and Gentamicin (Sigma) at a final concentration of 8µg/ml and 100U/ml pen/strep). For some experiments, IL-2 (Proleukin, Novartis) was added to give a final concentration of 175U/ml.

Culture of primary NK cells

Primary NK cells were cultured in CellGro® SCGM (stem cell growth medium - CellGenix™), supplemented with 10% heat-inactivated (56ºC for 45 minutes), sterile-filtered (0.22µm), human serum (HS) (Sigma), pen/strep 50U/ml and IL-2 175u/ml.

Culture of SNK and SNT cell lines

The SNK and SNT cell lines[562] were kindly provided by Dr Norio Shimizu, Tokyo and cultured in conditions recommended by the originators: RPMI-1640 supplemented with 10% HS (prepared as for primary NK cells), 6mM L-glutamine (Invitrogen), 50U/ml pen/strep, 1mM Sodium Pyruvate (Sigma), 0.13mg/ml Oxalacetic Acid (Sigma) and IL-2 700U/ml (Novartis). The cell suspensions were 'split' twice a week at a ratio of 1:2 - 1:4 according to growth rate and density.

293FT epithelial cells

The lentivirus-packaging cell line 293FT was cultured in 100mm plates (Iwaki) in RPMI-1640 with 10% v/v FCS with the aminoglycoside antibiotic G418 (Invitrogen) added at 200 µg/ml for maintenance.

Cryopreservation of cells

Where appropriate, $5x10^6$ – 200 x 10^6 cells were pelleted by centrifugation at 350g-force (g) for 5 minutes and re-suspended in 0.5-1.5ml of 'freezing mix': RPMI-1640 supplemented with 30% v/v FCS, 10% v/v dimethylsulphoxide (DMSO) and 2mM glutamine. Vials were subjected to controlled freezing by placing in a 'Mr Frosty' container surrounded by a sponge soaked in propan-2-ol and stored overnight at -80°C, followed by transfer to the vapour stage of a liquid nitrogen freezer at -180°C for long term storage.

In vitro EBV infection experiments

Production of 2089 virus from 293 cells

293 cell clones stably transfected with the recombinant 2089 B95.8 EBV genome[678] were plated into 6 well plates 24-48 hours before transfection. When the cells were 70% confluent they were transfected with 0.5μg of BZLF1 and 0.5μg of BALF4 (gp110) plasmid[678-679] per well using Lipofectamine (Invitrogen) reagent in Optimem minimal medium (Gibco) according to manufacturer's instructions, and incubated for 3 hours at 37°C, 5% CO_2. After 72 hours, the supernatant was centrifuged at 900g for 5 minutes and sterile-filtered to 0.45μm. If not immediately used it was stored in aliquots at -80°C. A 50μl aliquot of virus supernatant was reserved for genome quantitation.

Production of Akata EBV from Akata-BL cells

Akata virus was generated from an EBV-loss clone of Akata-BL, which had previously been re-infected with recombinant GFP-expressing EBV ('Akata virus')[680]. Approximately $50x10^6$ cells (at $4x10^6$ cells/mL) were stimulated to produce virus by 2 hours incubation with 0.5% v/v anti-human IgG Fab 2 antibody at 37˚C, 5% CO_2. The 'cross-linked' cells were cultured at a density of $3x10^5$ cells/ml in RPMI 10%FCS at 37˚C, 5% CO_2, for 72 hours. Viral supernatant was harvested and stored as described for 2089 EBV.

Quantitation of EBV titre by Q-PCR

An equal volume of lysis buffer (100mg/ml proteinase K (Roche), 10mM Tris-HCL pH 8.8, 1.5ml $MgCl_2$, 50mM KC:, 0.1% v/v Triton X-100) was added to the virus supernatant and incubated at 55°C for 1 hour. Q-PCR for EBV BALF5 (page 101 and reference[560]) was undertaken using a PCR input volume of 5µl of processed viral supernatant per well.

Infection of cell lines and primary cells with EBV

Infection of $1x10^7 – 10^8$ target cells was achieved by incubating in a volume of PCR-quantitated viral supernatant, calculated to equate to a multiplicity of infection (m.o.i) of 10-100, supplemented by 20% v/v FCS, for 4-8 hours in 50ml tubes (Falcon) at 37˚C, 5% CO_2. The incubated cells were gently mixed at 1-2 hourly intervals to augment viral binding. The cells were pelleted by centrifugation at 1400rpm and resuspended in the relevant culture media at an appropriate density.

Flow cytometry and cell sorting

MACS buffer (PBS with 2mM EDTA, adjusted to pH 7.5) with 5% v/v HInGS (heat-inactivated normal goat serum) was used as a staining and suspension solution throughout.

Surface staining

Table 2 details the conjugated monoclonal antibodies used for FACS analysis and cell sorting on the MoFlo. Target cells were re-suspended in microcentrifuge tubes at staining volumes of 5 $x10^7$/ml for MoFlo sorting and 1-5 x 10^7/ml for FACS analysis. Staining was performed for 30 minutes at 0-4°C and cells were washed once with MACS buffer prior to transfer to FACS tubes and analysis.

Flow cytometric analysis

One and two-colour flow cytometric analysis was performed on a Coulter Epics XL-MCL™ Flow Cytometer (Beckman Coulter). The appropriate MAb isotypes were used as negative controls and individual MAbs for surface antigens were used separately for positive controls and to allow for fluorochrome compensation. Data was analysed using FlowJo software version 7.6 (Treestar).

Table 2 Conjugated monoclonal antibodies used for flow cytometry analysis and cell sorting

Target	Clone‡	Conjugated fluorochrome	Working Dilution	Incubation	Isotype
CD19	SJ25-C1	PE-Cy5	1:20	30 minutes 0-4°C	IgG1
CD19	LT19	APC	1:20	30 minutes 0-4°C	IgG1
CD3	LT8	FITC	1:20	30 minutes 0-4°C	IgG1
CD3	UCHT1	PE-Cy5	1:20	30 minutes 0-4°C	IgG1
CD4	RPA-T4	APC	1:20	30 minutes 0-4°C	IgG1
CD8	LT8	FITC	1:20	30 minutes 0-4°C	IgG1
CD16	DJ130c	PE	1:20	30 minutes 0-4°C	IgG1
CD56	C5.9	PE	1:20	30 minutes 0-4°C	IgG2b
CD138	BA-38	FITC	1:20	30 minutes 0-4°C	IgG1
CD7	MEM-186	PE-Cy5	1:20	30 minutes 0-4°C	IgG1

‡All conjugated antibodies were purchased from AbD Serotec, UK

Fluorescence activated cell sorting

Three or four-colour fluorescence activated cell sorting (FACS) was used to sort PBMC (typically 20 - 50x10^6) or T-MNC (typically 50 -150 x10^6) into lymphocyte sub-populations using the desired combination of MAbs and gating strategy (see Results Part I). Following surface staining and prior to sorting, cell suspensions were strained though 40µm filters (Partec). After sorting, where cell numbers permitted, a small aliquot of each sorted population was reanalysed by flow cytometry to assess purity. For GFP-expressing NK cells transduced with the TREX-regulated LMP1-lentiviral vector (see Results Part II), 10ng/µl Doxycycline was added 48 hours prior to sorting. All cell sorting was performed courtesy of the MoFlo^{TM} (Beckman Coulter) cell-sorting service (operated by Dr Roger Bird) at the Institute of Biomedical Research (IBR), University of Birmingham.

Protein analysis

Western blotting

Preparation of gel samples

Protein preparations were prepared from whole cell lysates of 1x10^6 - 1x10^7 cells according to availability. Cells were washed twice in PBS to remove FCS and the cell pellet lysed in 100µL urea buffer (9M urea, 50mM Tris, pH 7.5). Disruption of genomic DNA by 10-30 seconds of sonication using an ultrasonic cell disruptor (Misonix) was undertaken to reduce viscosity. The protein concentration was determined using a Bio-Rad DC protein assay kit according to the manufacturer's instructions.

For Western blot analysis of all proteins except LMP2A, the following procedure was performed: An equal volume of 2x urea gel sample buffer (62.5mM Tris pH6.8, 4% w/v sodium dodecyl sulphate (SDS), 5% B-mercaptoethanol, 0.01% w/v bromophenol blue, 5M urea, 10% glycerol) was used to dilute the samples to 1-2mg/ml and diluted samples were denatured at 100˚C for 3 minutes.

For Western blot analysis of LMP2A protein, the following procedure was undertaken: Sonicated cell lysates were denatured with NuPAGE® LDS Sample Buffer (4X) (Invitrogen Cat. No. NP0007) heated to 70°C for 5 minutes and reduced with NuPAGE® sample reducing agent (10X) (Invitrogen NP0004) immediately prior to loading into the wells. Cell lysates were stored at -80°C, whilst protein samples in gel sample buffer were stored at -20°C.

SDS-PAGE (sodium dodocyl sulphate-polyacrylamide gel electrophoresis)

SDS-PAGE and Western blotting were undertaken in XCell Surelock™ mini cells (Invitrogen). Pre-cast NuPAGE® Novex® Bis-Tris 10 well gels (Invitrogen) were loaded with 20-40µg of protein alongside 15µl of Seeblue plus 2 pre-stained protein standards (Invitrogen). Electrophoresis was carried out in MOPS SDS running buffer (Invitrogen) at 78V for 3 hours in the XCell *Sure*Lock™ Mini-Cell (Invitrogen).

Blotting

Invitrolon™ polyvinylidine difluoride (PVDF) membranes (Invitrogen) were briefly exposed to 100% methanol prior to soaking in blotting buffer (25mM TRIS-BASE, 192mM glycine and 20% v/v methanol) for 5 minutes. Once the dye front had progressed sufficiently down the gel, 2 sponges and a sheet of filter paper (Invitrogen) soaked in blotting buffer were placed on the cathode side of the blotting cassette. The gel was layered onto the filter paper, followed by the PVDF membrane and a second pre-soaked filter paper. Air bubbles were carefully eliminated between the gel and the PVDF membrane and 3 further wet sponges were placed on top. The blotting cassette was filled with blotting buffer and the outer chamber of the tank filled with distilled water to prevent over-heating of the module during transfer. The gel was typically blotted at 30V, 250mA for 120 minutes. The PVDF membrane was then incubated in I-Block/Tween buffer (IBT) (0.4% w/v casein powder (I-Block™, Applied Biosystems), 0.05% v/v Tween-20, 0.02% sodium azide (NaN3)) on a shaker at room temperature for 1 hour to prevent non-specific antibody binding. PVDF membranes were then incubated with primary antibody (see table 3), diluted in blocking buffer overnight at 4°C. PVDF membranes were then washed 3 times, each 2 minutes, in PBS/0.1% Tween-20 detergent. The appropriate secondary antibody conjugated to

alkaline phosphatise (Biorad) diluted in blocking buffer was then incubated with the PVDF membrane at room temperature on a rocker for 30 minutes. The membrane was then washed ≥5 times, each for 5 minutes, in PBS/Tween-20.

Table 3 Primary and secondary antibodies used for Western blotting, immunofluorescence and immunohistochemistry

Target	Primary Antibody	Species	Application	Working Dilution	Incubation	Secondary Antibody	Working dilution
EBNA1	'AM' sera[36]	human	Western	1/200	4°C overnight	AP-anti-human IgG (Biorad)	1/10,000
EBNA2	PE2[39]	mouse	Western	1/25	4°C, overnight	AP-anti-mouse IgG	1/10,000
LMP1	CS1-4[681]	mouse	Western	1/300	4°C overnight	AP-anti-mouse IgG	1/10,000
LMP1	CS1-4[681]	mouse	IHC	1/50	4°C overnight	HRP-anti-mouse	1/500
LMP1	CS1-4[681]	mouse	IF	1/100	37°C for 30 mins	Anti-mouse-Alexafluor594	1/500
LMP2A	14B7[130]	rat	Western	1/25,000	4°C overnight	AP-anti-rat IgG	1/10,000
LMP2A	15F9[131]	rat	IHC	1/50	4°C overnight	HRP-anti-rat IgG	1/500
TAP 1	148.3[682]	mouse	Western	1/100 (supernatant)	4°C overnight	AP-anti-mouse IgG	1/10,000
TAP 2	435.3[§]	mouse	Western	1/100 (supernatant)	4°C overnight	AP-anti-mouse IgG	1/10,000
HLA class I heavy chain	HC-10[683]	mouse	Western	0.2µg/ml	4°C overnight	Ap-anti-mouse IgG	1/10,000
Calregulin	H-170**	Rabbit	Western	1µg/ml	4°C overnight	AP-anti-goat IgG	1/100,000
BZLF 1	BZ1[213]	Rabbit	IF	1/300 (supernatant)	37°C for 30 mins	Anti-rabbit Alexafluor488	1/500
Minor capsid protein (p18)	OT15E[††]	Rat	IF	1/300 (supernatant)	37°C for 30 mins	Anti-rat-Alexafluor594	1/500

[§] The murine mAbs specific for the TAP1, 148.3 and TAP2, 435.3, were kindly provided by R. Tampe (Wolfgang Goethe-University, Frankfurt) and by P.M. van Endert (Institute Necker, Paris) respectively.

** H-170 was purchased from Santa Cruz Biotechnology

[††] The OT15E antibody to p18 was a kind gift from Professor Jaap Middeldorp (Vrije Universiteit Medical Centre, Amsterdam, Netherlands.)

Detection by chemiluminescence

Alkaline Phosphatase (AP) chemiluminescence was used to visualise antibody bound proteins probed with an AP-conjugated secondary antibody. PVDF membranes were incubated twice in 10ml AP buffer (0.1M diethanolamine pH 9.5, 1mM MgCl2,) and transferred to Saranwrap where 0.8ml of CDP-star™ developer (Invitrogen) was uniformly applied to the membrane and incubated for 15 minutes. Excess reagent was removed, the Saranwrap sealed and placed in an autoradiograph cassette, with Kodak X-omat film, for detection.

Stripping PVDF membranes

The secondary antibody used during western blotting can be removed (stripped) to allow re-probing of the same blot with a further MAb and secondary antibody raised in an alternative species. The blots were stripped by incubation with Ponceau S stain (1% Ponseau S w/v (Sigma) in 3% v/v trichloroacetic acid (TCA)) at room temperature for 3 minutes with rocking. The membrane was washed with tap water initially followed by ≥3 times, each for 5 minutes, in PBS Tween and subsequently re-blocked in blocking buffer for 1 hour. Blots were either re-probed immediately or stored at 4°C in Saranwrap.

Immunohistochemistry

4µm sections of formalin-fixed paraffin embedded (FFPE) tissue mounted on slides (courtesy of Mrs Sue Sharpe, Histopathology department, University Hospitals Birmingham) were de-waxed in Histo-clear® (Fisher Scientific) for 10 minutes followed by a series of rehydration steps through gradated ethanol (100% for 5 min, 90% for 5 min, 70% for 5min).The slides were rinsed in running tap water for 5 minutes followed by endogenous peroxidise blocking in 0.3% hydrogen peroxide (H_2O_2) in water for 15 minutes and further rinsed in tap water.

Antigen retrieval

The process of formalin-fixation can render proteins within tissue to be relatively inaccessible to detection with antibodies. The process of antigen-retrieval, based on heat treatment at optimised acidic or alkaline pH, serves to ‘re-expose’ masked antigens, with varying efficiency, to allow

access for primary antibody binding. The previously published Agitated Low Temperature Epitope Retrieval (ALTER) method[684] was used. Briefly, EDTA buffer (pH 8) with 0.1% Tween-20 was pre-heated on hot-plate-stirrer (600rpm) to a stable temperature of 65°C and the slides incubated herein overnight (16-20 hours). The slides were then allowed to cool down slowly ≥1 hour at RT, before washing in very slow running tap water.

Immunostaining

Each slide was carefully dried and each section marked using the hydrophobic 'pap pen' (Abcam), followed by conditioning in TBS buffer (pH 7.6) for 5 minutes. Each section was then blocked with 100-300µl of 1% casein (in TBS) and incubated for ≥10 minutes. The primary antibody (see table 3, page 94) was diluted 1:50 in PBS, 100µl applied to each section and incubated overnight at 4°C. The slides were then washed in TBS-tween (0.1%) buffer (pH 7.6) thrice, 5 minutes each and the appropriate secondary antibody (Dako, 1:500) in PBS was applied for 30 minutes. Further washing in TBS-tween was followed by the application of Real Envision DAB (950µl supplied diluent and 20µl DAB) (Dako) for 1-2 minutes. For counterstaining, the slides were washed in TBS-tween for 5 minutes, then in tap water, followed by counterstaining in Mayer's Haematoxylin solution (2 minutes). A further wash in warm tap water (5 minutes) was followed by gradated dehydration in ethanol (70% for 5 mins, 90% for 5mins, and 100% for 5 mins). Finally, the slides were placed in Histo-clear® (Fisher Scientific) for 10 minutes and mounted in DPX (Surgipath).

Staining was visualised with a Nikon Eclipse E400 microscope (optical magnification x100-400) and images captured with a Nikon Coolpix E995 digital camera.

Immunofluorescence

Cell fixation

Cells were washed once and re-suspended at 10^6/ml in PBS. Aliquots of 10µl (10,000 cells) were spotted onto each window of a 10 window multisport microscope slide (Hendley-Essex) and air

dried. The slide was incubated at -20°C in a pre-cooled fixative solution of methanol and acetone (1:1 v/v) for 30 minutes, followed by air drying and either immediate use or storage at -20°C.

Cell staining

Slides were not permitted to dry out from this stage of the protocol onwards. To rehydrate the cells, 30µl of PBS/10% HINGS was added to each window spot and incubated at room temperature for 20 minutes. This was aspirated and carefully replaced with 30µl of the primary antibody (Table 3, page 94), or 30µl of HInGS alone for control staining, and incubated in a moist container for 30 minutes at 37°C. Following 2 washes in PBS, 30µl of secondary antibody (Alexa Fluor^{TM} 488 or 594: Zenon kit, Invitrogen) was applied to each window, incubated as before and visualised immediately after mounting in Vectashield mounting medium with DAPI (Vector Laboratories) or 1 drop of 90% glycerol and prior to application of a 20x70mm cover slip.

T cell assays

IFNγ ELISA

The epitope specificities and HLA-restriction of the LMP2-specific $CD8^+$ T cell clones tested are detailed in table 4. Known numbers of T cells (100-2500) were incubated in U-bottom or V-bottom microtest 96-well plates with specified numbers of target cells to achieve the desired effector: target ratio (autologous LCL and HLA -mismatched LCLs as controls), with/without pre-loading for 1 hr with 5µM peptide (or an equivalent concentration of DMSO solvent as a control).

In blocking assays, targets were pre-incubated with saturating concentrations of mAbs specific for HLA class I (clone W6-32[683]) or HLA-DR as a control (YE2/36-HLK antibodies[685]) for 1 hr before T cell addition to the assay. The supernatant medium harvested after 18 hrs was assayed for IFNγ by ELISA (Endogen) following the manufacturer's recommended protocol. 96-well Maxisorp plates (Nunc) were coated with 50µl of a 0.75µg/ml solution of a mouse IgG1K anti-human IFNγ mAb (Pierce, Endogen) overnight at 4°C. The following morning the antibody was tapped out of the wells which were subsequently blocked at RT for 1hr with 1% BSA, 0.05% Tween 20 in PBS. The plates were then meticulously washed x6 in wash buffer (0.05% Tween 20 in PBS) and 50µl

of neat or a specific dilution of supernatant from the T cell assay was added to each well and incubated at RT for 2-4hrs. The supernatant was discarded, plates washed x6 in wash buffer and a biotinylated mouse IgG1K anti-human IFNγ Mab (Pierce, Endogen) applied at 0.375µg/ml for incubation at RT for 1-2hrs. Further washing was performed and extravidin peroxidase conjugate (SIGMA) applied for 30mins. After a final 8 washes of the plate in wash buffer, 100µl of the developing solution TMB (tetramethyl benzidine, Tebu-Bio laboratories) was added and left at RT for typically 1-3 minutes to allow the colour to develop. The reaction was terminated by the addition of 100µl 1M HCL per well and absorbencies were read at 450nm, using a microplate reader (Bio-Rad model 680) with software Microplate Manager v. 5.2.1. All results represent the mean value of samples assayed in triplicate.

^{51}Cr-release killing assays

$CD8^+$ T cell clones were tested for cytotoxicity against target cells by chromium-release assays. Pelleted target cells ($1x10^6$) were resuspended in15µl (75µCi) of ^{51}Cr- sodium chromate (Perkin Elmer) and incubated at 37°C, 5% CO_2 for 90 minutes to allow intracellular uptake of the radioisotope. Where appropriate, 5µM of epitope peptide (or an equivalent volume of DMSO solvent as a control) was added for the final hour of the incubation. In the meantime, the effector T cell clones were diluted in RPMI-1640/10% FCS and aliquoted in 100µl into 96-well, V-bottom plates at the requisite number to achieve specified effector: target ratios. The target cells were washed twice with RPMI/10% FCS and diluted to achieve 2500 cells per 100µl aliquot and added to each well containing the T cell clones. For each target, the radioactivity spontaneously released from the labelled cells when incubated with 100µl of medium alone and the maximum radioactivity released when incubated with 100µl 1% SDS solution served as controls. The plates were centrifuged at 1200rpm for 3mins and incubated for 5hrs at 37°C, 5% CO_2. Aliquots (100µl) of supernatant were then harvested from each well into LP2 tubes (Luckman), the radioactivity of which was measured on a gamma-counter (Cobra Auto-Gamma counting system, Hewlett Packard). Results for the CTL assays are expressed as percent specific lysis whereby:

% specific lysis = [(release from target with T cells - spontaneous release)/maximum release]x100

Table 4 Epitope specificities and HLA-restriction of LMP2-specific $CD8^+$ T cell clones

Minimal epitope sequence	Peptide position‡‡	HLA class I restriction
FLYALALLL	356-364	HLA-A*0201
TYGPVFMCL	419-27	HLA-A24
LLWTLVVLL	329-337	HLA-A*0201
CLGGLLTMV	426-434	HLA-A*0201
SSCSSCPLSK	340-349	HLA A*1101

DNA and RNA work

DNA extraction

DNA was extracted from between $1x10^4$ and $5x10^6$ cells according to availability. Cells were washed in 1x PBS to remove serum, and DNA was extracted using the Qiagen DNeasy kit by adhering strictly to the manufacturer's protocol. DNA was eluted in 30-100µL nuclease-free water (Ambion) and the resultant concentration determined using a Nanodrop (Thermo Scientific). Where low DNA yields were anticipated due to low cell numbers, the elution step was repeated, re-using the 'first-pass' eluate. For samples with high DNA concentrations, an aliquot was diluted with nuclease-free water to achieve ≤50ng/µL for use in Q-PCR assays.

RNA extraction

RNA was extracted from $5 \times 10^5 – 5 \times 10^6$ cells after washing in 1x PBS to remove serum. The RNeasy™ kit (Qiagen) was used in compliance with the manufacturer's instructions. RNA was

‡‡ Based on the B95-8 LMP2A protein sequence

eluted in 30µL water (Ambion) and the concentration determined using a Nanodrop (Thermo Scientific). For samples >100ng/µL, aliquots were diluted to 100ng/µL and stored at - 80˚C.

DNase treatment of RNA

The RNeasy RNA extraction kit is designed to minimise DNA contamination in the eluted RNA. However, although a majority of the downstream RT-Q-PCR assays involve either a primer or probe designed to span an exon-exon splice boundary, this was not the case for all assays; some were designed within an exon. Thus, to ensure that only cDNA transcripts were amplified during the PCR reaction, 1µg aliquots of RNA were treated with DNase I using the Turbo DNase kit (Ambion) according to the manufacturer's instructions, prior to reverse transcription.

Reverse Transcription

400ng of RNA was heated to 90˚C for 3 minutes within a thin walled, 0.5ml PCR tube and then immediately placed on ice. Reverse transcription of denatured RNA was carried out in a 20µL reaction containing 1x avian myeloblastosis virus reverse transcriptase (AMV-RT), reaction buffer (Roche), 200µM each of dATP, dCTP, dGTP and dTTP (Roche), 50pmoles of random hexamers (Promega) and left at RT for 5 minutes prior to a 42˚C incubation for 1 hour. cDNA was diluted to a total of 80µl and stored at -20˚C until required. Negative controls with no reverse transcriptase, EBV-negative RNA and nuclease-free water were routinely employed.

TaqMan quantitative polymerase chain reaction (Q-PCR)

Real time PCR allows the relative or absolute quantitation of a specific gene – against a known standard - during the exponential phase of cDNA/DNA amplification, when the quantity of PCR product is directly proportional to the amount of nucleic acid template.

Each well of a RT-Q-PCR assay typically comprised a 25µl reaction volume[133] including 5µl of input cDNA/DNA. However, where cDNA from primary tissue was limited, 2ul input cDNA (with identical final concentrations of primers/probe) in a reaction volume of 20ul was used. For the EBV-encoded gene/transcript of interest, the probe was labelled with FAM at the 5' end and a TAMRA quencher at the 3' end (Eurogentec). For endogenous control genes, the probe was

labelled with VIC® at the 5' end and BHQ (black hole quencher) at the 3' end (Applied Biosystems). Thermocycling and fluorescence detection were carried out in a 7500 Real-Time PCR System (Applied Biosystems). Initial heating to 95°C for 10 minutes activated the ApliTaq® Gold polymerase enzyme, followed by 40 cycles of denaturation at 95°C for 15 seconds and primer annealing and extension at 60°C for 60 seconds. Data was analysed using 7500 system software v1.4.0 (Applied Biosystems) and Excel (Microsoft 2007).

EBV genome load was assayed by RT-Q-PCR as previously described[560]. Triplicate aliquots of DNA (maximum 250ng per well) were subjected to a multiplex PCR using primer/probe combinations specific for the EBV BALF5 (POL) and the human β2 microglobulin (β2m) sequences. In parallel, serial DNA dilutions of the Namalwa BL line (known to contain 2 integrated EBV copies per cell[686]) were also amplified and used to create standard curves for absolute quantitation in the test samples. In each case, the number of POL copies per sample was normalized using the corresponding β2m value and the final result expressed as EBV genomes per 10^6 cells

QRT-PCR assays for the EBV latent transcripts[133] and the EBER1 and EBER2 transcripts[82] have been previously described, whilst new assays were designed for LMP2 using Primer Express software v2.0. A list of primer and probe sequences for the new LMP2 assays are shown in table 5. EBV coordinates are based on the revised B95.8 sequence[687].

5′ Rapid amplification of cDNA ends (RACE)

5´ RACE is a procedure for amplification of an mRNA template between a defined internal site and unknown sequence at 5´ -end of the mRNA[688], allowing sequencing and identification of unknown 5´ origins of mRNA. Figure 9 shows a summary schematic of the method. The manufacturer's protocol (5´ RACE System for Rapid Amplification of cDNA Ends,v 2.0 Invitrogen) was followed without adaptation. To summarise: First strand cDNA synthesis was primed using a

Table 5 Oligonucleotide sequences of LMP2 primer and probe combinations used for RT-Q-PCR assays

LMP2 assay	Primer/probe combination	Oligonucleotide sequence (5'-3')	EBV genome coordinates[§§]
LMP2A conventional	F LMP2A	CGGGATGACTCATCTCAACACATA	166412-35
	R LMP2	GGCGGTCACAACGGTACTAACT	163-142
	LMP2 probe	CAGTATGCCTGCCTGTAATTGTTGCGC	66-92
LMP2B conventional	F LMP2B	CGGGAGGCCGTGCTTTA	169421-37
	R LMP2	GGCGGTCACAACGGTACTAACT	163-142
	LMP2 probe	CAGTATGCCTGCCTGTAATTGTTGCGC	66-92
Exon 2	F2 primer	CTGTAATTGTTGCGCCCTACCT	78-99
	R2 primer	TGCTGCCAAGAGTAGAAGTGAGA	199-177
	Exon 2 probe	CTATTGCCGCCTCGTGTTTCACGG	114-137
TR-LMP2	TR-F primer	ACTTTTCTTCTTGCCCGTTCTCT	28-50
	TR-R primer	GAAACACGAGGCGGCAATAG	133-114
	TR exon 2 probe	CAGTATGCCTGCCTGTAATTGTTGCGC	66-92
Exon 6	F5-6 primer (exon5/6 junction)	TGGACACTTGTGGTTCTCCTGAT	942-951/1026-1038
	F6 primer	GGTTCTCCTGATTTGCTCTTCGT	1027-1049
	R6 primer	CGCGGAGGCTAGCAACA	1129-1113
	Exon 6 probe	TCCTTCTGGCACGACTGTTCCTATATGCTC	1074-1103

[§§] Location of primer and probe sequences based on EBV genome sequence accession number NC007605.

gene-specific primer (GSP1= LMP2 R6, see table 5). The first strand product was then purified from unincorporated dNTPs and GSP1. TdT (Terminal deoxynucleotidyl transferase) was used to adjoin homopolymeric tails to the 3' ends of the cDNA. Tailed cDNA was then subjected to first round PCR amplification used a combination of the Abridged Anchor Primer (AAP) and GSP2 (LMP2 R5, 3′ primer sequence AGTGACGCTAGCAGTGCCAGA), followed by a second round of amplification using the Abridged Universal Amplification Primer (AUAP) and nested LMP2 R4 (3′ primer sequence AGAGGACGAAAGCCAGTAGCAG). PCR products were analysed by agarose gel electrophoresis and cDNA recovered from excised bands (QIAquick Gel Extraction Kit, Qiagen) for cloning and sequencing.

Cloning of cDNA sequences

To allow efficient and accurate sequencing of individual cDNA products obtained following the RACE procedure, the pGEM®-T Easy Vector System I (Promega) kit was used to clone the PCR products. The manufacturer's instructions were followed. pGEM®-T is a linearised vector with a single 3′ terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases (such as high-fidelity Taq, Roche). The vectors contain T7 and SP6 RNA polymerase promoters that flank a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates. Selected white colonies were inoculated into 3mls of Ampicillin-containing LB-broth, incubated at 37°C on a shaker overnight and plasmid DNA extracted the following morning using the Qiagen miniprep kit. Sequencing of products was performed using a M13 (reverse) universal primer, and courtesy of Dr Anthony Jones, Functional Genomics, Biosciences, University of Birmingham.

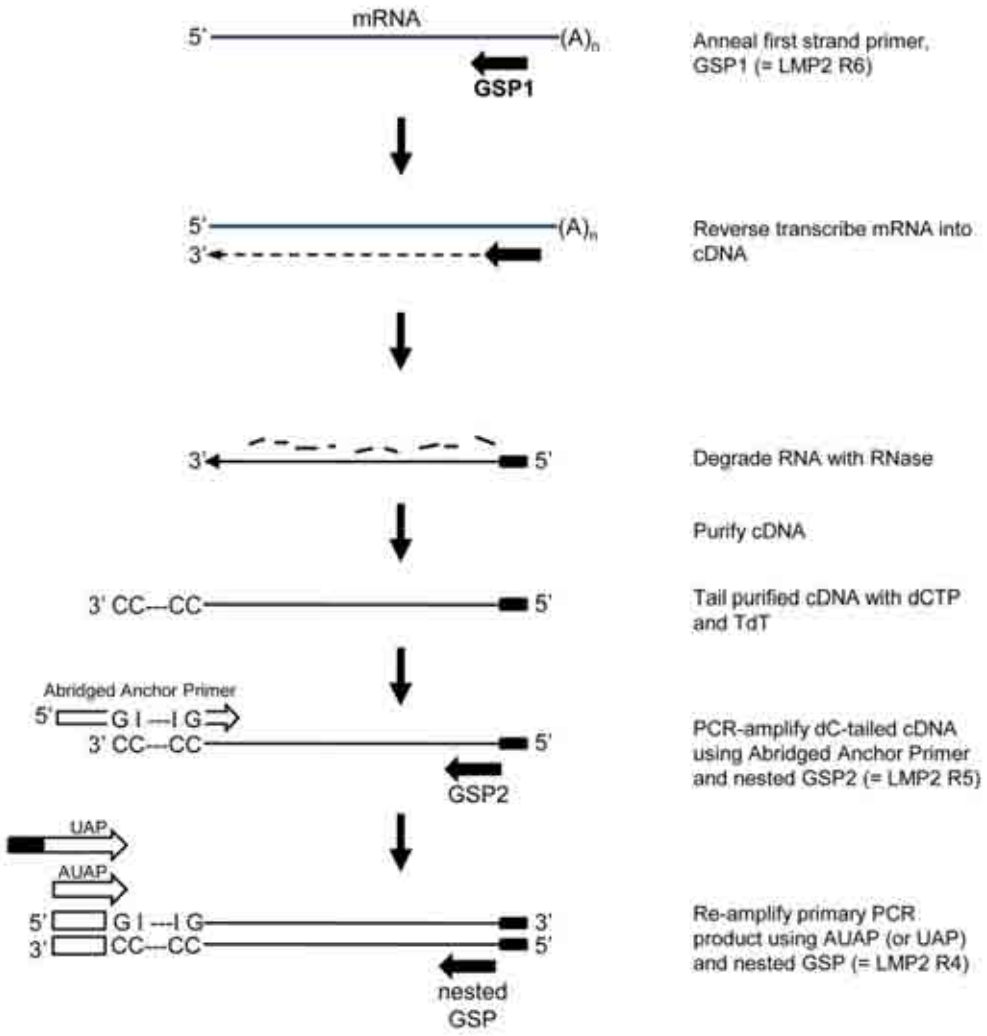


Figure 9 5′ rapid amplification of cDNA ends

Overview of the 5' RACE procedure (adapted from Invitrogen 5′ RACE System for Rapid Amplification of cDNA ends, version 2.0, catalog no. 18374-058 Version E 6 December 2004)

EBER in-situ hybridisation on fixed cells

A commercially-available (Dako) EBER PNA (peptide nucleic acid) probe, complementary to sequences common to EBER1 and EBER2 was used to detect EBER expression in sorted tonsillar lymphocyte populations. Primary cells and control cell lines suspended at 10^4 cells/10µl (due to small numbers of available purified primary cells) were spotted onto each window of a 10 window multisport microscope slide and fixed in methanol/acetone (as for immunofluorescence, pages 96-7) prior to the EBER in-situ hybridisation protocol.The Dako EBER kit was used according to the manufacturer's instructions. Briefly, initial digestion at RT with diluted Proteinase K (1:10,000 – note that this is 100-1000 times less than the concentration typically used for FFPE sections), followed by washing, dehydration in ethanol and drying, before adding 1-2 drops of fluorescein-conjugated PNA probe or control probes. Hybridisation was achieved during 90 minutes incubation at a humidified 55°C, followed by a prolonged washing with a stringent wash solution sustained at 55°C. AP-conjugated anti-FITC antibody was then added to each section and incubated for 30 minutes followed by washing and addition of the substrate (BCIP/NBT/levamisole, as supplied in kit) to allow detection of EBER-positive cells by microscopy. Strict precautions to avoid RNAse contamination were adhered to throughout.

Conventional RT-PCR

To a thin-walled PCR tube was added 50µl reaction mix containing 20ng of random-primed cDNA, 1x PCR reaction buffer with magnesium(Roche), 5µl of 2mM dNTPs (Roche), 5µl of 10µM gene specific primers (Table 5, page 102) and 1µl of high-fidelity Taq polymerase (Roche). The Taq enzyme was typically added after initial heating to 90°C for 1 minute (i.e. a 'hot-start' PCR). Samples were heated to 94˚C for 5 minutes in an Eppendorf Thermocycler and incubated through 35 cycles of 94˚C for 1 minute to denature the DNA strands, 50-60˚C for 1 minute to allow the primers to anneal to the DNA and 72˚C to allow extension of the product. The annealing temperature and extension time at each stage varied according to the specific primers and the anticipated product size of each PCR. Products were separated by agarose gel electrophoresis.

Agarose gel electrophoresis

DNA samples were mixed with 1:6 v/v with gel loading buffer (0.25%w/v xylene cyanol, 0.25% w/v bromophenol blue, 30% v/v glycerol) and separated by gel electrophoresis at 90-120 volts through a 1 x TBE (0.09M Tris-borate, 0.002M EDTA), 1% agarose gel (Eurogentec). The '1kb plus' DNA ladder (Invitrogen) was loaded into one lane to determine PCR product size. The gel was then stained in a bath of 1 x TBE containing 0.5µg/ml ethidium bromide to allow visualisation of DNAbands via UV light on a transilluminator. A Polaroid photograph of the gel was taken where appropriate.

Extraction of DNA from Agarose gel

PCR products visualised using a UV transilluminator allowed the desired band to be accurately excised from the gel using a sterile scalpel. DNA was extracted using the DNA gel extraction kit (Qiagen) according to the manufacturer's instructions.

Digestion of DNA with restriction endonucleases

Plasmid DNA was digested by addition of 10x restriction endonuclease buffer (New England Biolabs or Roche), 1µL of the appropriate restriction endonuclease, and water (Ambion). A final volume of 20µL was typical and the quantity of restriction endonuclease never exceeded 10% of the total reaction volume. Reactions were incubated at 37˚C for 2-14 hours and an aliquot separated by gel electrophoresis. Digested DNA products required for DNA ligation were purified by gel extraction (Qiagen).

Bacteriology

All lentiviral plasmid vectors were grown in STBL3 bacteria (Invitrogen) and other plasmids in DH5α bacterial cells.

Bacterial growth medium

Liquid bacterial cultures were grown in Lennox Broth (L-Broth) medium. 2.5% w/v L-broth (Fisher Scientific) was dissolved in 400ml distilled water and sterilised before use by autoclaving at 121°C, 15psi for 15 minutes. Bacterial colonies were cultivated on L-Broth Agar (L-agar) plates.

1.5% w/v of agar (Fisher Scientific) was dissolved in 400ml of deionised water and sterilised by autoclaving at 121°C, 15psi for 15 minutes. The L-agar was re-melted by microwaving, cooled, and ampicillin added to achieve 100µg/ml. 20ml of L-agar was poured into Petri dishes and used following solidification or stored at 4°C for a maximum of one week. Before use plates were warmed/dried at 37°C.

Back-transformation of competent bacteria

Aliquots of competent bacteria (DH5α or STBL3 (Invitrogen)) were thawed on ice and 100µl transferred to an autoclaved 1.5ml microcentrifuge tube containing 10ng of plasmid DNA or the product of a DNA ligation reaction. The mixture was incubated on ice for 30 minutes and then heat-shocked for 90 seconds at 42°C. 900µl of S.O.C medium (Invitrogen) was added and incubated at 37°C for ≥1 hour. The bacteria were briefly centrifuged and an aliquot – according to expected colony density - spread onto L-agar/ampicillin plates and incubated overnight at 37°C.

Preparation of plasmid DNA

A single bacterial colony was picked with a sterile loop and used to inoculate 3ml of L-Broth containing the appropriate antibiotic (typically ampicillin at 100µg/ml). Cultures were incubated with shaking at 37°C for 6 hours. A fraction of the culture (typically 200µl) was inoculated into 200ml of L-Broth containing the appropriate antibiotic in a 250ml conical flask and incubated at 37°C in a shaker overnight. The next day the bacteria were pelleted by centrifugation at 75,000g for 10 minutes and plasmid DNA extracted using the midi-prep system (Jetstar) according to the manufacturer's instructions. The DNA pellet was then air dried before re-suspension in 200µl of TE buffer. The concentration of the plasmid DNA was determined using a Nanodrop (Thermo Scientific) according to manufacturer's instruction. Plasmid DNA was typically diluted to 1µg/µl and stored at -20°C until required.

Transfection of cultured eukaryotic cells by electroporation

Cell lines in the exponential growth phase were used. Target cells were pelleted by centrifugation (350g) and resuspended to a density of $2x10^7$ per ml in Optimem serum-free media (Invitrogen).

1-5μg of the plasmid of interest was added to 0.4 mm gap sterile electroporation cuvettes (Geneflow) with 0.5ml cell suspension (10^7 cells) and carefully mixed by gentle pipetting. Electroporation was undertaken with a Bio-Rad Gene Pulser II with a capacitance of 975μF and a voltage of between 210 and 270, optimised for individual cell lines. Electroporated cells were transferred immediately to 8ml of warmed appropriate medium in one well of a 6-well plate and incubated at 37˚C, 5% CO_2 overnight. At 24hours, the cells were often centrifuged on a density gradient (Lymphoprep, Axis Shield) to eliminate dead cells and debris. Transfection efficiency and viability was determined by flow cytometry after 48 hours. For some difficult to transfect lines, similar experiments were done using the Amaxa®Nucleofector™ (Lonza) with supplied reagents and in accordance with the manufacturer's optimised protocol (VX-001).

Lentiviral vectors

Generation of a lentivirus expressing inducible LMP 1 is discussed Results part II, pages 178-184)

Lentivirus packaging and synthesis

Typically, 3 x 10^6 293FT epithelial cells (Invitrogen) were placed into 10cm plates in RPMI-1640/10% heat-inactivated (HI) FCS (no G418), to result in 70-90% cell confluence the following day, ready for plasmid transfection. The culture medium was carefully replaced 2 hours prior to transfection with 10ml pre-warmed RPMI/10% HI FCS. For each plate, 36μl Lipofectamine L2000 was mixed with 1.5ml OptiMEM (Invitrogen). A further 1.5ml OptiMEM per plate containing 4μg LMP1 plasmid (FTREX(LMP1)UTG), 2μg envelope plasmid (*pMD2G* codes for the broad range VSV-G envelope)and 6μg packaging plasmid psPAX2 was prepared. Both were incubated for 5 minutes at RT, mixed together and incubated for a further 20 minutes at RT. 3ml of the transfection mix was carefully added to the 10ml medium already on each plate of 293 FT cells. The following day, the media was replaced with 10ml pre-warmed RPMI/10% HI FCS supplemented with 100μl 1M sodium pyruvate (Sigma). The medium was harvested 3 days post transfection and centrifuged for 5 minutes at 350g to pellet cell debris. The supernatant was then

sterile-filtered through 0.45μm syringe filter (Millipore) and either frozen in aliquots at -80°C for future use, or concentrated and used immediately.

Virus concentration

Virus supernatants were concentrated by ultracentrifugation (19,500rpm) in a Beckman SW40 swing-out rotor for 2 hours at 16°C in a Beckman 14ml polyallomer tube. A friable, near-translucent pellet was just visible after centrifugation. The supernatant was carefully removed and the pellet resuspended in 200μl media, appropriate for the target cell population, and immediately used.

Lentiviral transduction of NK cells

Pure populations of IL2-stimulated primary NK cells were infected with the FTREX(LMP1)UTG lentivirus (LMP1-LV) typically at least 2 weeks after initial isolation to ensure the majority of cells displayed a $CD56^{HIGH}$ $CD16^{DIM/NEG}$ phenotype, as ascertained by flow cytometry. Aliquots of 5 x 10^5 NK cells were suspended in 150μl of NK media (see page 88, culture of primary NK cells) and placed in one well of a 48-well plate (Iwaki), combined with 100μl of re-suspended, concentrated LMP1-LV. Control wells comprised 5 x 10^5 NK cells without LV and, in initial experiments, 5 x 10^5 LCL to serve as a comparator for transduction efficiency. The plate was incubated overnight at 37°C and, the following morning, a further 500μl of relevant media was added to each well. Transduction efficiency was assessed by flow cytometry for GFP after at least 72 hours and the cell populations subsequently expanded in culture to attain sufficient numbers for cell-sorting, guided by GFP. At 48 hours prior to cell sorting, 10ng/μl of Doxycycline was added to the media to induce expression of LMP1. This was to allow sufficient time for LMP1 to be expressed and influence the cellular transcriptome, in readiness for RNA extraction from the purified GFP^+ population immediately after sorting.

Results (I): *In vivo* and *in vitro* EBV infection of non-malignant NK and T cells

Introduction

The intimate association of EBV with a diverse spectrum of rare but well-defined lymphoproliferations of NK and T cell origin raises important questions about the biology of these particular virus-cell relationships. Of fundamental importance is consideration of the mechanism by which the virus enters these cell lineages. Related to this, of equal importance, is understanding the frequency with which EBV infection of NK and T cells occurs in the absence of disease. Is this a rare event, with erroneous infection of such cells provoking a relatively high likelihood of clonal proliferation/malignancy? Alternatively, is T or NK infection quite common *in vivo*, but in most such cases the infected cell is destined to die by apoptosis or lay exposed to EBV-specific CTL attack?

We initially set out to investigate the occurrence and frequency of NK and T cell infection *in vivo* by separating peripheral blood and tonsillar lymphocytes, from a range of donors, to high purity and quantitating the EBV genome number in each population by an established Q-PCR method[560]. This method has advantages over those used in previous studies attempting to address similar questions in which immunostaining combined with EBER ISH was performed on fixed tissue sections[244,246,249]. In particular, applying our approach allows a substantially higher number of cells to be processed (10^6-10^8) and analysed for the presence of EBV; with a sensitivity of 2 EBV genomes (1 Namalwa BL cell equivalent) amongst 10^5 EBV-negative cells.

The overall aim of the experiments presented in this chapter was to investigate occurrences of EBV infection in NK and T cells *in vivo*, in the context of healthy viral persistence, primary infection and immune dysfunction. Although *in vitro* infection of NK and T cells with recombinant EBV was not a primary goal of this work, a limited amount of such data will also be presented, as relevant to the ex-vivo findings.

In the early stages of the study, whilst recruiting and screening healthy tonsil donors, we were fortunate to identify 3 instances of adult patients presenting with clinical and laboratory features consistent with a diagnosis of EBV-HLH. In addition to affording a unique opportunity to analyse the clinical features of a disease rarely presenting in adulthood, we were also able to apply the same cell-sorting and Q-PCR strategy to peripheral blood lymphocytes from these patients.

EBV-HLH in adults

As reviewed earlier (pages 56-65), EBV-HLH is a clinicopathological syndrome, encompassing a dramatically dysregulated immune response and hypercytokinaemia. The disease is characterised clinically by fever, splenomegaly and cytopenias accompanied by histological evidence of haemophagocytosis - and confers a high mortality[476]. Most descriptive reports of EBV-HLH have arisen from study centres in East Asia with the majority occurring in the context of primary EBV infection in children or adolescents. Of the rare cases of adult EBV-HLH reported, the majority have been in individuals of East Asian origin[495] although isolated cases arising in adults of other ethnicities have been described[245,492].

Previous studies of EBV-HLH, incorporating analysis of both tissue biopsies and of circulating lymphocyte subsets, demonstrated $CD3^+$ T cells (usually the $CD8^+$ subset) to be the predominantly infected population, with isolated cases suggesting less frequent, co-infection of $CD16^+$ cells[471]. This contrasts markedly with CAEBV where the presence of EBV in NK cells occurs in approximately half the cases[472].

Clinical features

We investigated 3 adult patients with suspected EBV-HLH (mean age 44). All patients had EBV loads of 10^5-10^6 genomes/ml whole blood, ascertained at the referring hospital, and fulfilled established clinical and laboratory diagnostic criteria for the diagnosis of EBV-HLH[476], including histological evidence of haemophagocytosis (Figure 10, page 114). There were no pre-existing clinical features indicative of a background of CAEBV.

Table 6 Clinical and laboratory characteristics of adult EBV-HLH patients

	Splenomegaly & Pancytopenia	Ferritin (μg/l)	LDH (IU/ml)	HLH criteria ***	EBV serology†††	Therapy‡‡‡	Outcome
HLH 1	Yes	>16,500	5602	6/8	IgM VCA - IgG VCA + IgG EA - IgG EBNA +	IVIG/RITUX/ GCV/DEX/ CSA	Died
HLH 2	Yes	22,699	1879	7/8	IgG VCA + IgG EBNA +	MP/IVIG/ VP16/CSA	Died
HLH 3	Yes	4203	2085	6/8	IgM VCA - IgG VCA + IgG EBNA +	DEX/VP16	CR

*** Diagnostic criteria from reference476. Henter JI, Horne A, Arico M, et al. HLH-2004: Diagnostic and therapeutic guidelines for hemophagocytic lymphohistiocytosis. *Pediatr Blood Cancer*. 2007;48(2):124-131.

††† Plus sign indicates antibody detected by enzyme immunoassay and/or immunofluorescence. Negative sign indicates not detected by one or both of these assays.

‡‡‡ IVIG= Pooled human immunoglobulin; GCV=Ganciclovir; CSA=Cyclosporin A; DEX=Dexamethasone; MP=Methylprednisolone; VP16=Etoposide; RITUX=Rituximab

Table 6 summarises the clinical and laboratory characteristics and described here in brief: **Patient HLH-1:** A previously fit and well 60 year old Caucasian man presented with a 3 week history of fevers and lethargy. Haemophagocytosis was demonstrable on a second diagnostic bone marrow biopsy. Despite a range of immunosuppressive therapies only a transient response was achieved and he died of hepatic failure 5 weeks after initial presentation. **Patient HLH-2:** A 50 year old Caucasian man with a history of ulcerative colitis, described several weeks of fevers, anorexia and weight loss. He subsequently developed respiratory failure associated with radiological evidence of pulmonary infiltrates and required mechanical ventilation. A transient clinical response was seen following therapy according to a modified HLH-2004 protocol[476] but the patient subsequently died of respiratory failure, two months after initial presentation. **Patient HLH-3**: A previously healthy 21 year old man, of Arab origin, presented with a 4 week history of fever, sweats, weight loss and cough – ultimately requiring intensive care support. He fulfilled the diagnostic criteria for EBV-HLH and a subsequent biopsy of a small lymph node confirmed coexisting EBV^+ classical HL, with $EBER^+$ RS cells identified. The HLH responded well to initial treatment with Etoposide and Dexamethasone, following which he was successfully treated with a standard chemotherapy protocol for Hodgkin's lymphoma.

Viral loads in peripheral blood lymphocyte populations in HLH

Figure 11, panel A represents an example of the 3-colour fluorescence-activated cell-sorting strategy applied to these cases. Populations of B ($CD19^+$, $CD3^-$, $CD16/56^-$), T ($CD3^+$, $CD19^-$, $CD16/56^-$) and NK cells (CD16 or $CD56^+$, $CD3^-$, $CD19^-$) were isolated by gating initially on total viable lymphocytes (forward versus side scatter plots), with subsequent gates allocated to exclusively isolate monofluorescent populations to high levels of purity (consistently 95-100% pure). DNA was then extracted from each sorted population and Q-PCR for EBV genome copy number performed. For patient 1 we employed CD16 as a peripheral blood (PB) NK marker (gating on $CD16^+CD3^-CD19^-$ cells), as these are usually the dominant PB NK population[312], but unexpectedly found the highest viral load in a population of small lymphocytes negative for all of CD16, CD3 and CD19.

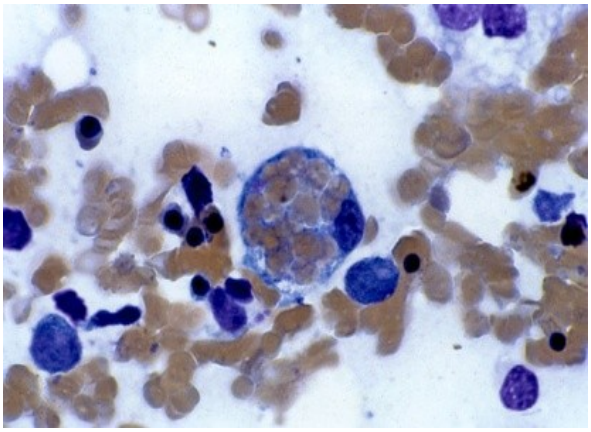
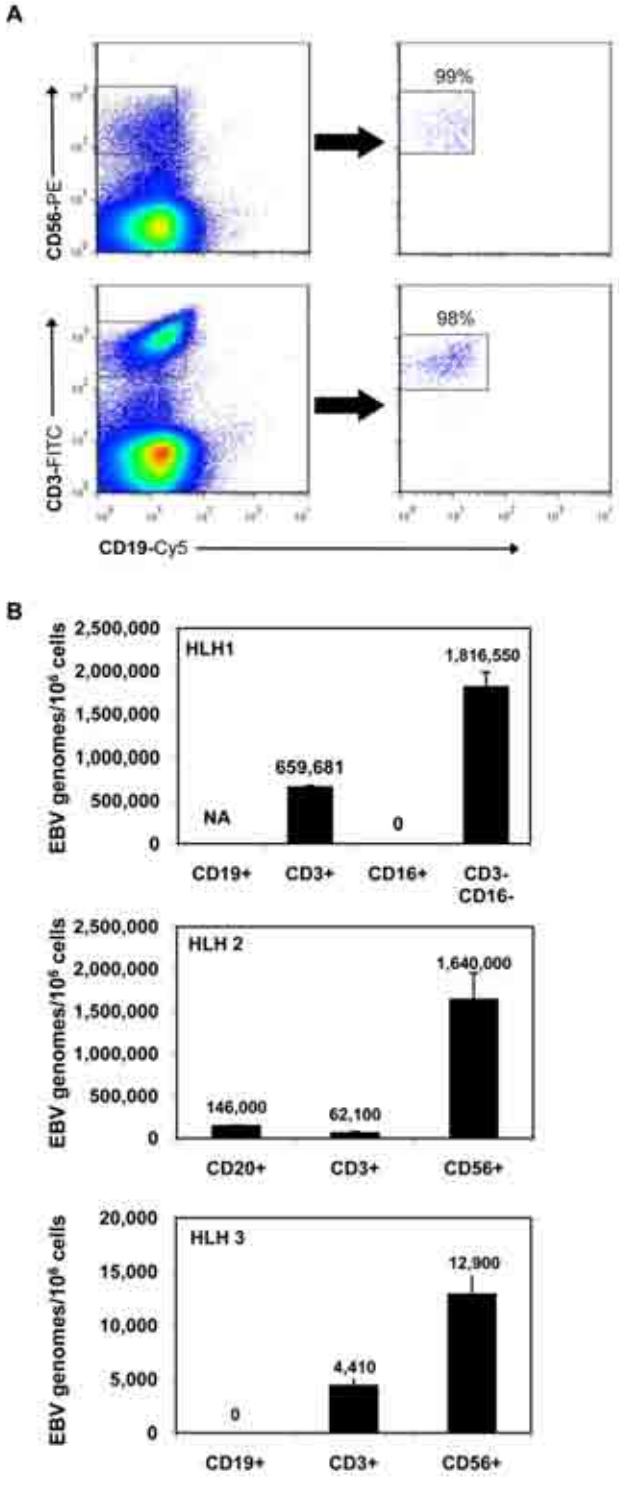


Figure 10 Cytopathological evidence of bone marrow erythrophagocytosis in EBV-HLH 2

Photomicrograph of a bone marrow aspirate slide preparation from HLH-2 showing erythrocytes engulfed by a macrophage (original magnification x200). Courtesy of Dr Philip Gothard, Department of Infectious Diseases, University College London Hospitals, London.

We hypothesised that this population may represent $CD56^{BRIGHT}CD16^{-}$ NK cells, a minority (~10%) NK cell subset in normal peripheral blood (introduction pages 34-37 and reference[312]). Consequently, we used CD56 as an alternative NK marker for the subsequent two patients. Figure 11, Panel B shows the high numbers of viral genomes within the NK cell populations, expressed as EBV copy number per million cells and compared to B and T cells from the same donor. In all three cases, the number of $CD56^{+}CD3^{-}CD19^{-}$ (or $CD16^{-}CD3^{-}CD19^{-}$ in patient 1) as a proportion of total peripheral blood mononuclear cells was not particularly high (2-5%). $CD3^{+}$ lymphocytes constituted the majority PBMC population and all patients were relatively B lymphopenic ($CD19^{+}$ cells ≤2%). Note that a caveat to interpretation of the relative and absolute lymphocyte numbers is that in all 3 cases, patients had received treatment with corticosteroids prior to PBMC analysis.



Panel A

Fluorescence-activated sorting strategy (HLH patient 3 shown as example).

Panel B

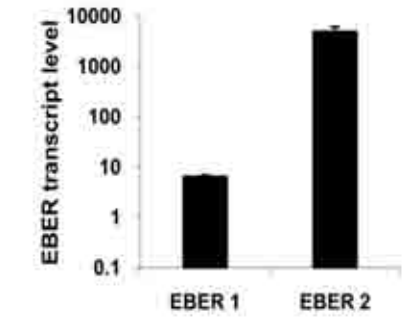
EBV genome numbers in sorted cell populations for HLH patients 1, 2, and 3. Histograms represent the mean number of viral copies per million cell equivalents. Each data-point was assayed at least in triplicate and error bars indicate standard deviations from the mean. NA = insufficient B cells for analysis from patient 1 as a result of *in vivo* rituximab therapy.

Figure 11 High EBV genome load within circulating CD56$^+$ natural killer cells in adult patients with EBV-HLH

EBV gene expression

The available cell numbers restricted analysis of viral gene expression to HLH1 and HLH2 and was performed on total PMBC rather than purified lymphocyte populations. RT-Q-PCR assays specific for viral antigen-coding transcripts (Wp, Cp, Qp, LMP1, LMP2A and BZLF1) were all negative, although the EBER transcripts were expressed in both cases and formally quantified by RT-Q-PCR in HLH-1, where levels greatly exceeded those seen in a LCL (Figure 12).

A



B

Patient	EBERs	Wp	Cp	Qp	LMP1	LMP2	BZLF1
HLH-1	+	-	-	-	-	-	-
HLH-2	(+)	-	-	-	-	-	-

Figure 12 High levels of EBER expression in PBMC from adult patients with EBV-HLH

RT-Q-PCR data for EBV-encoded transcripts in PBMC from HLH 1 and 2.

Panel A: EBER transcript quantitation for HLH 1 expressed relative to levels in a LCL.

Panel B: Summary of RT-PCR assays for latent and lytic transcripts in HLH1 and 2. A plus sign indicates detectable transcripts, a negative sign indicates not detected. The parenthesis (+) for HLH-2 indicates a non-quantitative (conventional end-point) PCR assay was used.

EBV genome load in lymphocyte subsets in health and immune deregulation

The next series of experiments investigated the occurrence and frequency of *in vivo* infected NK and T cells in the absence of clinically-apparent lymphoproliferative disease. Following person-to-person transmission of EBV, immediate contact with tissues of the oro- and nasopharynx represents the virus' first encounter with its target B cells and adversarial immune effectors. Within such SLT is where the $CD56^{BRIGHT}$ NK subset dominates[312] and, coincidentally, is the anatomical site of the majority of ENKTL cases[689]; all of which display a $CD56^{+}$ phenotype[473]. Against this background, and given the striking EBV load in $CD56^{BRIGHT}$ NK cells in the peripheral blood of adult HLH patients, we focused our attention on tonsillar, as well as peripheral blood, lymphocytes.

The limited data available on infection of non-malignant T and NK infection *in vivo*, primarily by IHC and EBER ISH staining, suggests that EBV-infected NK and T cells can be seen – albeit very rarely – in the context of healthy persistence and symptomatic primary infection[230,246,249]. Due to technical difficulties encountered with CD56 antibodies in fixed tissues, such studies adopted PEN5 as an NK cell marker. However, a major limitation of PEN5 is that its expression is restricted to the $CD56^{DIM}$ subset (and is down-regulated in response proliferative stimuli[316]), limiting its usefulness as a NK marker- particularly in SLT. As discussed earlier (page 34), as an optimum NK-specific marker is not currently available, the widely accepted phenotype for their identification in SLT has been $CD56^{+}CD3^{-}$ [308]. A subset of T cells is known to co-express CD56[313] but can be easily identified by surface expression of CD3 or CD8, both absent on NK cells.

With this in mind we elected to employ a combination of conjugated MAbs together with a sorting strategy aimed at delineating NK cells, $CD4^{+}$ and $CD8^{+}$ T cell subsets (given the data on 'cell-of-origin' of CAEBV and HLH respectively[471]) and B cells. For the majority of the following experiments, 4-colour fluorescence cell-sorting was undertaken. The initial gate, based on forward and side scatter plots, allowed identification of lymphocytes and excluded the majority of

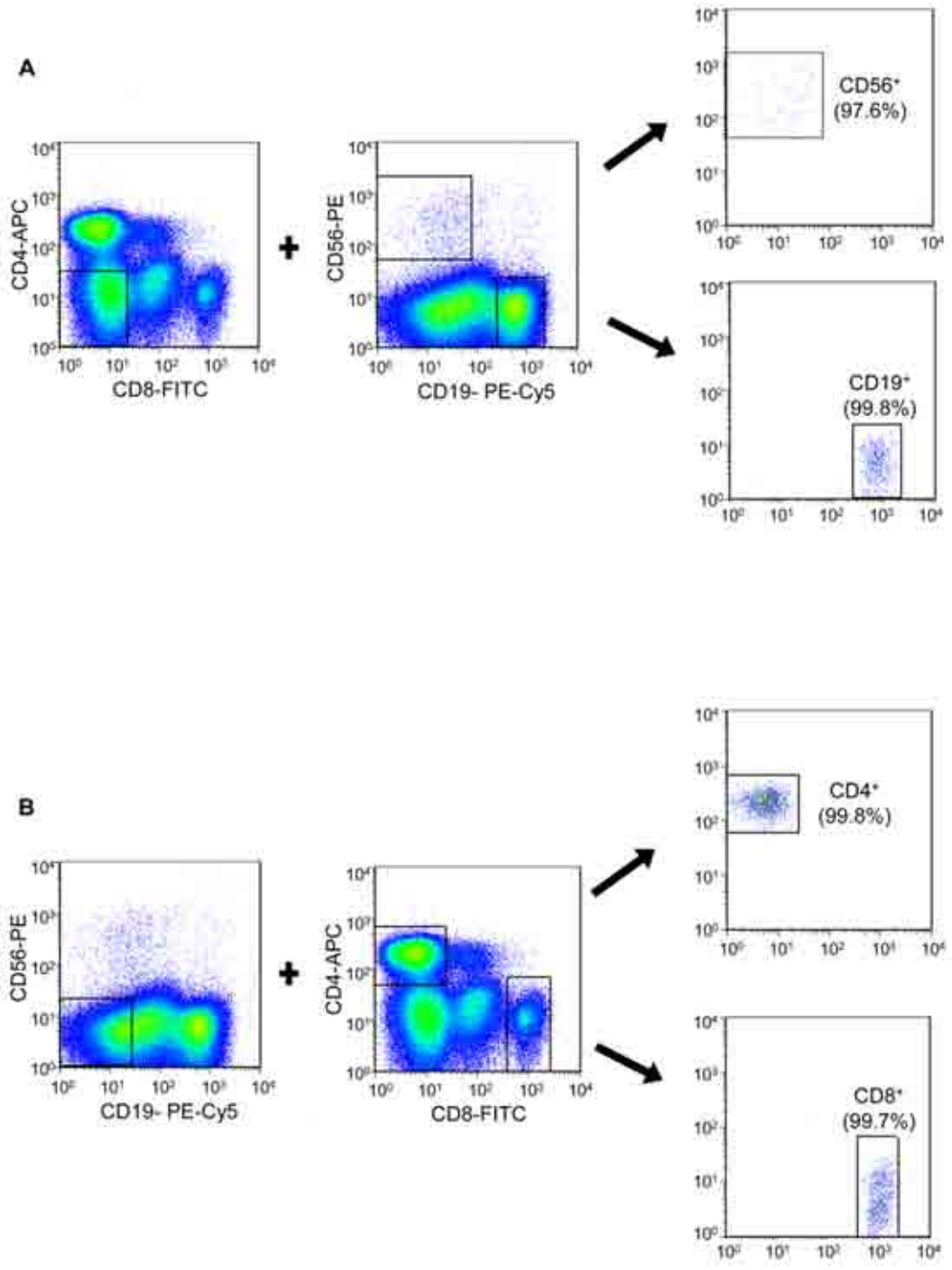


Figure 13 Isolation of highly pure lymphocyte subsets from blood and tonsil

Four-colour fluorescence sorting strategy for isolation of pure monofluorescent lymphocyte populations from tonsil and blood mononuclear cells, as discussed in main text. Initial gating on small lymphocytes by forward/side scatter (not shown) followed by gating logic as indicated. Reanalysis of individual populations following sorting allowed purity/contamination analysis

larger cells, such as monocytes and cell-conjugates. Thereafter, monofluorescent populations were identified and purified: B cells defined by $CD19^{+}CD4^{-}CD8^{-}CD56^{-}$; major T cells subsets $CD4^{+}$ or $CD8^{+}CD56^{-}$ $CD19^{-}$; and NK cells $CD56^{+}CD4^{-}CD8^{-}CD19^{-}$ (figure 13, previous page). Note that for peripheral blood samples, antibodies to CD16 and CD56 (both conjugated to PE-fluorochromes) were used together (alongside CD19, CD4 and CD8) in order to capture both major NK subsets.

Tonsils donated by healthy donors undergoing routine tonsillectomy, patients with acute IM and patients receiving immunosuppressant therapy following allogeneic solid-organ transplantation were studied. Figure 14 shows viral load values quantitated in unsorted tonsil mononuclear cells (MNCs) from each patient group. Each individual symbol represents a separate tonsil donor, and the viral load - plotted on the y axis as a $\log_{10}$ scale - is presented as EBV genome number per million cells. Horizontal bars correspond to median values within a patient group. The highest values, seen in the IM group, were 3 orders of magnitude higher than in the other tonsil donors whilst healthy donors had the lowest median values – in the range of 10^{2}-10^{3} genomes per million cells.

Viral genome load in blood and tonsil lymphocyte subsets in healthy EBV persistence

This series of experiments focused on healthy donors and initially confirmed that EBV is strictly confined to B lymphocytes in the peripheral blood, consistent with current understanding of the virus' biology[56]. Peripheral blood lymphocytes from a healthy donor, known to have a persistently high but stable viral load, were sorted to high purity and each population assessed by quantitative PCR (Q-PCR) for EBV copy number. Figure 15 (page 121) shows the viral load values in total PBMC alongside the sorted lymphocyte populations, demonstrating confinement of EBV to the circulating B cell population as anticipated. In keeping with their status as a minority peripheral lymphocyte population, the B lymphocyte viral load is several times greater than that measured in unsorted PBMC. The inset pie-chart displays relative proportions of cell populations present amongst analysed viable lymphocytes.

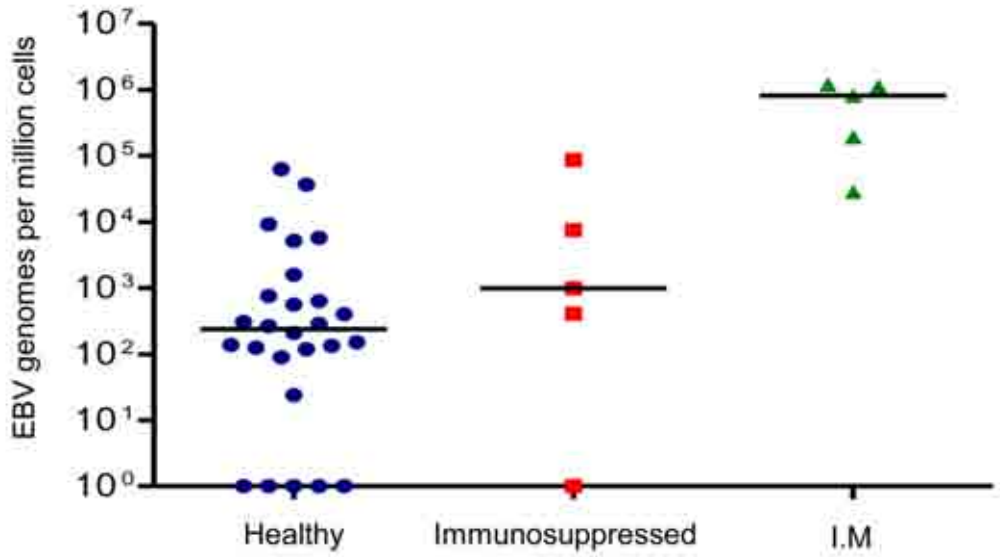


Figure 14 Distribution of EBV genome load in total tonsil mononuclear cells from different patient groups

EBV genome numbers per million tonsil mononuclear cells. Each symbol represents an individual tonsil donor and colours indicate the different patient groups. The horizontal line indicates the median viral load value of each group. Note the y axis is a $\log_{10}$ scale.

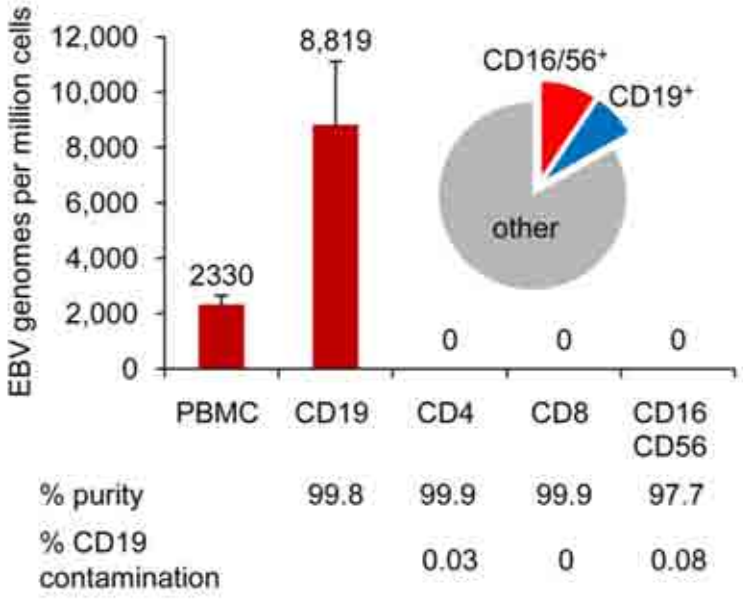


Figure 15 EBV is exclusively restricted to B lymphocytes in peripheral blood from healthy donors

Viral load data in total PBMC and purified lymphocyte populations, expressed as EBV genomes per million cell equivalents. Numerical values above each histogram indicate the mean value of Q-PCR performed in triplicate with standard deviations as indicated by capped vertical bars.

The pie chart shows the relative proportions of NK cells, B cells and other (predominantly T lineage) cells within the gated lymphocyte population, as indicated by red, blue and grey segments respectively.

The percentage figures beneath the x axis result from re-analysis of sorted populations: the top row indicates purity of each respective population whilst the bottom row is the percent of $CD19^+$ cells within each sorted fraction.

Our attention then turned to tonsillar lymphocytes, obtained from healthy individuals undergoing elective tonsillectomy for chronic or recurrent tonsillitis. Although, by virtue of the indication for surgery, these tonsils cannot be considered entirely 'normal', all patients were otherwise healthy with no history of immunodeficiency or IM. Moreover, there were no instances of clinically apparent infection or acute inflammation at the time of excision.

In total, 26 separate tonsil donations were obtained and the viral load in total mononuclear cells ascertained by Q-PCR. The viral load distribution is shown in Figure 14 (page 120): five patients (19%) were EBV negative by Q-PCR, whilst the median value for the remaining 21 patients was approximately 300 viral copies per 10^6 mononuclear cells. Anticipating that EBV-infection of NK or T cells is likely to be a rare event in this context, we elected to focus on those tonsils with a relatively high viral load. Lymphocytes from such donors were subject to high-purity cell sorting into 4 monofluorescent populations: $CD19^+$, $CD4^+$, $CD8^+$ and $CD56^+$ as described above (and see Figure 13, page 118). Unless numbers of purified cells were critically low, small aliquots of all 4 sorted populations were re-analysed to confirm purity (values of 97-100% were typical) and to identify the phenotype of contaminating cells.

Representative viral load data obtained from five normal tonsil (NT) sorts are shown in Figure 16. A consistent finding in most analysed tonsils was that the viral load in the T-MNC was equivalent to, or sometimes exceeded, that seen in the CD19 fraction. This contrasts with the situation in peripheral blood and is partly attributable to the substantially greater proportion of B cells in T-MNC (approximately 30-70% compared to 5-10% in PBMC) and thus proportionally less enrichment of EBV-containing B cells after sorting. The inset pie-charts for each tonsil represent relative proportions of lymphocytes in the pre-sort MNC but note that tonsils were *partially* depleted of B cells immediately prior to sorting, to improve non-B cell purities (see materials and methods pages 85-86). This resulted in an underestimation of the *in-vivo* % of tonsillar B lymphocytes, but this approach was consistently applied to all tonsil sorts and improved purity yields of T and NK fractions.

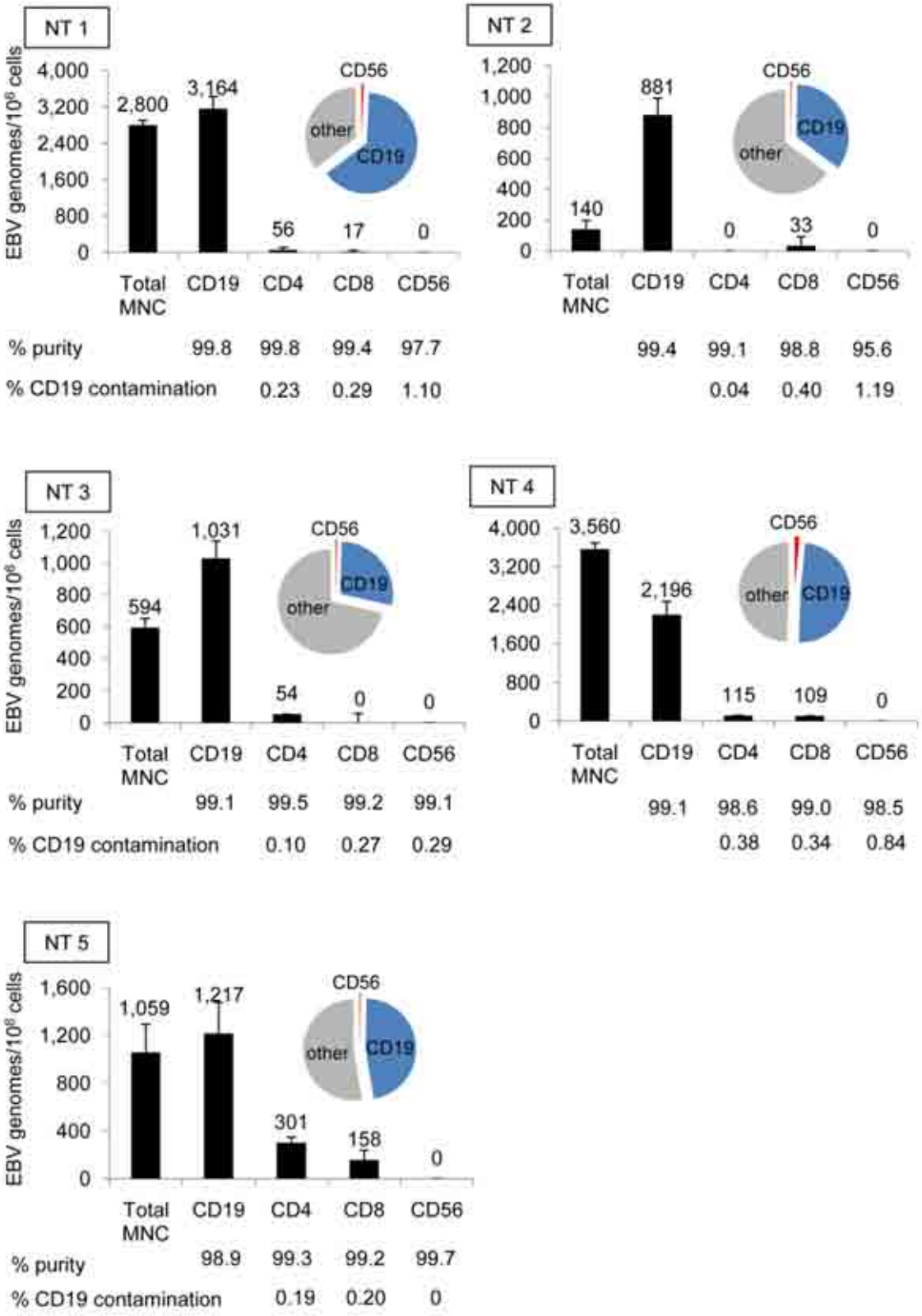


Figure 16 EBV is predominantly restricted to B lymphocytes in tonsils from healthy donors

In all cases of NT analysed, EBV genomes were predominantly restricted to the $CD19^+$ B lymphocyte population. In the majority of cases, no substantial numbers of EBV were identified in the $CD4^+$ or $CD8^+$ T lymphocyte populations, although low levels of virus not numerically explicable by contaminating B cells was often appreciable. On one occasion (NT5) there was stronger evidence of T cells harbouring EBV with values approaching 25% of the CD19 load. In this, and all cases of apparent T cell infection, a confirmatory cell-sort was repeated on at least one, and sometimes two, further occasion(s) using a further aliquot of T-MNC from the same tonsil. However, in no cases of NT analysed did $CD56^+$ NK cells appear to harbour any virus.

EBV genome load within lymphocyte subsets during infectious mononucleosis

IM peripheral blood

Although phenotypic analyses of EBV-infected B-cell subsets in IM has been extensively investigated[690-691], much less is known about the occurrence of T or NK cell infection in the context of symptomatic primary infection. That a numerically and functionally robust T cell response - alongside elevated numbers of peripheral blood NK cells - consistently occurs in IM (pages 41-46) implies that ample opportunities are afforded for erroneous NK or T cell infection to occur via engagement with B cells. Indeed, EBV-HLH is frequently seen to occur in the immediate aftermath of symptomatic primary infection[240,485-486,490].

The next phase of investigation therefore focused on peripheral blood from patients presenting with IM, diagnosed locally in Birmingham on the basis of characteristic clinical features (pages 41-42 and reference[375]) coincident with a positive heterophile antibody test (Monospot). Figure 17 summarises the viral load data of peripheral blood lymphocyte

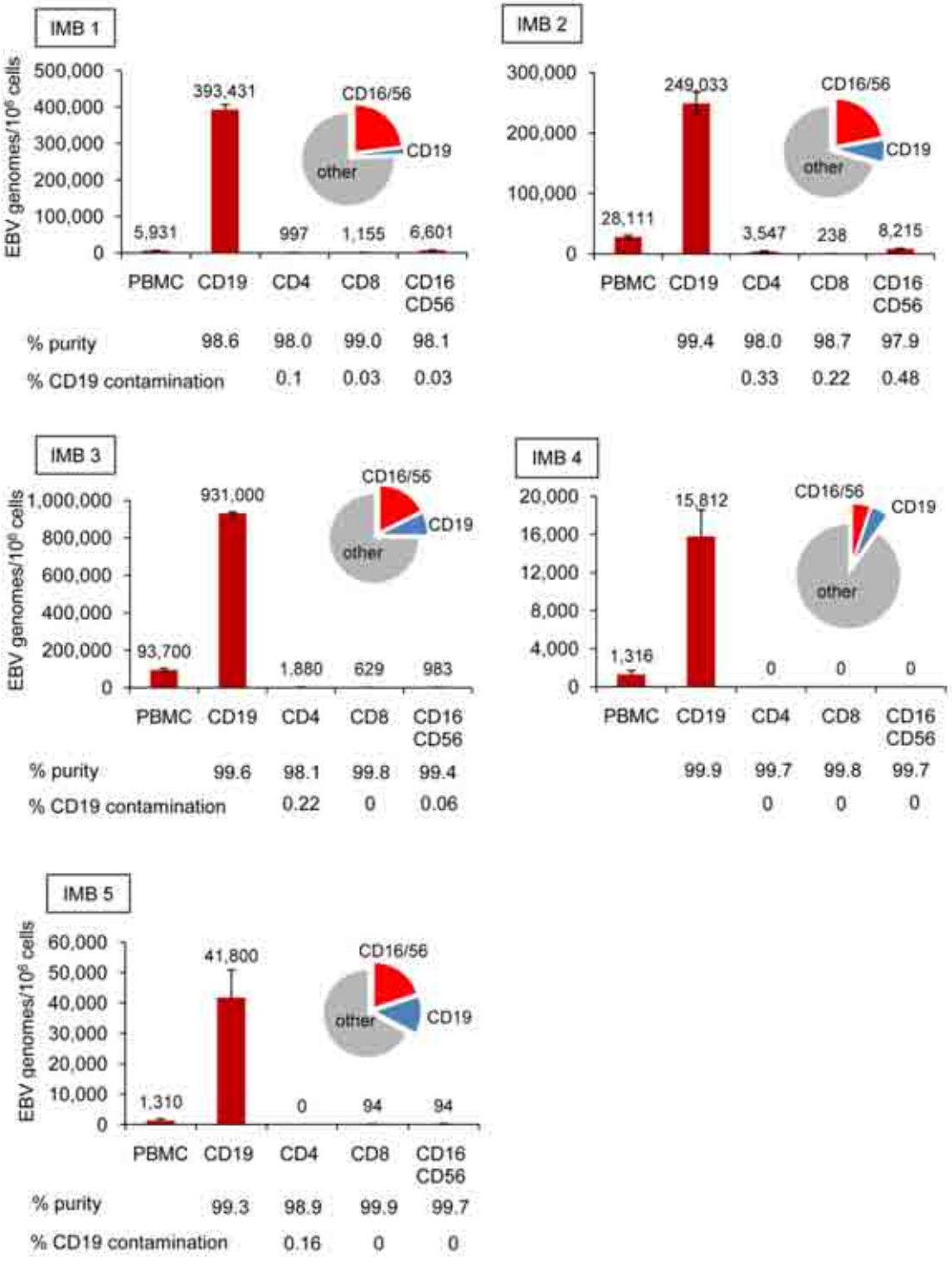


Figure 17 EBV genome load is principally restricted to B lymphocytes in the peripheral blood of individuals with infectious mononucleosis

populations sorted, as before, from five IM donors. In acute IM, the number of EBV-infected B cells in the blood is known to be increased by 10^3-10^4 fold, equating to 1-10% of all circulating B cells infected[383]. In three of our cases (IMB1-3), the blood sample was donated at the height of IM symptomatology, and within 24 hours of the diagnostic Monospot test, whereas IMB4 and IMB5 were analysed in the immediate recovery period (approximately 10-14 days following a positive Monospot). Accordingly, the viral load values for the latter two donors are ten-fold lower than IMB1-3. As expected, in all cases the $CD8^+$ T cell population was markedly expanded, alongside less marked increases in NK cell numbers. In some cases this was reflected in proportionally fewer B cells. As for normal blood, and contrasting with the situation in tonsils, the viral load in the purified CD19 population in IM was substantially higher than in total PBMC.

In all 5 cases of IM blood examined, the viral load was almost entirely restricted to $CD19^+$ B lymphocytes. As indicated previously, the NK cell population was sorted using a combination of CD16 and CD56 MAbs. In the case of IMB2 and 3, the viral load appreciable in these total NK fractions could potentially be attributable to 2% and 3% contaminating B cells, respectively, although the observed $CD19^+$ contamination was less than this.

IM Tonsils

As discussed earlier (pages 26-27 and 110), although the published data is somewhat conflicting it is overall in keeping with infrequent infection events of T and NK cells in IM tonsils. We applied our cell-sorting and sensitive Q-PCR assay to try and address this issue more quantitatively. IM tonsil (IMT) MNC, obtained through collaboration with Dr Wolfgang Bergler (University Hospital Mannheim, Germany) were recovered from liquid-nitrogen storage and partially depleted of B cells as for NT. Figure 18 is a summary of data obtained from five such IM tonsils, presented as for NT. Akin to the situation with IM blood samples, the range of viral loads between IMT MNC could vary by 1-2 orders of magnitude. This could potentially be explained by the timing of tonsillectomy in relation to symptomatic presentation of IM and/or the occurrence of peak viral load, although this information was not available to us.

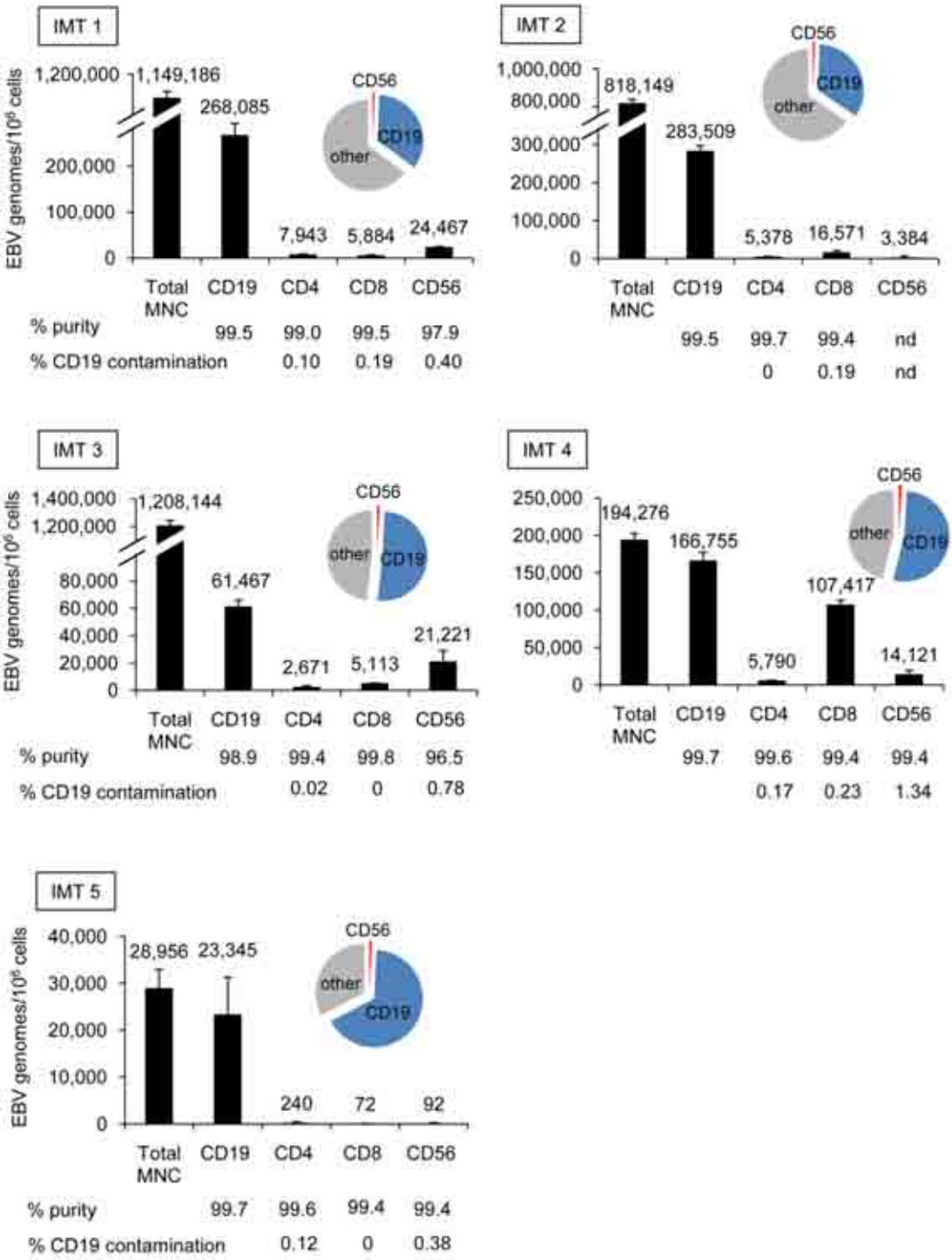


Figure 18 EBV genome load is not exclusively restricted to CD19$^+$ B cells in the tonsils of patients with infectious mononucleosis

An initial observation of the data in Figure 18 was the striking discrepancy in some cases (IMT 1-3), between viral load values in total MNC and $CD19^+$ B cells. This difference, amounting to 3-20-fold, is substantially greater than that seen in NT and is highly suggestive of loss, or exclusion, during the sorting process, of a B cell population with a high viral load. For example, B cells committed to plasma cell differentiation with low or absent CD19 expression could potentially be implicated here. Examining the non-B cell fractions, there were three main observations. Firstly, in most cases examined and similarly to NT, a low level viral load was evident in the $CD4^+$ and/or $CD8^+$ T cells that could not be numerically accounted for by contaminating B cells. Secondly, one case (IMT4) displayed an impressively high viral load in the $CD8^+$ population, approaching the CD19 value. Lastly, there were three instances of apparent infection of $CD56^+$ cells, approximating to 10-30% of the corresponding $CD19^+$ load (IMT1, 3 and 4). Although definitive evidence of viral lytic cycle occurring in IM tonsil B cells has not been available we considered the possibility that cell-free virus, arising from replicative infection, may adhere to neighbouring T and NK cells. To ascertain evidence of such replicative infection, we undertook RT-Q-PCR to detect an immediate early (BZLF1) and a late (gp350) viral lytic transcript in total RNA extracted from purified $CD19^+$ tonsillar populations. With assay sensitivity permitting detection of 1 lytic cell in 10^3-10^4 latent cells, only IMT 3 manifest detectable EBV lytic transcripts (equivalent to 14% and 1% of $CD19^+$ cells expressing $BZLF1^+$ and $gp350^+$ respectively); the other tonsils tested were negative (data not shown).

EBV genome load in blood and tonsil lymphocyte subsets from immunosuppressed individuals

As discussed in the introduction (page 47-50), patients with compromised T cell effector function are at risk of developing EBV-driven lymphoproliferations of B cell origin. By contrast, considerably fewer cases of EBV^+ NK or T cell lymphoproliferation are reported[408]; no increased incidence in immunocompromised persons is readily apparent.

Most patients who develop NK and T cell lymphoproliferative diseases are considered to be immunocompetent; there is no antecedent history of clinically evident immune dysfunction.

However, the dramatically dysregulated and ineffective immune response seen in EBV-HLH, and the seeming inability of CAEBV patients to mount a meaningful immune response to EBV-infected T or NK cells, intimates that host immune status may be important in pathogenesis.

However, little data has been available on the frequency or occurrence of EBV-infected lymphocytes in the context of impaired T cell function. On this note, we were able to identify and analyse tonsillar lymphocytes (with matched peripheral blood samples in two cases) from four patients receiving immunosuppressive therapy with Tacrolimus (or FK-506, a calcineurin inhibitor) following solid-organ transplantation (SOT). Pharmacological inhibition of calcineurin results in suppression of signal transduction and IL-2 transcription within T lymphocytes, impairing their activation and proliferation[692]. Available clinical information on our patients was somewhat limited: IST1 and IST2 had undergone previous renal transplantation but their EBV status was not available at the time of surgery. IST3 and IST4 had both undergone heart/lung transplantation and IST4 had experienced an episode of documented 'EBV viraemia' 2 years previously. Tonsillectomy was indicated for symptomatic enlargement, in one case presenting with acute airway compromise and another with a longer history suggestive of obstructive sleep apnoea. It is important to note that the intensity of immunosuppression in each case could be quite different, influenced by the transplanted organ type, nature of additional peri-transplant immunosuppression, serum levels of Tacrolimus and the time elapsed since transplantation.

For the first immunosuppressed tonsil (IST1) we investigated, numbers of available tonsillar cells were limited and we therefore used 3-colour FACS (MAbs against CD19, CD3 and CD56) to allow sufficient numbers of purified populations to be collected. The sorting procedure, viral load analysis and representation of data (Figure 19) were otherwise unmodified. Notwithstanding an essentially normal T-MNC viral load, comparable to the median value of the NT samples (Figure 14, page 120), we were struck by an unexpectedly high genome number in the $CD56^{+}CD3^{-}CD19^{-}$ population; 70-fold higher than in the corresponding B cells. This pattern had not been thus far observed in NT or IMT and we therefore sought to investigate this further. IST2 and IST3, with normal to moderate T-MNC viral loads showed essentially the same pattern; the viral load per

$CD56^+$ cell patently dominated and was approximately 20 to 100-fold higher than the B cell counterparts. The final 'immunosuppressed tonsil' we analysed (IST4) also manifest a high load in the $CD56^+$ compartment, although the pattern in this case was more akin to IM tonsils 1,3 and 4 (Figure 18, page 127); amounting to approximately one-third of the CD19 load. For IST2-4, the sorting procedure was repeated on at least one further occasion in each case, using separate aliquots of T-MNC, and the results confirmed. Note that the proportion of $CD56^+$ $CD19^-$ $CD4^-CD8^-$ cells in IST cases was not increased; amounting to approximately 0.4% of viable lymphocytes.

For the donors of IST3 and IST4, we had the opportunity to analyse a matched peripheral blood sample taken at the time of tonsillectomy (designated ISB3 and ISB4 respectively). By distinct contrast to the matched tonsil samples, the sorted peripheral blood lymphocytes from these immunosuppressed patients showed EBV present almost exclusively within the B cell compartment. No EBV genomes were detectable in the peripheral blood NK fraction. In the case of ISB4, the discernible viral load in the CD8 fraction could be explained mathematically by 0.6% CD19 contamination (although only 0.14% contamination was evident on re-analysis) and is therefore best regarded as of uncertain significance.

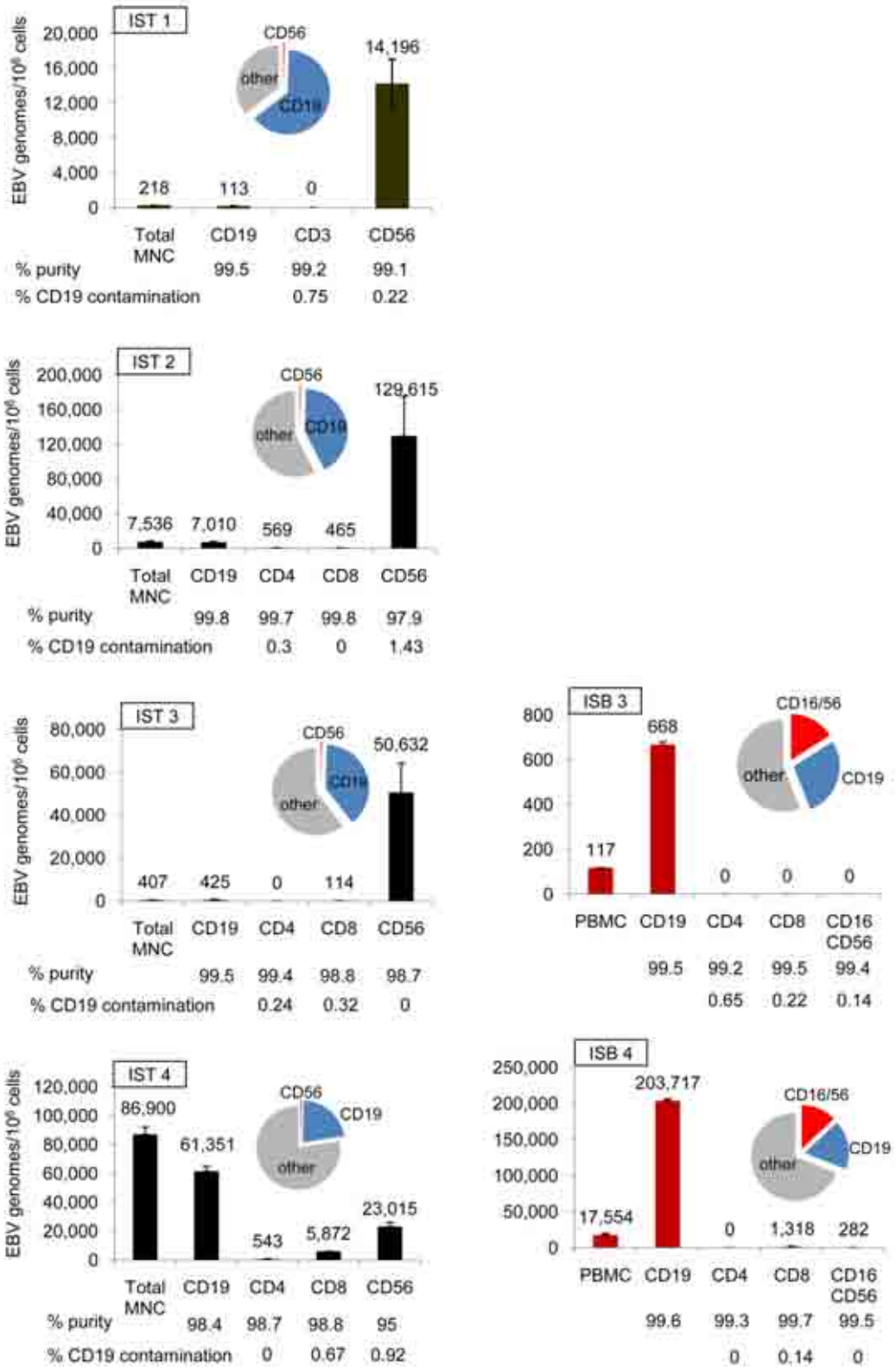


Figure 19 High EBV genome copy number in tonsillar, but not peripheral blood, CD56$^+$ lymphocytes from immunosuppressed patients

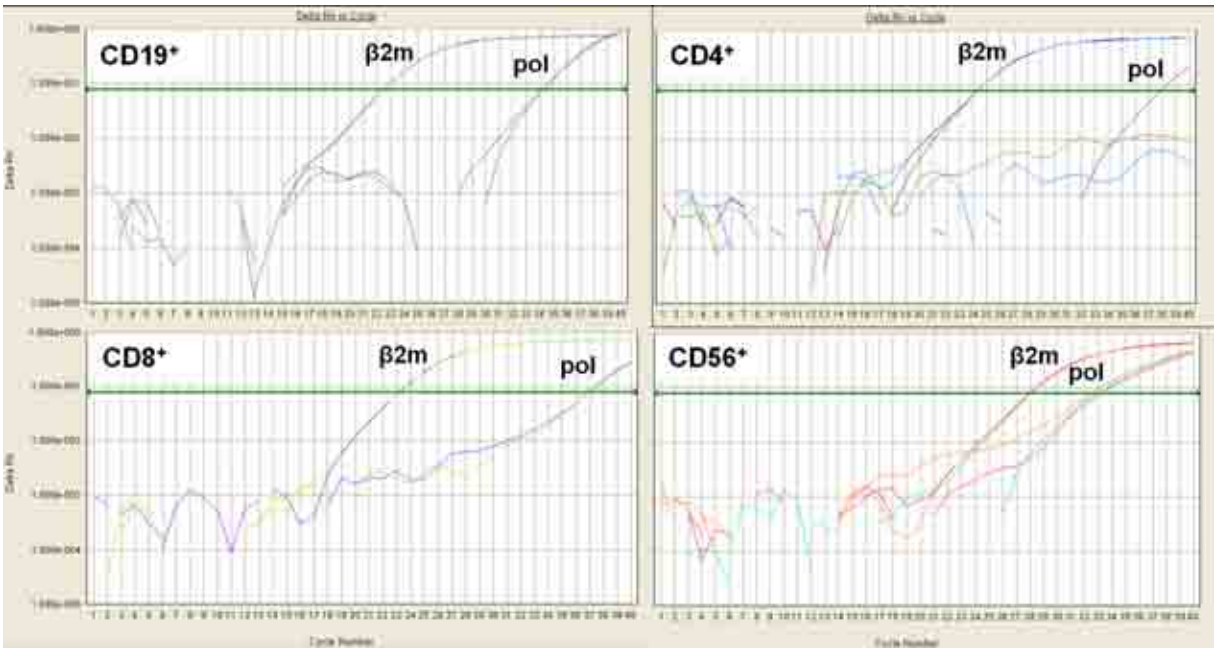


Figure 19b Taqman PCR data for viral load ascertainment in sorted tonsil lymphocytes (IST 3).

Representative raw data from Taqman PCR amplification plots; relative quantitation of viral genomes (pol) and a cellular control gene (β2-microglobulin). Each quadrant displays amplification data from DNA extracted from a specified purified lymphocyte population from tonsil IST3 (see figure 19). The x axes represent the number of amplification cycles and the coloured horizontal line is the assigned detection threshold.

Cell yield for each purified population:

$CD19^+$ ($CD4^-CD8^-CD56^-$) = $4x10^6$ cells

$CD4^+$ ($CD19^-CD8^-CD56^-$) = $1x10^6$ cells

$CD8^+$ ($CD19^-CD4^-CD56^-$) = $5x10^5$ cells

$CD56^+$ ($CD19^-CD4^-CD8^-$) = $1x10^4$ cells

(Note that no more than $1x10^6$ cells from each population were used for DNA extraction and, where necessary, DNA was diluted to ≤50ng/µl prior to qPCR analysis; the β2m value served as a control for input DNA. See Materials and Methods, page 99)

One key question regarding the very high genome numbers in the $CD56^+$ tonsillar fraction was whether this represented true infection of these cells, rather than surface-bound virus. As described for the IM tonsils, there was no discernible lytic activity in the $CD19^+$ fraction of the IST. One approach to address this question relating to surface virus would be to establish whether any viral mRNAs were transcribed in the $CD56^+$ population. As the EBERs are abundantly present in all latently infected cells, this represented an attractive target. However, we were seriously challenged by limited IST material and, moreover, by very small numbers of $CD56^+$ cells yielded from cell sorting (typically 10^3-10^4); insufficient for extraction of good quality RNA. I therefore took an existing protocol for EBER-ISH on formalin-fixed tissues and optimised it for use on small numbers of cells fixed in methanol/acetone, dried onto slides (page 105). EBV^+ cell lines served as controls and showed intense nuclear staining, whereas only very rare $EBER^+$ cells (<1/500) were seen in the $CD56^+$ fraction purified from IST3; no appreciable EBER expression was seen in the overwhelming majority of cells (data not shown). Using antibodies directed to early (BZLF1) and late (p18, a minor capsid protein) lytic antigens immunofluorescence staining was performed on cells from this same $CD56^+$ fraction and highlighted an isolated cell undergoing replicative infection (Figure 20).

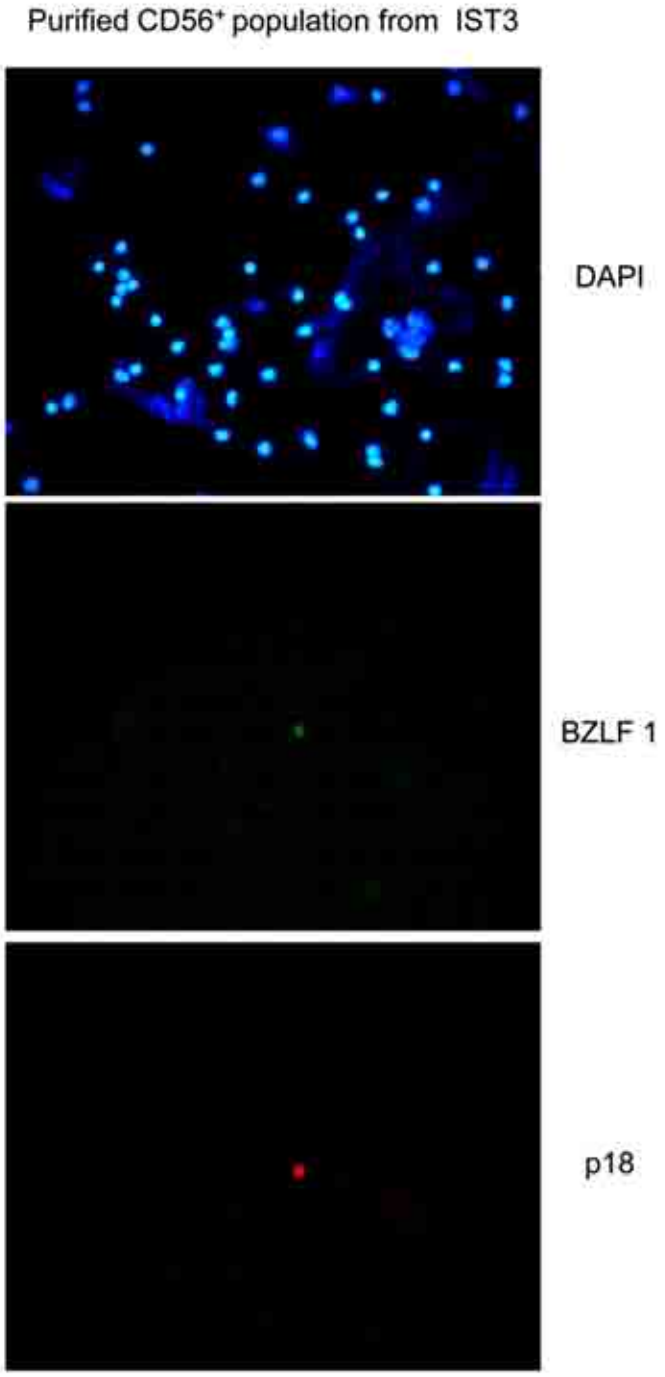


Figure 20 Evidence of EBV full lytic cycle in rare cells within tonsillar CD56$^+$ cells from an immunosuppressed patient

Photomicrographs of phase-contrast, fluorescent images of purified CD56$^+$ cells from donor IST3. DAPI staining shows all nucleated cells. An isolated cell is seen to express both early (BZLF1) and late (p18, a minor capsid protein) lytic antigens.

EBV infection of T and NK cells in vitro

In vitro infection of primary NK cells and T/NK lines with cell-free EBV

Experimental infection of T and NK cells *in vitro* was not a major focus of this thesis; an allied body of such work was being undertaken concurrently within our laboratory by Dr Claire Shannon-Lowe and Miss Emily Adland. However, given the *in vivo* findings described above: high EBV genome numbers in circulating $CD56^{+}$ cells in HLH; similar findings in $CD56^{+}$ tonsillar cells from immunosuppressed individuals; and to a lesser extent in $CD56^{+}$ tonsillar lymphocytes in IM, we elected to undertake a series of complementary *in vitro* studies.

The solitary report of remarkably efficient *in vitro* infection of human NK cells[301] (published in 2004 and discussed on pages 31-33) used freshly isolated primary NK cells, the majority of which have a $CD16^{HIGH}$ $CD56^{DIM/NEG}$ phenotype (Figure 4). Our interest in $CD56^{HIGH}$ cells was stimulated by our *in vivo* findings in HLH and immunosuppression. We therefore initially set-out to reproduce the published data from Isobe *et al* but extend these studies to include IL-2 stimulated primary NK cells; where the majority display a $CD56^{BRIGHT}$ phenotype in culture. NK cells were isolated from peripheral blood and either used immediately or cultured in IL-2-containing medium (page 86-87) until a $CD56^{BRIGHT}$ phenotype dominated (typically 2 weeks). Primary B cells from the same donor served as a positive control. We also included NKL (an NK leukaemia line also infectable in the study of Isobe et al[301]) the T cell lines MOLT4 (published studies consistent with virus-binding but no entry[53,285]) and Jurkat (known to express low levels of surface CD21[287]). Infection experiments with these individual cell populations using cell-free EBV were performed independently with both Akata EBV and B95-8(2089) viruses, used at an m.o.i of 100 as described earlier (pages 89-90). The data in Figure 21 represent a 10 day time-course experiment whereby RNA from an aliquot of each cell population was extracted on the indicated day following infection and RT-Q-PCR undertaken to quantitate EBER1, 2 and

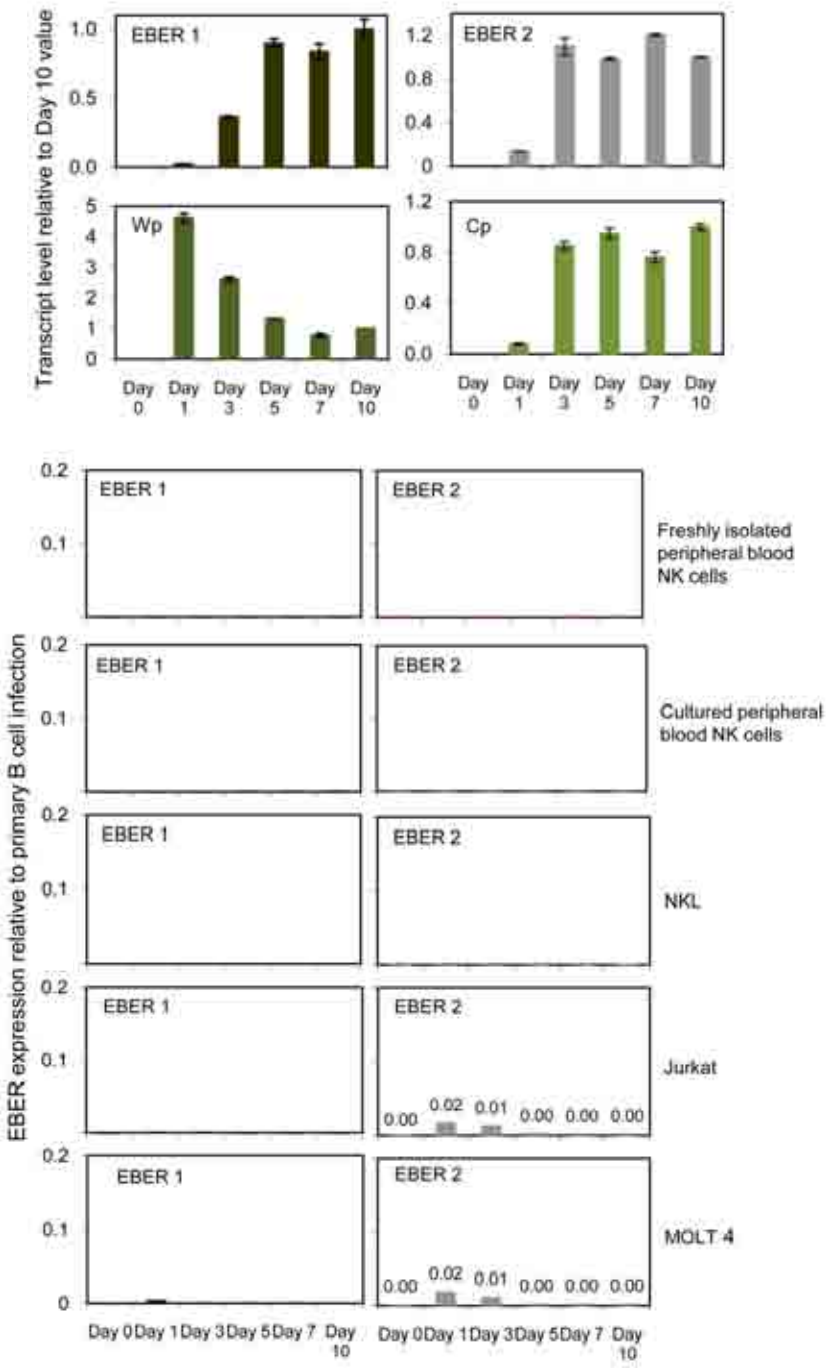


Figure 21 Quantitation of EBV-encoded transcripts in primary lymphocytes and cell lines.
Panel A shows expression of EBV transcripts over a 10 day period following infection of primary B cells. Histograms represent normalised transcript levels relative to a Day 10 value.
Panel B shows expression of EBV transcripts over a 10 day period following infection of primary NK cells and T/NK lines. Histograms represent normalised transcript levels relative to the corresponding B cell value on each day. Note the different y axis scale between panels A and B

EBNA1 transcripts. Data for B cells is expressed relative to the Day 10 value (arbitrarily set at 1) whilst normalised transcript levels for the T and NK cells are shown relative to the corresponding B cell time-point. Panel A shows the results from primary B cell infection where, consistent with published results[82], significant expression levels of EBERs are evident from Day 3 onwards whilst prompt initial activity from the Wp promoter is quickly superseded by Cp transcription.

Panel B shows the results of infection of primary NK cells and T cells and demonstrates that infection of primary NK cells and T/NK cell lines is extremely inefficient with cell-free virus. In spite of a high m.o.i, no appreciable EBER transcripts are detected in freshly isolated, or in IL2-stimulated $CD56^{BRIGHT}$ NK cells - almost certainly indicating absence of infection. For the MOLT4 and Jurkat T cell lines, EBER2 transcript levels are seen on day 1 and day 3 following infection. This equates to approximately 1-2% efficiency of the primary B cell population infected in parallel and suggests small numbers of transiently infected T cells.

In vitro infection of tonsillar T and NK cells: cell transfer and immunological synapse experiments

The data discussed above, together with complementary *in vitro* infection studies by Dr Claire Shannon-Lowe in our laboratory (including engineered expression of a recombinant CD21 molecule in primary NK cells, that facilitated virus binding: Claire Shannon-Lowe, unpublished data 2008), strongly suggested that infection of NK and T cells *in vitro* with cell-free virus was extremely inefficient. Reflecting that this approach was not optimally physiological - NK and T cell infection *in vivo* is perhaps more likely to occur as a result of 'transfer infection'[280] or during immunological encounters[299,693] with B lymphocytes or epithelial cells - we decided to take a more 'physiological' approach.

The first series of experiments involved 'bulk infection' of total tonsil mononuclear cells (ascertained to be EBV-negative or $\leq 10^2$ copies/10^6 cells *ex-vivo*) with the B95-8 strain of EBV at different m.o.i: 10, 50 and 100. A standard infection method was used to allow initial virus binding

to cell surfaces (pages 89-90). Following two days culture (37°C in RPMI 10% FCS supplemented with 175U/ml IL-2), the T-MNC were subjected to high-purity cell sorting by MoFlo™ as for the *ex-vivo* analyses.

Figure 22 shows data from 3 such experiments undertaken with an m.o.i of 10 (Panel A), 50 (Panel B) and 100 (Panel C). Sample A and B were sorted by 3-colour fluorescence into B cells ($CD19^+CD3^-CD56^-$), T cells ($CD3^+CD19^-CD56^-$) and NK cells ($CD56^+CD3^-CD19^-$), whilst for sample C, CD4 and CD8 T cell populations were further delineated. Viral loads were analysed by Q-PCR as before but, for this experiment, data are represented as EBV genome number per cell equivalent. In all three cases a similar pattern of results was seen: the dominant viral load was always seen in the $CD19^+$ population; ranging from 10-300 fold higher than their T or NK counterparts. However in some cases, EBV genome number in the T and/or NK populations was higher than could be attributed to B cell contamination alone. For example, the 98% pure $CD3^+$ population in panel A appeared to contain approximately 10% of the B cell load. Similarly, the viral loads in the CD8 and CD56 fractions shown in panel C exceed that expected from the degree of B cell contamination. Recognising that this could represent viral genomes or DNA adhering to the cell surface, aliquots of the sorted cells were stripped using chymotrypsin (as previously described[280]). This treatment resulted in a 66% reduction in T cell viral load, compared to 33% of B cell virus (data not shown). Moreover, where cell numbers permitted, the purified T cells were maintained in culture following sorting and the viral load was observed to fall by >80% within a week (data not shown). The limitations of this experimental approach then became apparent: the levels of EBV DNA observed in T and NK cell fractions could be potentially explained by cell surface virus or membrane-bound viral DNA, or even small numbers of contaminating high-load B cells.

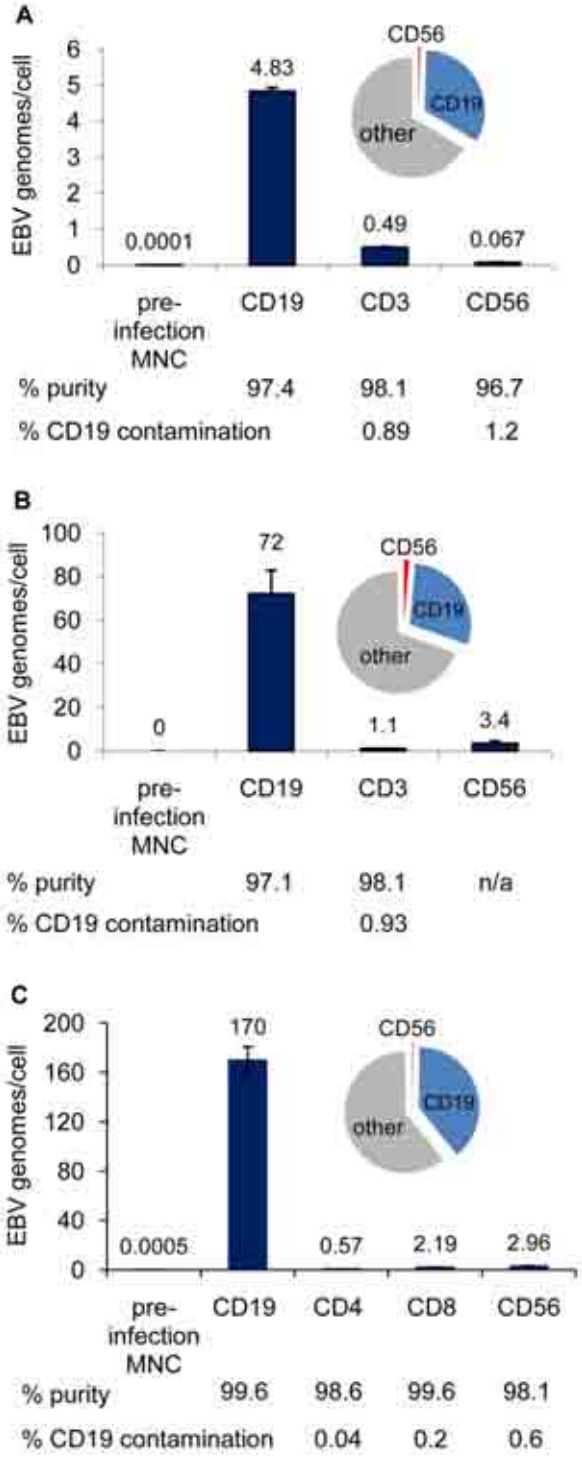


Figure 22 EBV predominantly infects B lymphocytes following 2 days co-culture with tonsil mononuclear cells.

Viral load data from purified tonsillar lymphocyte populations following *in vitro* infection with EBV at an m.o.i. of 10 (A), 50 (B) and 100 (C). Note that y axis is EBV genomes per cell.

We therefore adopted a different approach: maintaining the experimental model of T-MNC co-culture, but on this occasion focusing on evidence of EBV gene expression at the single cell level, by virtue of GFP expression from the recombinant Akata virus. B cells were isolated from freshly harvested tonsil mononuclear cells (using $CD19^+$ Dynabeads^{TM} pages 85-86) and incubated at 37°C with Akata-EBV (m.o.i of 50). Aliquots of these B cells with surface-bound EBV (B/EBV) were either cultured separately or added back to the (B-depleted) T-MNC at ratios approximating to 1: 20 to 1: 2 (ie 5% to 50% B cells amongst tonsillar non-B cells). Parallel cultures with T-MNC infected with cell-free virus were also performed. The cultures were analysed by two-colour flow cytometry from day 3 onwards post-infection, as this is the earliest time of B lymphocyte proliferation and GFP expression following infection with EBV[53]. MAbs to CD19, CD3 and CD56 were used to identify the phenotype of the GFP-expressing, EBV-infected cells.

Figure 23 is a representative summary of such an infection experiment, showing data from days 5, 7 and 10. Panel A is the positive control sample of purified tonsillar B cells (identified by CD19 staining), infected with EBV, and demonstrates emergence of a GFP-positive population in the absence of T cell effectors. Panel B summarises data from co-cultured tonsillar B/EBV with autologous non-B cells at a ratio of 1: 10. The proportion of GFP^+ $CD19^+$ cells emerging in this co-culture is reduced compared to the purified B cell population in panel A. This is likely due to autologous T and NK effectors killing EBV-infected B cells within the culture, via recognition of latent and lytic viral antigens. In spite of this indirect evidence of immunological engagement of T/NK cells with EBV-infected B cells, we could see no clear evidence of GFP^+CD3^+ or GFP^+CD56^+ cells throughout the time-course to day 10. Where occasional GFP^+ events were seen in the CD3-PECy5 or CD56-PE quadrants (panel B), no GFP^+ cells were evident in the corresponding CD19-negative quadrant (panel A); all GFP^+ cells were $CD19^+$. This infers that any apparently GFP^+ T or NK cells were simultaneously engaged with B cells, i.e. conjugate formation. We did not formally prove this by undertaking multi-colour analyses as the evidence for T and NK infection was not convincing or, at most, these were extremely rare events.

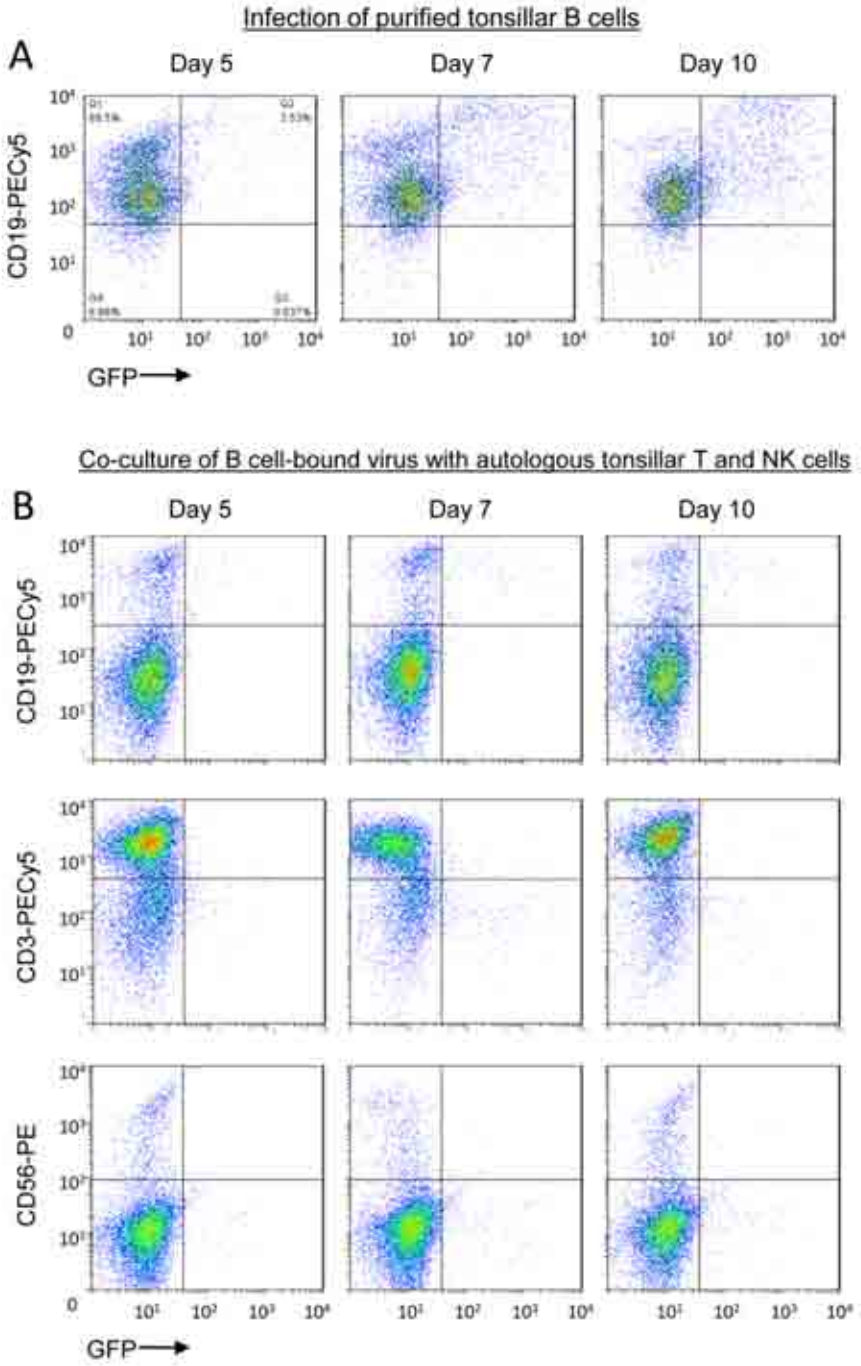


Figure 23 No evidence of T or NK cell infection with EBV despite autologous T/NK-mediated killing of EBV-infected B lymphocytes

Two-colour flow cytometric (compensated) analysis. GFP^+ lymphocyte populations are identified in the top-right quadrant of each individual plot. **Panel A** comprises an analysis of purified tonsillar B lymphocytes 3-10 days following *in vitro* infection, on days indicated. **Panel B** represents parallel cultures of infected B cells co-cultured with the autologous non-B cell tonsillar fraction, to give an initial ratio of 1: 10 (B: non-B).

High viral load in CD56$^+$ tonsillar lymphocytes following ex-vivo culture

The *in vitro* data described above - extremely inefficient, or absent, T and NK cell infection under a range of optimised experimental conditions – could be considered in keeping with the rarity of EBV-driven NK and T cell proliferations *in vivo* and the relative infrequency of T and NK infection events in primary and persistent infection (figures 15-18 and references[246,249]). However, we remained intrigued by our *in vivo* findings in tonsillar lymphocytes from immunosuppressed patients; high EBV genome numbers in CD56$^+$CD3$^-$CD19$^-$ tonsillar, but not peripheral blood, lymphocytes (Figure 19, page 131).

Thus, in an attempt to recapitulate the *in vivo* observation of infected CD56$^+$ tonsillar lymphocytes *in-vitro*, we initially undertook an experiment similar to that already presented in Figure 22; infection of freshly isolated T-MNC (from a healthy donor) using B95-8 EBV at an m.o.i of 50. However, in this instance we also treated the EBV-infected co-culture with 1µg/ml Cyclosporin A (CSA, a calcineurin inhibitor) in order to pharmacologically influence the *in vitro* environment, akin to the Tacrolimus therapy received by our immunosuppressed tonsil donors. As a control, tonsil MNCs from the same donor were cultured in the presence of CSA but without the addition of exogenous virus. As before, the cultured cells were subject to high-purity MoFlo sorting on day 2 followed by DNA extraction and Q-PCR analysis for EBV genome load.

The results did not demonstrate any augmentation of infection of tonsillar T or NK cells in the CSA-treated, EBV-infected T-MNC culture; the viral load data was almost identical to those shown in Figure 22, and as discussed above. However, entirely unexpectedly, the control CSA-treated T-MNC culture showed an extremely high EBV genome load in the sorted CD56$^+$ population; without being exposed to exogenous virus (Figure 24, Panel A, CT1).

There was no evidence of numerical expansion of the gated CD56$^+$ population; these cells amounted to 0.3-0.4% of the total live MNC, similar to - or less than - previous results. This is reflected in the minimal increase in the total MNC load after 2 days in culture; although the EBV

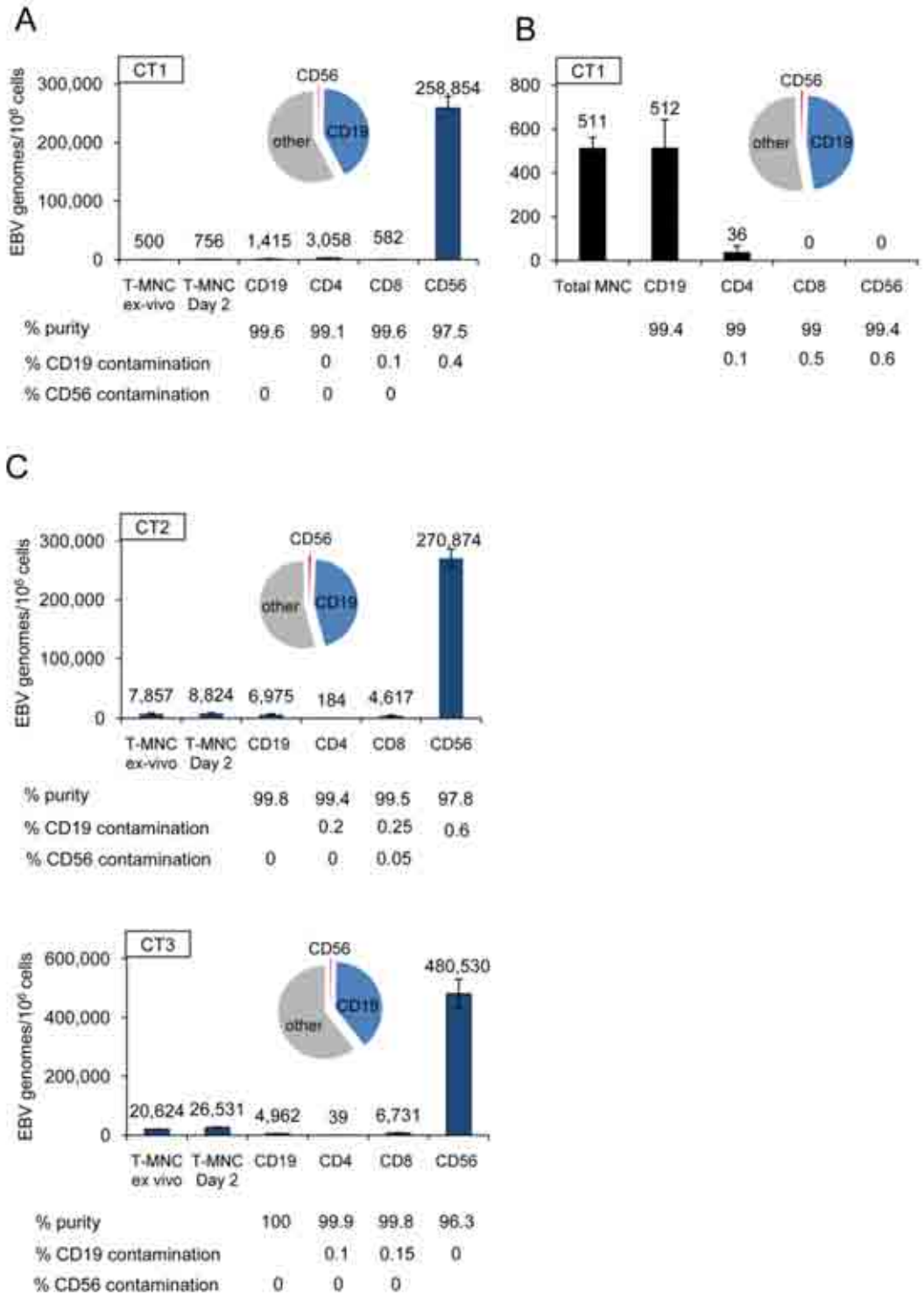


Figure 24 High viral genome load in CD56$^+$ tonsillar cells following *ex-vivo* culture

Panel A is data from a tonsil lymphocyte culture treated *in-vitro* with Cyclosporin A.

Panel B is data from the same tonsil donor as A, sorted immediately ex-vivo, without culture

Panel C shows data from 2 further tonsil donors, cultured without Cyclosporin.

copy number per $CD56^+$ cell is high, the absolute EBV numbers are relatively small when judged against the entire T-MNC population.

To exclude the possibility that the observed phenomenon had already occurred *in vivo* rather than following *in vitro* culture, an aliquot of the original T-MNC from the same donor was recovered from liquid nitrogen and immediately sorted. Panel B (Figure 24) shows the viral load data: exclusively restricted to B cells, reminiscent of the NT results presented in Figure 16 (page 123), and consistent with an *in vitro* phenomenon resulting in the high viral load in the $CD56^+$ cells.

To confirm these data, the same experiment was performed on two further healthy tonsil donors. For CT2 and CT3, an identical method was followed (that is, without exogenous virus) but, for each donor, the effect of CSA was tested by culturing half the T-MNC in the absence of CSA and performing two cell sorts on day 2. Both these donors had higher *ex-vivo* T-MNC viral loads than CT1, but precisely the same distribution of viral load was observed; a very high load in the CD56 population. The data shown in Panel C, Figure 24, are from those T-MNC cultures *not* treated with CSA. This indicated that the observed phenomenon of *in vitro* viral entry, reactivation or replication in the $CD56^+$ fraction was independent of CSA treatment.

Characterisation and re-analysis of EBV-harbouring tonsillar $CD56^+$ lymphocytes

The results described were both striking and unexpected. The phenomenon appeared to be a consequence of *in vitro* culture but it remained unclear why, and how, tonsillar NK cells (apparently EBV negative *in vivo* - panel B, figure 24) should develop such a viral load, whereas efforts at *in vitro* infection with exogenous virus were futile. The first priority was to further characterise the $CD56^+$ population.

Characterisation of CD56-expressing cultured tonsillar lymphocytes

As discussed earlier (introduction, pages 34-35), although there exists no entirely specific NK marker, CD56 has been widely used and the judicious combination of non-NK specific markers, $CD56^+CD3^-$, has been the accepted phenotypic definition[308]. Other cell types known to express CD56 include cytotoxic $CD8^+$ T cells (excluded on our sorting strategy) and muscle and neuronal cells – absent in lymphoid tissue. Malignant diseases such as multiple myeloma are well recognised to frequently express CD56, but normal and reactive plasma cells from blood, bone marrow and tonsil have been shown to be universally negative[694-695].

In the first instance, we used 2-colour FACS to confirm the phenotype of ex-vivo cultured tonsillar $CD56^+$ cells. A range of B cell, plasma cell, T and NK cell markers were used together with CD56. We were initially struck by the emergence of a CD138-expressing population of cells after 24 hours in culture, initially comprising ≤0.5% of T-MNC *ex-vivo* and increasing to 3-8% (according to tonsil donor) over 24-48 hours (Figure 25). Moreover, the majority of these CD138-expressing cells appeared to co-express CD56. CD138 (Syndecan-1) is a plasma cell specific marker, a differentiation antigen that appears after the plasmablastic stage and increases in intensity in concert with plasma cell maturation[695]. This strongly suggested upregulation of the CD56 antigen on a small population of tonsillar plasma cell-like lymphocytes during *in vitro* culture. A majority of these cells also expressed low but appreciable levels of CD19. NK cells are not known to express CD138 and we confirmed the non-NK nature of this emergent $CD138^+CD56^+$ population by demonstrating mutual exclusivity of CD94 and CD138 expression in this same tonsil culture (data not shown).

We were thus prompted to repeat the cell sorting and viral load experiment, applying a different sorting strategy and alternate combination of MAbs.

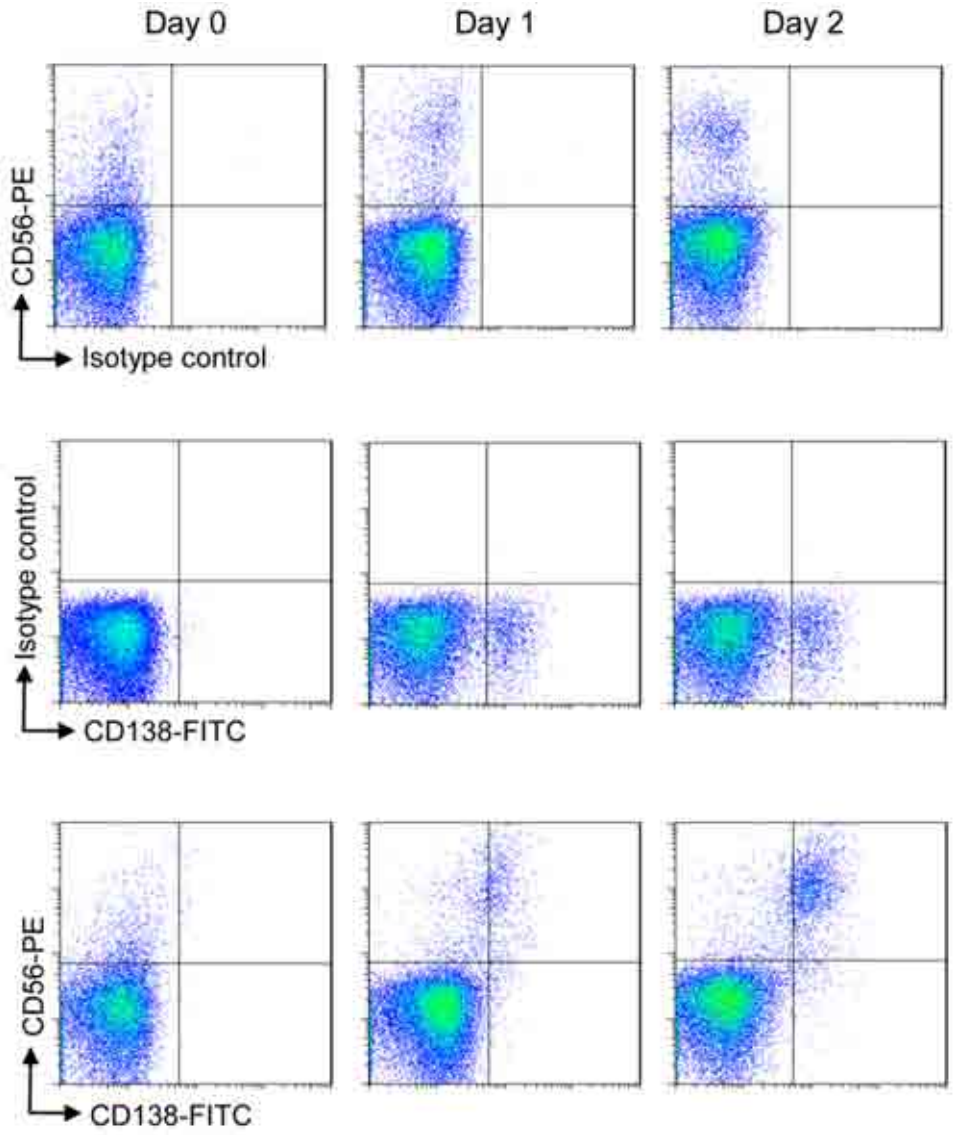


Figure 25 A subset of CD56$^+$ tonsillar lymphocytes co-express the plasma cell marker CD138 following short-duration ex-vivo culture

Flow cytometry analysis (fluorescence-compensated) showing CD138 and CD56 expression on cultured tonsillar lymphocytes from a healthy donor. The top two rows are plots of CD56 or CD138 versus isotype controls, showing the total respective populations. The lower row is a two-colour plot of CD56 and CD138, with cells expressing both markers identified in the top-right quadrant of each plot.

Re-analysis of EBV loads in tonsillar lymphocyte populations *in vitro* and *in vivo*

During the final stages of these investigations, a report was published that described a minor $CD56^+$ population that were not NK cells, but phenotypically and functionally resembled cells from the monocyte/DC lineage[696]. The authors noted that co-expression of CD7, expressed on virtually all NK cells, was the optimal phenotypic marker to distinguish NK cells from this rare DC/monocyte population. With this in mind, we decided to use co-expression of CD56 and CD7 to reliably identify NK cells, whilst the combination of CD138 and CD56 would capture the newly identified 'plasma cell-like' emergent population.

Figure 26 shows the viral load data from such a sort, performed, as before, after 2 days culture (again, without CSA) – using MNC from the CT3 donor denoted in Figure 24 (page 142). The first observation is that the previously striking load in the $CD56^+$ fraction is not evident when co-expression of CD7 is used to capture 'true' NK cells. This $CD7^+CD56^+$ NK fraction comprised 0.8% of viable lymphocytes, in keeping with our previously described tonsil data, and consistent with the published literature[327]. The small viral load now seen in the NK population could be accounted for by 5% B cell contamination and therefore can be regarded as negative. A population of $CD56^+138^+$ cells were again clearly evident, in this case amounting to 6.7% of viable lymphocytes when sorted on day 2. Co-expression of CD19 on these cells was variable: a proportion appeared CD19 negative, whilst some expressed low levels of CD19. These populations were collected and analysed separately and contained equivalent viral loads, greater than that seen in the $CD19^+$-only fraction (Figure 26).

Together these data appeared to provide an explanation for the high EBV copy number in $CD56^+$ tonsillar cells after 2 days in culture; these were not in fact NK cells, but rather, an emergent sub-population of 'plasma-like' B cells co-expressing CD56.

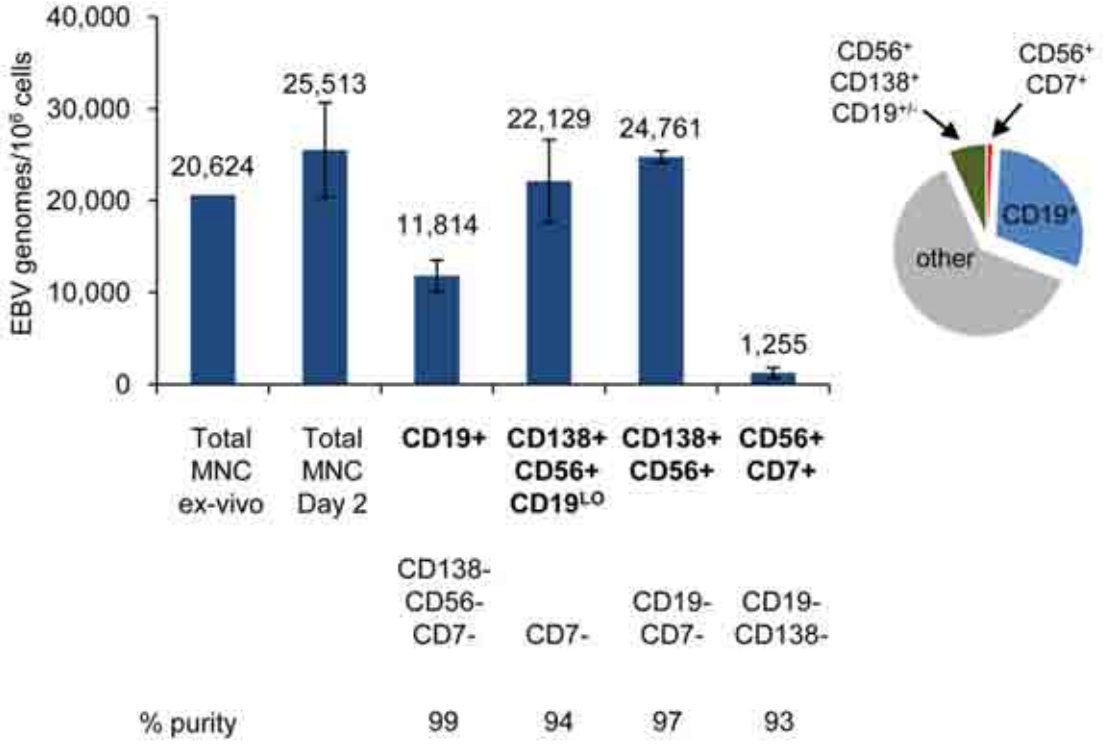


Figure 26 Cultured CD56$^+$ tonsillar lymphocytes with high EBV load are not natural killer cells. Viral load data from total mononuclear and purified tonsillar lymphocyte populations, from a healthy donor, sorted after 2 days of ex-vivo culture. Each population was phenotypically defined as indicated by $^+$ (presence) or – (absence) of a specified markers, as discussed in the main text. The pie chart shows the relative proportions of NK cells (red), B cells (blue), CD56$^+$CD138$^+$ population (green) and other (predominantly T lineage) cells (grey) within the total gated lymphocyte population.
The percentage figures beneath the x axis labels indicate the purity of each respective population on re-analysis.

This prompted reappraisal of our *in-vivo* findings of high genome numbers in $CD56^+$ tonsillar lymphocytes donated by immunosuppressed patients: do these cells, isolated on the basis of $CD56^+CD4/8^-CD19^-$, truly represent NK cells? To address this, we adopted a similar sorting strategy – using CD7 as an NK co-marker - for re-analysis of an IST2 (Figure 19, page 131) immediately following recovery from liquid nitrogen (without culture). Figure 27 comprises viral load data from the sorted populations. By contrast to the cultured tonsil data, initially obtained from a healthy donor (Figures 25 and 26), there appeared to be no substantial population of $CD138^+CD56^+$ cells; these rare cells accounted for 0.03% of viable lymphocytes. However a small population (1.1%) of $CD56^+CD19^+$ cells, not previously appreciated (although contaminated with $CD19^+$only cells), was seen to contain a viral load several times that of the $CD19^+$-only population (Figure 27). NK cells (defined by $CD56^+CD7^+CD19^-CD138^-$) comprised 0.43% of T-MNC and did not contain the substantial viral load previously seen (IST2, Figure 19, page 131); the small load shown could potentially be explained by 7% contamination by $CD19^+CD56^+$ cells. The rare $CD138^+56^+$ cells did not appear to harbour any EBV genomes but this should be interpreted with caution in view of the tiny cell numbers and lack of purity data for this population.

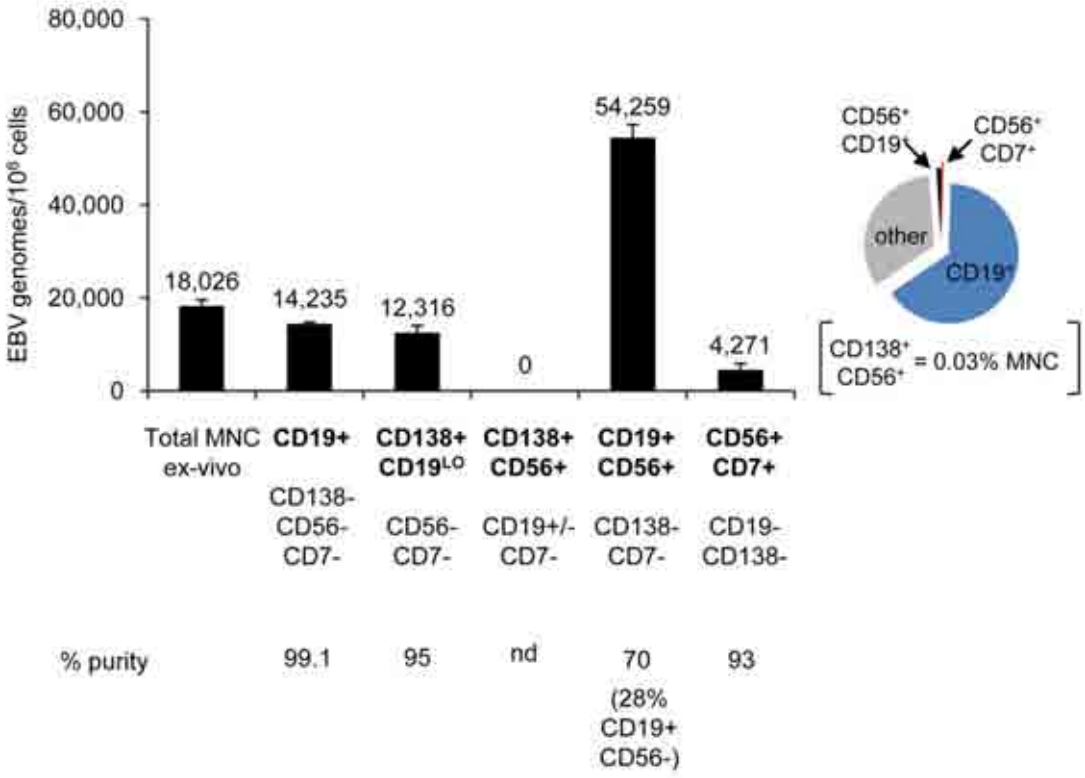


Figure 27 CD56$^+$ tonsillar lymphocytes from immunosuppressed patients with high EBV genome copy number are not natural killer cells.

Viral load data from total mononuclear and purified tonsillar lymphocyte populations, from immunosuppressed donor IST2, sorted *ex-vivo* without manipulation/culture. Each population was phenotypically defined as indicated by $^+$ (presence) or – (absence) of a specified markers, as discussed in the main text. Values are expressed as EBV genomes per 10^6 cells. Numerical values above each histogram indicate the mean value of Q-PCR performed in triplicate, with standard deviations as indicated by capped vertical bars. The pie chart shows the relative proportions of NK cells (red), B cells (blue), CD56$^+$CD19$^+$ population (black) and other (predominantly T lineage) cells (grey) within the total gated lymphocyte population. The percentage figures beneath the x axis labels indicate the purity of each respective population on re-analysis. nd = purity not determined due to insufficient cell numbers.

Discussion (I)

EBV-HLH presenting in adult patients in the UK

Clinical aspects

A rare disease[374], EBV-HLH occurs most commonly in the context of primary EBV infection[240,485-486,490] and, accordingly, is predominantly encountered in immunocompetent children and adolescents[498-500] where it is the commonest cause of HLH[374,487-488]. By contrast, rare adult cases of HLH usually have an underlying cause other than EBV infection, usually attributable to malignancy and most commonly lymphoma[374,501]. Furthermore, most reported cases are described in persons of East Asian ethnicity[499] although occurrences in patients of European[240,494], Middle Eastern[492], North American and Hispanic[245] origin have been described.

Our description of three cases of EBV-HLH occurring in adult patients in the UK highlights some uncertainties regarding disease pathogenesis. In common with more than 90% of adults worldwide, our three cases had serological evidence of prior EBV infection although their T-cell specific immunity was not tested. Whether acquired immune dysfunction is required to trigger the onset of EBV-HLH in EBV-immune adults is not clear. Although well-defined inherited immune defects[502,508] are recognised to predispose to EBV-HLH, most patients are apparently 'immunocompetent'[501]. In the context of iatrogenic immunodeficiency, CMV appears to be a more frequently implicated agent, although EBV-HLH has been reported and can be associated with a poor outcome[511]. In our cases, HLH-1 had no history to suggest immune dysfunction, HLH-2 was receiving therapy with 5-aminosalicylic acid (mesalazine) for inflammatory bowel disease (previously treated with Azathioprine) and HLH-3 was subsequently diagnosed with EBV^{+} Hodgkin's Lymphoma (mixed cellularity subtype). This has recently been recognised to be associated with HLH[697], although EBV infection of NK cells in the context of HL has not been previously described. Thus, an influence of relative immunosuppression in some cases may be

contributory, but this issue requires formal investigation including analyses of EBV-specific T cells.

We have also been aware of three further cases of EBV-HLH in adults from the UK Midlands region between 2007 and 2009, two of which were associated with EBV-positive HL in the context of HIV infection. In these cases, the clinical features of HLH were quite distinct from the HL and likely due to a coincident T or NK infection (as in patient HLH3), although we were unable to investigate these additional patients using the methodology described here. The remaining case was more 'typical', immediately following symptomatic primary EBV infection in a young adult.

The high mortality in EBV-HLH is attributable, at least in part, to delays in diagnosis - the initial clinical presentation can be almost indistinguishable from a wide range of infective and inflammatory conditions[487], a difficulty often compounded by the apparent absence of haemophagocytosis on initial biopsy. Although undoubtedly a rare disease, we believe that the incidence in adults in western countries is underestimated. The criteria established by Henter et al[476], alongside a high clinical suspicion, remain key to diagnosis. Delineating the phenotype of the virus-harbouring cell to inform the application of targeted therapies[698] is an attractive concept, but requires larger collaborative studies of adults and children with EBV-HLH.

EBV-infected NK cells in HLH

Published data has shown that $CD3^+$ T cells, most often the $CD8^+$ subset, both in tissue biopsies[240,245,464-465,467,469,493] and circulating lymphocytes[471,494], are the dominant infected population in EBV-HLH. Focusing on studies examining peripheral blood lymphocytes, Kasahara *et al* relied upon semi-quantitative EBER in-situ hybridisation[471] and, consistent with the majority of reports, identified $CD8^+$ T cells as the dominant target cell. The same study identified a smaller number of EBER positive $CD16^+$ cells seen but the cells were not co-stained with CD3 to delineate between cytotoxic T cells and NK cells. Whilst our study of HLH patients was ongoing, a report was published describing 2 adult cases of EBV-HLH which stated that one case involved infection of NK cells – but details of data supporting this finding were not provided[699].

Interestingly, a relatively large study of non-Asian children with EBV-HLH used Q-PCR on purified peripheral blood lymphocytes and found the dominant viral load in $CD19^+$ cells, followed by $CD3^+$ T cells. However, the median total PBMC load exceeded the median CD19 load, suggesting an unidentified population. It is notable that NK cells were excluded on their isolation protocol[494].

The advantages of our approach of investigating EBV-HLH cases are the use of three-colour flow cytometry to clearly define peripheral lymphocyte populations (Figure 11A, page 115), in conjunction with a sensitive Q-PCR assay. Applying the conventional definition of a NK cell, $CD56^+CD3^-$[308], two of three cases we investigated had a high viral genome load in circulating NK cells (Figure 11B, page 115). For HLH1, although we cannot be absolutely certain of the identity of the $CD3^-CD16^-CD19^-$ small lymphocyte population – we believe this most likely represents the $CD56^{BRIGHT}$ NK subset. That our data is the first to clearly show peripheral blood $CD56^+$ cells as the dominant EBV target may be a 'real' finding - for example relating to host immuno-genetic factors or acquired immune-deregulation - or may have simply become apparent because this cell population was not specifically addressed in previous studies.

As a result of our subsequent findings of $CD56^+$ cells of B lymphoid origin in tonsils both *in vitro* and *in vivo* (discussed later) we reflected on whether the infected cells in our HLH patients were indeed NK cells. Unfortunately there was no remaining primary material to address this directly; for example by looking for B-cell specific mRNA or Ig VDJ rearrangements. However, the circumstantial evidence weighs against a B cell origin in our HLH patients. Firstly, in contrast to the tonsil data, we did not find significant levels of virus in peripheral blood $CD56^+$ cells *ex-vivo* in IM or immunosuppression. Secondly, again in contrast to tonsillar cells, when we cultured PBMC from a healthy donor and performed the same cell sort on day 2 we did not find any virus in the $CD56^+$ compartment; the viral load data were akin to that seen in *ex-vivo* PBMC (one experiment, data not shown). Thus it seems likely that the $CD56^+CD3^-$ cells in the HLH patients are of NK origin, but future analyses should attempt to confirm this with cellular RNA or DNA studies and by applying CD7 as a co-marker alongside CD56 to accurately identify NK cells.

Available data on viral gene expression in the context of EBV-HLH remains ill-defined[122]. In two of our cases, sufficient cell numbers were available for RNA extraction, albeit from total PBMC rather than separated lymphocyte subsets. This could potentially result in interpretative difficulties if any EBV-infected B cells present were transcriptionally active[122]. Nonetheless we interpret our data as meaningful because PBMCs were devoid of B cells in HLH-1, and for HLH-2 the viral load in the NK cell fraction was a log higher than in the B cells. Our results showed an apparently tightly restricted pattern of viral gene expression with high levels of EBERs exclusively expressed – greatly exceeding transcript levels seen in an LCL - but an absence of protein-encoding transcripts (Figure 12A and B, page 116). The significance of this observation is unclear but may be of relevance to a recent study suggesting a role for EBERs, present in serum from patients with HLH, in disease pathogenesis[198].

EBV genome load in blood and tonsillar lymphocyte subsets in health and immune dysfunction

Studies in primary infection and healthy persistence, predominantly based on immunohistochemistry combined with EBER ISH for identification of EBV-infected cells, have in some instances demonstrated rare T[229] and NK[230,246,249] cell infection in tonsillar tissue, whilst other studies have not[230,247-248]. The limitations of these studies, as discussed (pages 27 and 34, introduction), include imperfect sensitivity and specificity of cell lineage markers, usually applied singly rather than in combination and thus further limiting specificity. For NK cell identification, the NK antigen PEN5 has been used[246,249], but this marker is not usually expressed on the $CD56^{BRIGHT}$ population that dominates in tonsillar tissue[259]. Moreover, the sensitivity of this semi-quantitative approach is limited by virtue of the manual approach of cell counting via a microscope.

Our approach of high-throughput, high-purity cell sorting of (typically) $\geq 10^8$ viable tonsil lymphocytes followed by a Q-PCR method able to detect as few as 2 EBV genomes in a background of 10^5 cells offered clear advantages, although it lacks evaluation at the single-cell

level. The lack of a sensitive and specific NK marker was addressed by using a combination of MAbs consistent with the customary phenotypic definition ($CD56^+CD3^-CD19^-$). Using four-colour cell sorting to isolate specific monofluorescent populations allowed us to achieve consistently high purities of total tonsillar NK cells and the major T cell subsets.

Normal tonsils

Our analyses of tonsils donated in the context of healthy persistence (Figure 16, page 123), albeit on a background of recurrent tonsillitis, allowed three main observations to be made. The first related to the discrepancy between T-MNC and CD19 viral loads, which is discussed below in the context of IM. The second observation was the occasional, albeit low level, finding of EBV genomes in the CD4 and CD8 T cell populations. Although, where seen, this was not explicable by CD19 contamination, definitive conclusions cannot be drawn. In particular, questions remain about whether this could be surface rather than intracellular virus and, importantly, how this data would appear at the single-cell level. The presence of surface virus however seems less likely when the $CD56^+$ population is considered, serving as an internal control; consistently negative for EBV genomes in NT. On this note, an immunohistochemical study[249] of routine tonsillectomy sections found frequent instances of conjugate formation between $EBER^+$ cells (of plasmacytoid morphology) with both CD4 and CD8 T cells. By virtue of forward scatter and side scatter criteria, our cell sorting strategy should, in principle, have excluded cell conjugate formation. Nonetheless this observation could be relevant to our findings, considering the transfer of cell membrane and associated receptors that can occur during immunological encounters[299,693]. Lastly, in our normal tonsils we found no evidence of EBV in the $CD56^+$ population.

Infectious mononucleosis

In the context of symptomatic primary infection, a previous study adopting a similar experimental approach included healthy children with acute symptomatic EBV infection. In these cases the viral load was confined to the CD19 cells with no evidence of CD4 or CD8 infection, although NK cells were not examined in this study[700]. Moreover, it was unclear whether those studied represented *bona fide* IM cases.

In our analyses, the diagnosis of IM was unequivocal – on the basis of characteristic symptoms and a positive Monospot test – blood was donated by 3 such cases at the height of the illness and 2 cases in the early convalescent phase (Figure 17, page 125). In these individuals the virus was almost exclusively restricted to $CD19^+$ B cells; small numbers of EBV genomes in the NK fractions could potentially be explained by 2-3% contamination by 'high-load' B cells, although by CD19 staining this was not manifestly the case. Thus, it remains unclear whether this represents a rare infection event in circulating NK cells or simply contamination by an undefined B cell population.

Turning to IM tonsils (Figure 18, page 127), we were initially struck by the finding of marked discrepancies in viral load between total MNC (harvested for DNA extraction immediately following recovery from liquid nitrogen) and the sorted CD19 population. In three cases this amounted to a 3-20 fold higher value in T-MNC and was much more marked in IMT than seen in NT. In one case a comparator EBV load was also measured in the T-MNC population after density centrifugation (Lymphoprep^{TM}) but prior to sorting. The load in this viable MNC population was approximately one-third lower than in the original ex-vivo MNC (data not shown) and suggests that dying/dead cells contributed proportionally more to the viral load value. With this in mind, another explanation may be the inadvertent exclusion of $CD19^{DIM}$ B cell populations (potentially containing higher EBV copy numbers) with this sorting strategy; which gated on the $CD19^{HIGH}$ cells in the interests of achieving high purities. An alternative but related explanation could be the demise of lytically-infected B cells during the sorting process.

In most analysed IM tonsils, we found a 'low level' viral load in the $CD4^+$ and/or $CD8^+$ T cells that could not be numerically accounted for by contaminating B cells (discussed under NT – above). However, in one case (IMT4, Figure 18, page 127) the $CD8^+$ fraction contained a substantial viral load, approaching the $CD19^+$ value. We do not have data at the single-cell level; in principle this could equate to 1 in 10 $CD8^+$ cells infected with an EBV genome or the presence of considerably fewer cells participating in replicative infection. $CD8^+$ T cells are greatly outnumbered by their $CD4^+$ counterparts in normal and IM tonsils so there were insufficient purified $CD8^+$ cells to

analyse viral gene expression in this population. However RT-Q-PCR analysis of RNA from $CD19^+$ cells in IMT4 (Figure 18, page 127) showed transcriptional evidence of full lytic cycle in the tonsillar B cell compartment and thereby susceptible to attack by lytic-epitope specific $CD8^+$ T cells[382]. Thus, in the case of IMT4, infection of the $CD8^+$ cells could be rationally explained immunological encounters with lytic cells. The subsequent fate of this infected $CD8^+$ population, however, remains challenging to determine in an *in vivo* context.

Lastly, we were interested to see apparent infection of $CD56^+$ cells (in 3 of 5 IM tonsils examined), equivalent to 10-30% of the corresponding $CD19^+$ load (IMT1, 3 and 4, Figure 18, page 127). Although these loads could not be accounted for by the level of CD19 contamination, uncertainties relating to the cause of the discrepant MNC/CD19 load caution that the CD56 data should be interpreted carefully; for tonsils IMT1 and IMT3, 1-2% contamination of an uncharacterised cell present in the T-MNC could potentially account for the CD56 viral loads.

Immunosuppressed patients

Both inherited and acquired, numerical and functional, defects of T cell immunity have long been recognised to substantially increase a person's risk of developing EBV-driven B cell lymphoproliferation[405-406,701-702]. A previous study has shown that EBV-harbouring memory cells, rather than lymphoblasts, accumulate in the peripheral blood of immunosuppressed patients[160]. For NK and T cell lymphoproliferations, usually seen to arise in the context of apparently intact T cell immunity, no data is available on the occurrence of EBV-infected T and NK cells. One recent report focused on HIV-infected children established on antiretroviral therapy, taking a similar approach of cell sorting and EBV genome quantitation in $CD19^+$, $CD4^+$ and $CD8^+$ peripheral blood fractions[700]. In a majority of patients assessed, there was convincing evidence of EBV in the highly purified $CD4^+$ and $CD8^+$ subsets, although the median T cell viral loads were 5-15 fold lower than of the comparator $CD19^+$ subset (NK cells were not assessed). Interestingly, given that participants' median CD4 counts were 500/μl, their level of immunosuppression would be considered relatively 'mild'. In fact, these viral load data are reminiscent of the viral load distributions we have described in our cases of NT and IMT.

We had the opportunity to analyse tonsillar lymphocytes donated by four paediatric patients who had previously undergone allogeneic solid-organ transplantation and were consequently receiving T cell suppressive therapy with Tacrolimus, a calcineurin inhibitor. Using the same sorting strategy as for NT and IMT, we were struck by the unexpected finding of high genome numbers in the non-B, non-T, $CD56^+$ fraction (Figure 19, page 131). Although this is a numerically minor (0.3-1%) population amongst tonsillar lymphocytes, on a 'per cell' basis this population harboured the dominant viral load. By contrast, in 2 cases where matched peripheral blood samples were available, the same phenomenon was not apparent; these data were indistinguishable from normal peripheral blood.

An important question was whether this phenomenon was due to adherent surface virus or viral DNA, rather than true infection. We attempted to answer this question by staining purified $CD56^+$ cells (from IST3, Figure 20, page 133) for EBER expression at the single-cell level and found that only a tiny minority of cells were $EBER^+$ (data not shown). We considered whether this could relate to the occurrence of lytic cycle, explaining the high viral load and shown to result in EBER downregulation[193]. In the same $CD56^+$ population, we identified only a single cell with evidence of lytic replication including detection of a late viral capsid protein. However this finding should be interpreted in the context of recent studies in our laboratory, where Q-PCR measurements of viral load in induced AKBM cells[703] have been undertaken. At both the single cell level and in sorted lytic populations, the estimated viral load is in excess of 10,000 copies per lytic cell (Dr A.I Bell personal communication, 2010). Thus, very small numbers of lytic cells amidst an EBV-negative (or latently infected) population could potentially explain our findings. However, we note an immunohistochemical study of 15 tonsillectomy samples from immunosuppressed patients that did not reveal any BZLF1 expression, or any substantial numbers of EBV-transformed cells[704].

Nevertheless, although this issue was not yet entirely resolved and with these provoking data in mind, we moved on to complementary studies investigating *in vitro* EBV infection of T and NK cells.

EBV infection of T and NK cells *in vitro*

We began by aiming to reproduce the findings of the only published study that has accomplished efficient *in vitro* infection of NK (leukaemia) lines and primary NK cells[301]. We extended these experiments to include IL2-stimulated $CD56^{BRIGHT}$ primary NK cells and applied sensitive RT-Q-PCR assays for EBER expression to look for evidence of infection. No clear evidence of NK cell infection was seen (Figure 21B, page 135), whilst B cells from the same donor were readily infectable, displayed the co-ordinated transcriptional program of latent gene expression and expressed the anticipated levels of EBERs (Figure 21A, page 135). Low levels of EBERs were evident after 24-72 hours in the MOLT4 and Jurkat T Cell lines but this was not sustained; presumably the virus was lost or the cells died by apoptosis.

As discussed in the introduction of this thesis (page 31), NK cells do not express CD21 and the mechanism of viral entry is entirely unclear. It is reasonable to speculate that *in vivo* infection could occur via immunological synapse formation with latent or lytic target cells. Indeed, conjugate formation between $PEN5^{+}$ and $EBER^{+}$ tonsillar cells are readily seen by immunohistochemistry in healthy viral persistence[249].

We therefore adopted a more physiological approach and undertook a series of tonsillar co-culture experiments to provoke T/NK immunological encounters with newly-infected, EBV-transformed autologous B cells. The first set of experiments, involving 'bulk infection' of tonsillar MNC followed by cell sorting on day 2, suggested a minority of T and NK cells may be infected (Figure 22). However, EBV Q-PCR was unable to delineate between adherent virus/viral DNA and genuine infection. Thus we changed our experimental approach to incorporate a more convincing indicator of infection at the single-cell level. However, despite seeing evidence of B cell infection, proliferation and subsequent containment of outgrowth by tonsillar T/NK effectors, we were unable to demonstrate unequivocal T or NK infection (Figure 23, page 140). This assay assumes that GFP will be expressed, as in B cells, if the recombinant Akata virus enters NK or T cells; as was reported in the study of Isobe et al[301].

Prompted by our *in vivo* data from IST (Figure 19, page 131), we next tested whether pharmacological impairment (via calcineurin inhibition) of T/NK function would provoke susceptibility to *in vitro* infection. This effect was not achieved, but serendipitously led to the unforeseen result of high viral loads in cultured $CD56^{+}$ tonsillar lymphocytes, independent of CSA.

Noting a recent paper that demonstrated a differential effect of calcineurin inhibition on NK cell subsets, where $CD56^{BRIGHT}$ cells appeared to display resistance to this drug compared to their $CD56^{DIM}$ counterparts[334], we considered whether such treatment may result in a reliance on $CD56^{BRIGHT}$ NK cells for containment of B cells transformed by EBV[363] or cells in lytic cycle[298]. However the magnitude of the genome load per cell, occurring within 48 hours of culture impelled us to better characterise the $CD56^{+}$ cells.

Characterisation and analysis of EBV-harbouring tonsillar $CD56^{+}$ lymphocytes

As discussed earlier (NK cell phenotype, page 34) CD56 is expressed across NK subtypes and, together with an absence of CD3 (or CD4 and CD8), can accurately identify NK cells *in vivo* and *in vitro*, particularly in tonsillar tissue[308]. However, CD56 is not NK-specific; expression on other lymphoid cells, including cytotoxic T cells[314] and malignant plasma cells[705] is seen. Previously, comprehensive analyses of bone marrow, blood and tonsillar tissue have demonstrated an absence of expression on normal and reactive plasma cells[694-695].

Notwithstanding this, our analyses of tonsillar lymphocytes *ex-vivo* have shown that within 24 hours of culture, a population of cells expressing the plasma cell differentiation antigen CD138 emerges that co-express CD56 (Figure 25, page 145). Such cells also manifest low surface levels of CD19 but not CD94, implicating their origin as B cell and not NK cell. This finding of aberrant CD56 expression on a population of tonsillar, plasma-like B cells provided a potential explanation for our viral load findings in cultured lymphocytes. Indeed, previous work has shown that *in vivo* differentiation of healthy tonsillar B cells into plasma cells (as defined by a $CD38^{HIGH}CD20^{LOW}$

phenotype) initiated viral replication[212]. In response to data from a paper published during the later stages of this work[696], we adapted our sorting strategy to identify NK cells with greater specificity (i.e. co-expression of CD7 and CD56) and also to delineate the newly identified $CD56^{+}CD138^{+}$ population. The viral load in the 'true' NK population was negligible (figure 26, page 147) in distinct contrast to initial results from the same tonsil; amounting to <1% of the originally observed genome load (CT3, Figure 24, page 142). This confirmed our suspicion that the cultured $CD56^{+}$ cells with a high genome load were not NK cells. However, although the viral load in the cultured $CD138^{+}CD56^{+}$ fraction was higher than that in the 'standard' B cell population (irrespective of CD19 expression), this was still lower than anticipated when considering the magnitude of the viral load in the initial $CD56^{+}$ population. This raised the question of whether the original $CD56^{+}$ cells with a high genome load had been excluded on the new sorting strategy. As we were restricted to collecting 4 populations of cells, defined by combinations of 4 different fluorochromes, clearly there would be 'lost' populations. Such relevant populations may include: $CD19^{LOW}CD56^{+}138^{-}$ or $CD19^{+}56^{+}138^{+}$. Nevertheless, the fundamental conclusion from these data is that a lymphocyte of B/plasma-cell phenotype, co-expressing CD56, is the likely identity of the EBV-harbouring cell.

As to the original findings in tonsillar $CD56^{+}$ cells from immunosuppressed patients (Figure 19, page 131), reanalysis showed that the viral load in the NK cell population, now defined by $CD56^{+}CD7^{+}$, was negligible. In this instance, a $CD56^{+}CD138^{+}$ population was much less prominent than in the cultured T-MNC, but a discernible $CD19^{+}CD56^{+}$ population was also collected and contained at least 4x the viral load of the $CD19^{+}$-only population (figure 27, page 149). Notably, a population of plasma cells (as defined by $CD19^{LOW}CD138^{+}$) had a viral load equivalent to a $CD19^{+}$ B cell, although a lack of data at the single-cell level limits interpretation here. Consistent with the *in vitro* findings, these data suggest the existence of a hitherto undefined $CD56^{+}$ population of B cell origin. Such cells appear to contain higher numbers of EBV genomes than a conventionally-defined B cell and, although the data in Figure 20 (page 133) lends support to the notion that this could be due to viral replication, this remains to be definitively

proven. In retrospect, examining the flow cytometric plots of previously analysed tonsillar lymphocytes *ex vivo* often reveals a small but definite population of cells co-expressing CD19 and CD56 (Figure 13A, page 118) – as was the case for IST2.

As mentioned, CD56 is thought to be absent on normal plasma cells but almost always expressed in the setting of malignant myeloma[695]. Interestingly, myeloma cells circulating into the peripheral blood usually lack CD56, whereas myeloma cells in bone marrow or pleural/ascitic effusions clearly express CD56[695,706]. However, analogies with myeloma are less relevant to the clinical setting in which we made our initial observation of high genome load $CD56^+$ cells. Such T cell-impaired patients are at risk of PTLD, not infrequently seen in tonsillar tissue[707] and often displaying evidence of plasmacytic differentiation[408].

During preparation of this thesis, a study was published that combined surface immunofluorescence and EBER ISH ('immuno-FISH') to investigate the phenotype of EBV^+ cells in the peripheral blood from a range of patients, including those with HIV-infection and following HSCT[708]. Consistent with our findings in peripheral blood, these analyses could find no EBV genomes in an identically-defined NK population ($CD56/16^+$ $CD3^-$). Using detection of intracellular κ and λ Ig light chains combined with an absence of CD20 expression to define plasma cells, the authors reported frequent EBV infection of these cells in the peripheral blood. Notably, the genome load per cell was actually lower than their B cell counterparts. Interestingly, a population of undefined EBV-harbouring cells was identified in a majority of patients; negative for T-cell, B-cell, and monocyte markers. This is reminiscent of an immunohistochemical study of EBV^+ tonsil sections in healthy persistence where 11% of $EBER^+$ cells were of unconfirmed phenotype. Although their morphology and occasional expression of $CD20^{LO}$ suggested they were of plasmacytoid in nature, this was not confirmed; no $CD138^+EBER^+$ cells were seen[249]. A similar phenomenon has been reported in IM, where a substantial proportion of cells could not be clearly assigned a B or T lineage, using CD20, CD79a, CD3 and CD45RO as markers[247].

These data are pertinent to our findings, where two main issues remain to be resolved. The first relates to the frequency, phenotype and significance of tonsillar-derived $CD56^+$ B lymphoid cells both *in vivo,* where co-expression of CD19 identifies them as of B-lymphoid origin and *in vitro,* where a population of $CD138^+CD56^+$ cells emerges rapidly during ex-vivo culture. Such an investigation would include the application of a wider panel of B cell and plasma cell markers, comprehensive analysis by multicolour flow cytometry and immunohistochemical staining of immunosuppressed tonsil sections. This interesting work is beyond the scope of this thesis. The second area to be addressed relates to the nature of EBV infection in these $CD56^+$ cells: the genome load per cell; whether this is lytic or latent infection; and what pattern of EBV gene expression is manifest.

Amongst these unanswered questions, we can summarise our investigations of *in vivo* and *in vitro* infection of NK and T cells by drawing the following conclusions: EBV infection of peripheral blood NK and T cells appear to be extremely rare events in healthy persistence, primary infection and immunosuppression. In tonsillar T cells, our data is consistent with infrequent infection events in health and immune-deregulation (with the exception of 1/5 IM cases where a high CD8 load was seen). Convincing evidence for infection of tonsillar NK cells was absent amongst our patient groups, and particular caution should now be attached to the conventional NK phenotypic definition of $CD56^+CD3^-$; a previously unrecognised population of $CD56^+$ tonsillar B lymphoid cells, associated with high EBV loads, has opened up a new avenue of investigation.

Taken together with the inability to infect NK and T cells *in vitro* with any degree of efficiency, our data lead us to speculate that the development of T/NK lymphoproliferative disease is likely to be 'an accident' of a rare *in vivo* infection event. However, the relative contribution of viral gene expression and cellular genetic changes (whether pre-existing, coincidental or EBV-induced) remain to be clarified. The lack of a good experimental model of NK and T cell infection, coupled with the rarity of such events *in vivo* in the absence of disease, severely hampers scientific progress in this area. Thus, it is likely to be more informative (at least in the short-term) to

examine cells and tissues from patients with established T/NK disease in order to better understand both disease pathogenesis and basic biology of these rare cellular-viral interactions.

Results (II): EBV gene expression in NK and T cell malignancies and studies with LMP1

Introduction

Published data on EBV gene expression in tumours of NK and T cell origin is limited to a small number of studies[73,564-565,610,614] (and pages 59-60, 67-68 and 74-75). Moreover, studies examining the impact of EBV-encoded gene products on cellular phenotype have not been forthcoming; their role in pathogenesis remains unclear. We began this chapter of work by characterising the pattern of gene expression in CAEBV and ENKTL and in particular wished to focus on the role of LMP1 in pathogenesis of NK cell disease. This EBV-encoded, *bona fide,* oncoprotein[86] is known to induce pleiotropic effects in B cells and epithelial cells in which it is thought to contribute substantially to the malignant phenotype of corresponding tumours[439]. By contrast, studies investigating LMP1 function in T/NK cells are scarce; limited to isolated *in vitro* studies in an EBV-negative T lymphoma cell background[120-121]. No data exist on its contribution to NK cell phenotype or lymphoma pathogenesis.

Although considerable time was invested in this area of study, we were severely hampered by technical difficulties relating to gene transfection/transduction in primary NK cells and NK cell lines. Ultimately, a useful experimental model to examine the effects of LMP1 in NK cells was established but, due to time constraints and prioritisation of other areas of study (see Results part III), no definitive phenotypic data was obtained. Nonetheless, this work facilitated important technical progress regarding the expression of viral genes in primary human NK cells and resulted in a cell model that can be directly taken forward for analyses of global gene expression changes and functional studies.

	SNK 6	SNT 8	SNK 10	SNT 16
Patient age at diagnosis (years)	62	48	17	13
Primary tumour	ENKTL	ENKTL	CAEBV	CAEBV
CD56	positive	positive	positive	negative
sCD3	negative	positive	negative	positive
TCR complex	-	γδ	-	αβ
TCR gene	germline	rearranged	germline	rearranged
EBV TR	clonal	clonal	clonal	clonal
Cytogenetic abnormalities	complex, incl. 6q abnormality	complex incl. 6q abnormality	complex	complex

Table 7 Characteristics of SNT and SNK cell lines established from ENKTL and CAEBV
Adapted from references[562,709]

Expression and regulation of EBV-encoded genes in T/NK lymphoproliferations

Published data, based on both primary material[73,564-565,610] and cell lines established from patients with ENKTL and CAEBV[562], are consistent with a restricted 'Latency II' pattern of viral gene expression. However, interpretation of these data are somewhat limited by the lack of quantitative analyses at the mRNA level. We therefore began by characterising a number of IL-2-dependent T/NK cell lines, originally established from Japanese patients and kindly provided by Dr Norio Shimizu, Tokyo, Japan. A summary of the clinical and phenotypic characteristics is shown in Table 7, with data derived from the original cell line descriptions[562,709].

Figure 28A shows Western blot data of cell lysates from these four cell lines, compared to a LCL control and an EBV-negative NK leukaemia line (NKL[303]) and figure 28B represents RT-Q-PCR results. As anticipated, and consistent with a restricted pattern of latent gene expression, EBNA2 is absent from all T/NK lines. Expression of EBNA1 protein is seen to be substantially lower in all four T/NK lines compared to a LCL, corroborated by proportionally lower levels of Qp-initiated transcripts. The difference in EBNA1 protein size is attributable to the variable Gly-Ala repeat domain[21].

LMP1 protein levels of the two ENKTL tumour lines (SNK6 and SNT8) greatly exceed that of a LCL, whilst the two CAEBV-derived lines exhibit considerably lower levels. Applying primers within conserved sequences spanning the exon 2–exon 3 junction region (identified by sequence analysis of a panel of virus isolates from different geographical regions[133]), initial Q-PCR assays for LMP1 mRNA were entirely negative. This was resolved by using an alternative probe[133] (based on the Chinese[25] rather than B95-8[21] sequence) for assaying the T and NK lines, whilst the standard probe was maintained for the LCL. We confirmed the presence of a corresponding polymorphism in the SNT and SNK lines by sequencing of LMP1 exon 2 and 3. No evidence of further sequence changes relevant to the PCR primers/probes were seen (data not shown).

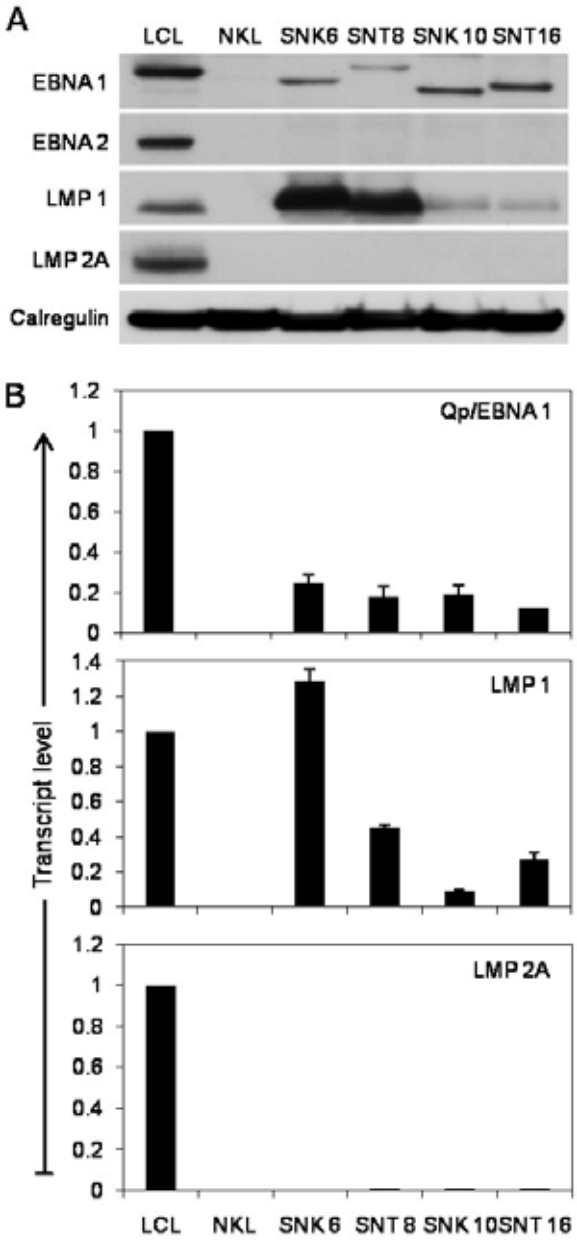


Figure 28 ENKTL and CAEBV cell lines display a Latency II pattern of viral gene expression
Panel A is a Western blot probed with antibodies to EBNA1, EBNA2, LMP1, and LMP2A compared with a LCL and an EBV-negative NK line (NKL). Calregulin served as a loading control.
Panel B shows RT-Q-PCR results for Qp-initiated EBNA1 and spliced LMP1 and LMP2A transcripts. All data is normalised to GAPDH transcript levels. Data for Qp is expressed relative to the BL line Rael, whereas LMP1 and LMP2A are relative to an a LCL, assigned a value of 1

Interestingly, the magnitude of difference in LMP1 protein levels between cell lines are not precisely mirrored at the mRNA level. SNK6 LMP1 transcript levels are only marginally higher

than the comparator LCL despite a much greater difference seen at the protein level. More strikingly, SNT8 LMP1 mRNA levels are lower than the LCL, whilst the protein appears more highly expressed.

With regard to LMP2A, using a monoclonal antibody to the N-terminus of the protein, no expression was evident in any of the 4 T/NK lines, concurring with a virtual absence ($\leq 10^{-3}$ of a LCL) of spliced LMP2A transcripts. This issue is explored further in the next chapter (Results part III).

Being interested in the high expression of LMP1 in the two ENKTL lines, we examined expression of this membrane protein at the single cell level. Immunofluorescence staining for LMP1 expression in SNK6 and SNT8 is shown in Figure 29 and, similarly to a LCL shows heterogeneous expression between cells, with focal, polarised membrane staining evident within individual cells. For SNK6, the majority of cells are seen to express LMP1, albeit at variable intensity, whilst levels in SNT8 are generally lower or just at the threshold of immunofluorescence detection. Notably, higher levels of LMP1 are expressed in the larger cells within the SNT8 culture. These data are pertinent as the published studies of primary ENKTL tumours describe LMP1 expression as limited to a subset of tumour cells[73] although the proportion of $LMP1^+$ cells can vary substantially from tumour-to-tumour[614]. For the CAEBV lines SNK10 and SNT16, less intense expression was seen in a majority (>50%) of cells (data not shown), similarly to the smaller cells in the SNT 8 culture.

We therefore turned our attention to primary ENKTL tissue sections, donated by patients diagnosed in the UK, and applied immunohistochemical stains for LMP1 using the CS1-4 antibody[681]. In 7 cases examined, by contrast with the cell line data but consistent with previous studies[73], LMP1 was restricted to minority of $EBER^+$ T/NK tumour cells (Figure 30).

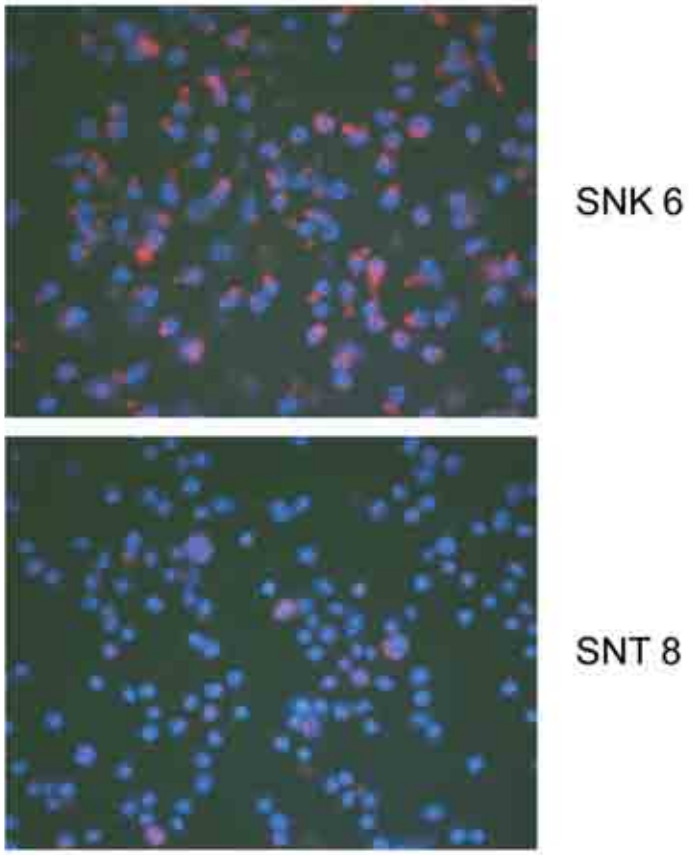


Figure 29 LMP1 is heterogeneously expressed in the majority of cells in ENKTL lines

Phase contrast immunofluorescence microscopy of SNK6 and SNT8 ENKTL tumour cells stained with MAbs CS1-4. DAPI is the blue nuclear stain and red fluorescence indicates LMP1 protein.

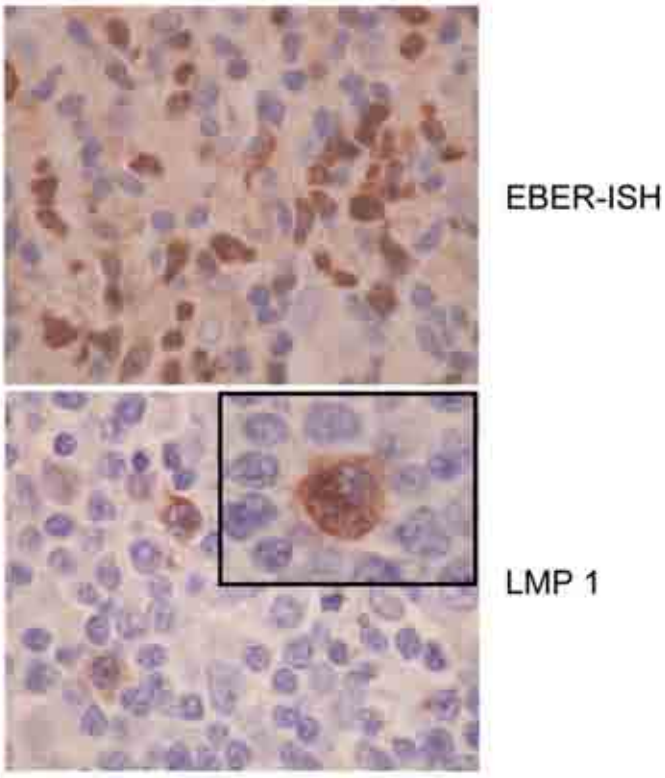


Figure 30 In primary ENKTL tissue, LMP1 expression is restricted to a sub-population of EBER$^+$ malignant cells

EBER in-situ hybridisation on a 4µm formalin-fixed, paraffin-embedded ENKTL tissue section (optical magnification x200). Courtesy of Dr Simon O'Connor, Department of Histopathology, Nottingham University Hospitals.

Immunohistochemical stain of a 4µm formalin-fixed, paraffin-embedded ENKTL tissue section using CS1-4 antibodies. The image was captured with a Nikon CoolpixE995 digital camera, via a Nikon Eclipse E400 microscope (optical magnification x400).

Together these data raised a number of questions concerning the role of LMP1 expression in initiation and/or potentiation of T/NK lymphomagenesis as well as factors governing its expression *in vivo* and *in vitro.* On this latter point, we were interested in work by Kis *et al* that examined cytokine-mediated regulation of LMP1 expression *in vitro* in the absence of EBNA2[114-115]. In the context of IL-2 dependent ENKTL cell lines, those authors showed that removal of IL-2 resulted in a fall in levels of LMP1 protein, and cytokines such as IL10[114], IL15 and IFNγ[115] could restore LMP1 protein expression in the absence of IL-2. Focusing our investigations on the role of LMP1 in the pathogenesis of ENKTL, we wished initially to reproduce and extend these findings to include a quantitative analysis at the transcriptional level. Figure 31 represents data from a time-course analysis following IL-2 depletion from the standard culture media of SNK6 (page 88). Immediately following the harvest of an aliquot of cells to represent day 0, the cells were repeatedly washed in RPMI/10% FCS, including 30 minutes incubation at 37°C, to ensure reliable depletion of IL2. Thereafter the cells were cultured in their original media (with 10% HS) but without IL2. Cell proliferation was clearly impaired and by day 6 the majority of cells appeared unhealthy by light microscopy. At this stage, the culture was divided into three: replenishment of IL-2, addition of IL-15 or no change. On the days indicated, cells were harvested and centrifuged on a density gradient (Lymphoprep^{TM}) in order that only viable cells were assessed for protein and mRNA analysis.

Panel A shows RT-Q-PCR data: the initial (day 0) LMP1 mRNA value was adjusted to 1 and subsequent values represented relative to this. A substantial fall in LMP1 transcripts is evident by 24 hours and does not recovery spontaneously in the absence of IL2. By day 6 LMP1 transcripts are less than 10% of the original value but are considerably restored, although not completely, by 48 hours of exposure to either IL2 or IL15. Panel B is a Western blot showing corresponding expression at the protein level. Consistent with previous data[115] a fall in LMP1 protein is seen and is mirrored by mRNA levels, although the fall in protein level appears to lag behind the transcript changes.

However, in terms of the observed cell phenotype – impaired proliferation and eventually cell death - we were unable to delineate the effect of IL2-withdrawal *per se* and loss of LMP1 expression. In order to resolve the relative contributions of LMP1 and IL2 to cell survival and proliferation, an experimental system was required whereby LMP1 expression could be regulated independently of IL-2.

Optimisation of *in vitro* gene delivery to primary NK cells and lines

With the above data in mind and with the aim of determining the contribution of LMP1 to the growth and survival of ENKTL tumour cells *in vitro,* we began by testing different ways of delivering LMP1-expression systems to the SNK and SNT cell lines. It quickly became clear that, using electroporation methods, the majority of these cell lines were very difficult to transfect either due to substantial cell death and poor viability post-transfection (SNT8) or extremely inefficient plasmid transfection (SNK10 and SNT16). Only the SNK6 line was sufficiently robust and 'transfectable' to allow conduct of these experiments.

Using our laboratory's standard method of electroporation (page 107-8) it was possible to deliver small expression plasmids into 10-20% of the SNK6 population, albeit with high rates (typically ≥60%) of associated cell death at 24 hours (data not shown). This could be further optimised using the Amaxa (Lonza) electroporation system. However, we wished to establish a stable, inducible system of LMP1 expression and so initially adopted the pRTS-CD2 vector used previously within our laboratory[55], a derivative of the pRTS-1 expression plasmid[710]. This plasmid carries a truncated rat CD2 gene, the EBV origin of replication (oriP) and the EBNA1 gene (for episomal maintenance), in addition to a bi-directional doxycycline (dox)-regulated promoter controlling expression of GFP and truncated NGF receptor in one direction and LMP1 in the other direction[711].

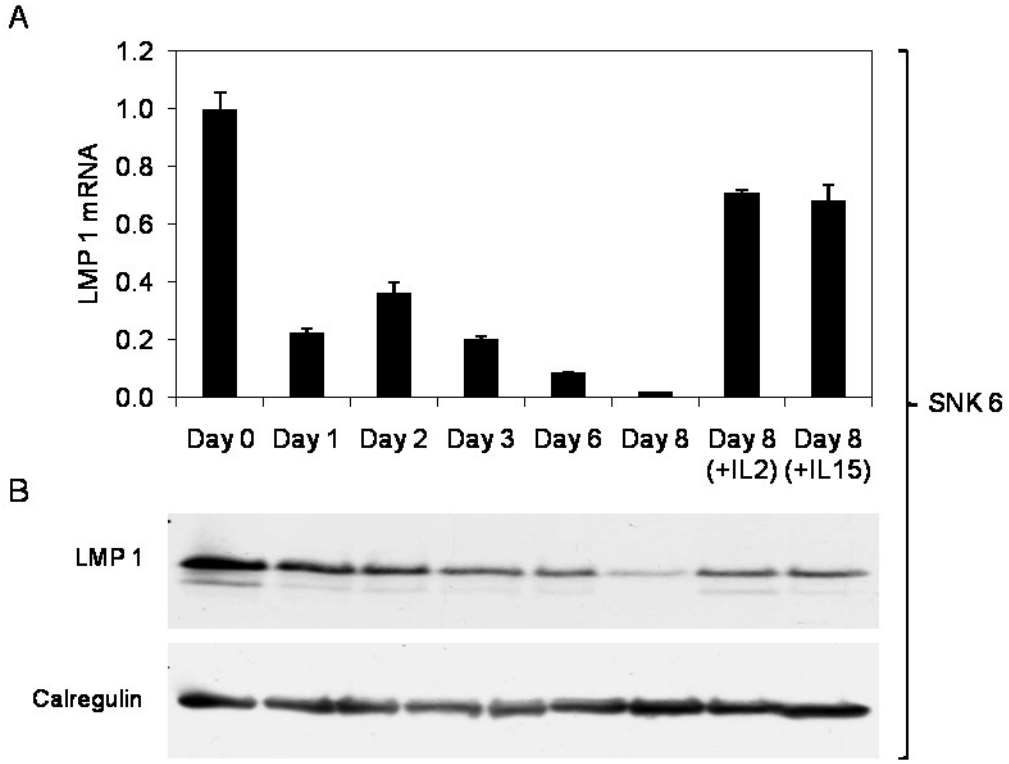


Figure 31 IL2 deprivation of ENKTL cell lines results in a fall in LMP1 mRNA and protein levels, that are restored by IL2 or IL15 treatment.

Panel A shows RT-Q-PCR data of LMP1 mRNA levels over a time-course of 8 days; RNA was extracted from harvested cells on days indicated. LMP1 transcripts were normalised to GAPDH and compared to the Day 0 level (ascribed a value of 1). Error bars indicate standard deviations from a mean value of PCR performed in triplicate.

Panel B is the corresponding protein expression data for identical time-points, assayed by Western blot with CS1-4 antibody. Calregulin serves as a loading control.

Figure 32, Panel A shows the most efficient transfection data from a large series of electroporation experiments. The small eGFP plasmid was efficiently transfected into SNK6 using the Amaxa nucleofection system, albeit associated with high cell mortality, but the substantially larger pRTS-CD2 plasmid was more challenging. An extensive series of optimisation experiments achieved a maximum transfection efficiency of approximately 8% (applying a combination of 220V, 950μF with 5μg pRTS-CD2-LMP1 plasmid). The next challenge was to purify the transfected population and an initial approach of using rCD2 as the selection antigen was greatly suboptimal due to very low surface levels of rCD2 apparent on transfected SNK6 cells. We therefore took the alternative approach of inducing GFP expression (by addition of doxycycline) followed by FACS. By undertaking multiple transfections in parallel (for example 10 cuvettes of 10^7 cells), we were able to collect a small but pure population (≥95%) of GFP-expressing cells for subsequent expansion in culture (without doxycycline) and experimental use. However, following a 10-14 day culture period after cell-sorting, the pRTS-CD2-LMP1 plasmid (and the pRTS-CD2 control vector) was consistently lost from the SNK6 cells; the cultured cells were <5% GFP-positive when reassessed by FACS (data not shown).

Faced with this setback, we abandoned the pRTS-CD2 system and turned to lentiviral constructs in an attempt to achieve efficient, stable and inducible LMP1 expression. Pilot experiments to test the feasibility and efficiency of this approach used a GFP-expressing lentiviral construct, originally generated by Dr Graham Taylor in our laboratory. Figure 32, panel B demonstrates evidence of efficient lentiviral infection of SNK6, as assessed by FACS at 48 hours.

These encouraging experiments coincided with collaborative discussions with Professor WC Chan, Omaha, Nebraska who had recently published data on genome-wide transcriptional changes of resting and IL2 stimulated NK cells[712] and subsequently compared this to cellular changes in NK malignancies[713]. These extensive transcriptional datasets provided a unique

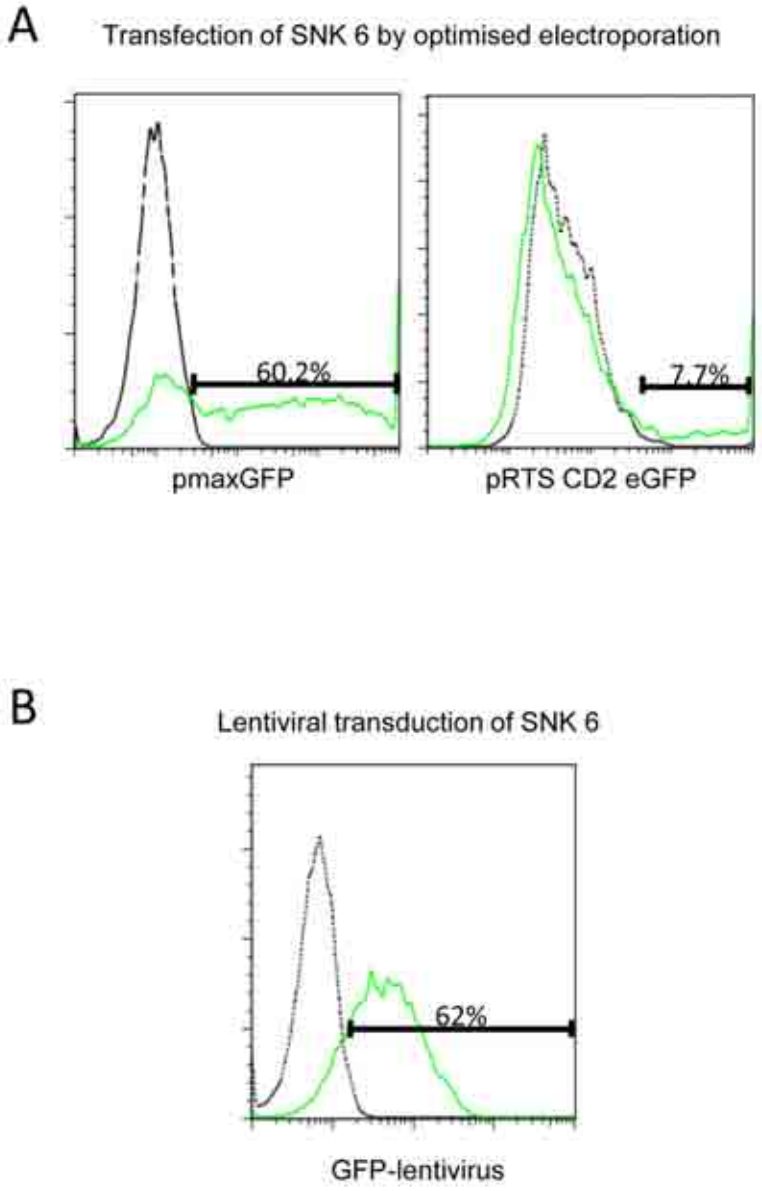


Figure 32 Efficiency of transfection/transduction of NK cell lines and primary cells:
Flow cytometric histograms of transfected/transduced SNK6 tumour cells with GFP-expressing vectors. The black bar indicates percent of GFP-expressing cells (green plot) compared to the mock transfected/transduced population (black plot).
Panel A shows data at 48 hours following electroporation using a small eGFP plasmid (left plot) and a larger, stable, inducible expression plasmid.
Panel B shows data at 72 hours following infection of SNK6 with a GFP-expressing lentivirus.

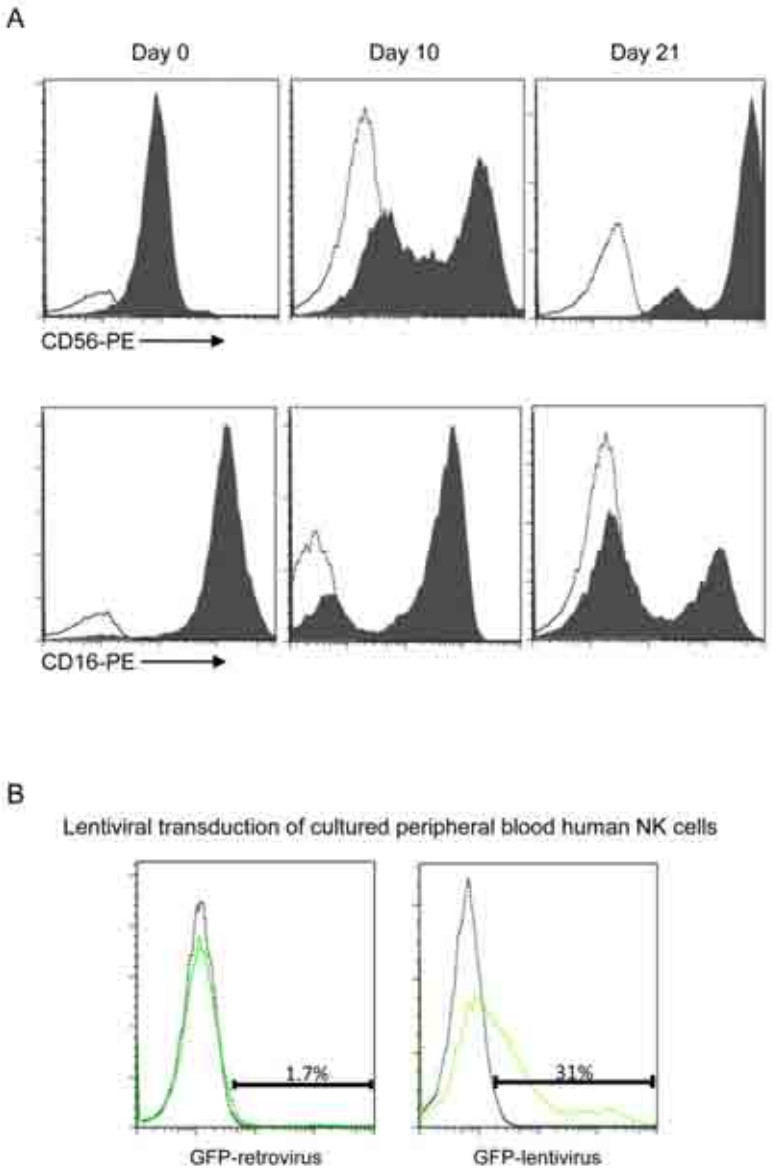


Figure 33 Phenotypic analysis and lentiviral transduction of IL-2-stimulated peripheral blood human NK cells

Panel A Single-colour flow cytometric histograms of primary NK cells stained with PE-conjugated CD56 (top plots) and CD16 (bottom plots) MAbs (x axis = $\log_{10}$ fluorescence).Black-shaded histograms represent the antigen-expressing population compared to the relevant isotype control (unshaded plots).

Panel B shows GFP expression data at 72 hours following infection of cultured primary NK cells with a GFP-expressing lentivirus. Green plots represent transduced population, compared to un-transduced NK cells (black plots).

opportunity to assess the role of LMP1 in NK lymphomagenesis, by comparison with a transcriptional profile of $LMP1^{+}$ NK cells. We planned to achieve this via lentiviral expression of LMP1, in IL-2 activated primary NK cells. This approach would enable us to delineate the effects of IL2 and LMP1 at the transcriptional level, assess LMP1's contribution to the tumour transcriptome and also provide an opportunity for follow-on functional studies of the biological effects of LMP1 in a normal NK cell background.

The schematic shown earlier in Figure 8 (page 87) summarises the protocol for NK cell isolation from peripheral blood (page 86). The principle of this protocol was straightforward: application of a cocktail of antibodies to allow elimination of all non-NK mononuclear cells from PBMC, resulting in a pure population of untouched NK cells. As described, the majority of peripheral blood NK cells comprise the cytotoxic $CD56^{DIM}$ $CD16^{BRIGHT}$ subset whilst the remaining 10% are $CD56^{BRIGHT}CD16^{DIM/NEG}$ and express the high-affinity heterotrimeric ($\alpha\beta\gamma$) IL-2 receptor[328-329]. Thus, following *in vitro* stimulation with IL-2 the latter population preferentially proliferate and eventually dominate the culture. Figure 33A comprises flow cytometric analyses of CD56 (top panels) and CD16 (bottom panels) expression on peripheral blood NK cells. The plots show relative levels of antigen expression immediately after isolation, followed by assessment at day 10 and day 21 by which time the $CD56^{BRIGHT}$ population had typically dominated the culture.

We next tested the efficiency of lentiviral transduction of these proliferating, IL2-sensitive NK cells using the constitutive GFP-expressing lentiviral vector. Figure 33B represents a typical flow cytometry analysis at 72 hours, where 30% transduction efficiency was routinely achieved. These encouraging data, together with the opportunity to maximise our understanding of LMP1's role in NK lymphomagenesis via collaboration with Prof WC Chan, directed us to focus efforts on achieving a stable, inducible system of LMP1 expression in primary human NK cells.

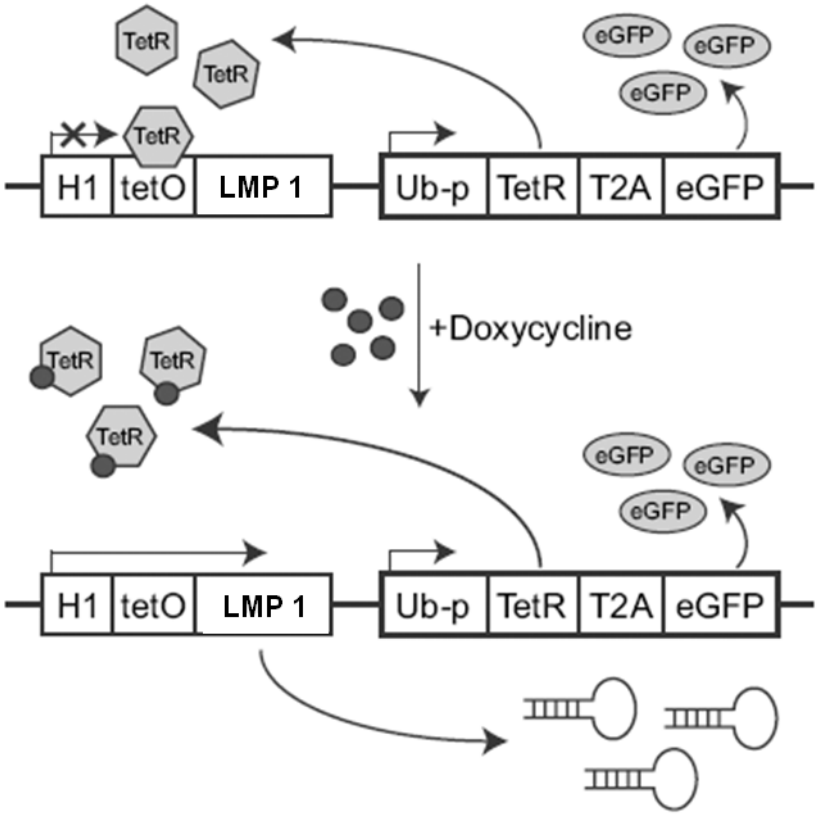


Figure 34 Principle of the FH1TIR5UTG vector (adapted from Herold et al[714])

A stable, inducible system of LMP1 expression in primary NK cells

Our aim was to incorporate inducible expression of LMP1 alongside a constitutively expressed fluorescent marker to allow identification and sorting of lentivirus-infected cells. We were interested to learn of such a lentiviral system, recently designed to express shRNAs in an animal model[714] and are grateful to Dr Marco Herold for providing the requisite vectors for expression of mRNA, prior to publication. Figure 34 schematically describes the principle of the FH1TIR5UTG vector system adapted from Herold *et al*[714]. Briefly: a codon-optimised tet-repressor (tetR) and eGFP were positioned downstream of an ubiquitin promoter and separated by a T2A peptide[715] to allow reliable and independent expression of both protein products. The gene of interest is placed under control of the H1 promoter with tetO (H1t) sequences present at the 3′ end of the TATA box. Constitutive expression of the tetR protein blocks transcription and upon addition of the inducer doxycycline, the tetR is released, thereby permitting expression of the gene of interest.

Figure 35 demonstrates the cloning strategy we used to engineer LMP1 expression in this lentiviral system, under the control of the TREX-promoter. PCR primers, to amplify LMP1 from SNK6[562] DNA, were designed to contain a BamHI site at the 5' end of the forward primer and an EcoRI site followed by a PACI site at the 5' end of the reverse primer. The LMP1 PCR product was first TA-cloned into a Topo vector (Invitrogen) and then released by digestion with EcoRI and BamHI restriction enzymes. This was ligated into an intermediate vector (FTGW, figure 35A), replacing eGFP and inserting downstream of the *in situ* TREX-p. Together with the TREX-p, LMP1 was cut from this intermediate vector and cloned into the final vector FHIT-IR5-UTG (figure 35B) which contained the tetracycline repressor protein gene and eGFP under the control of an ubiquitin promoter. This final vector was packaged into a lentivirus and used to transduce cultured primary NK cells, as described in materials and methods (pages 108-9).

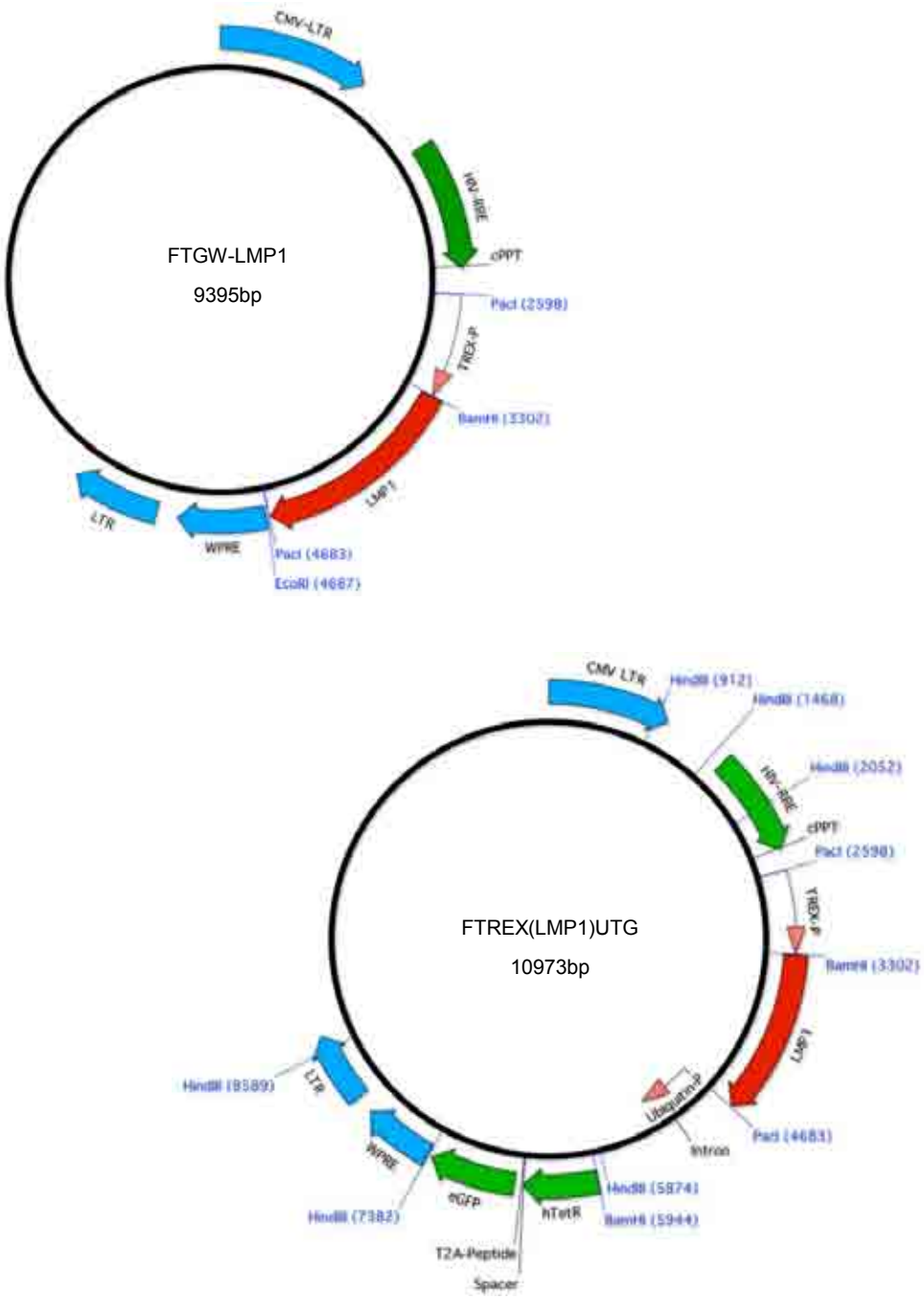


Figure 35 Cloning strategy for inducible LMP1 expression in a lentiviral vector

Simplified schematic map of the LMP1 lentiviral vectors, FTGW-LMP1(intermediate vector, top panel) and FTREX(LMP1)UTG (final vector, bottom panel). LMP1 expression was controlled by virtue of a tetracycline-regulated promoter (TREX-P) whilst eGFP and the tetracycline repressor protein (hTetR) were constitutively expressed from the ubiquitin promoter (ubiquitin-P).

Figure 36 comprises a series of flow cytometric analysis for GFP-expression which confirmed the ability of the LMP1-lentirus to infect NK cells and hijack the host cellular transcriptional machinery. Transduction efficiencies of 20-40% were consistently achieved in proliferating primary NK cells. As for the cell sorting (discussed next) it was found advantageous to gate on cells using both FL1 and FL2 fluorescence channels without compensation, in order to distinguish genuine GFP expression from auto-fluorescence (Figure 36, lower panels). Concomitant with a fall in the proportion of GFP^+ cells in the culture, the intensity of GFP-expression increased and both parameters ultimately stabilised.

Inducible expression of LMP1 in primary NK cells

We next wished to examine the stringency of repression and the dynamics of doxycycline-induced LMP1 expression within this lentiviral system. Aliquots of cultured, lentiviral-transduced primary NK cells in which approximately 15% of the cells were GFP^+ by FACS analysis, were treated with incremental concentrations of doxycycline as indicated in Figure 37A. The Western blot shown includes SNK6 (the ENKTL cell line from which the LMP1 gene was cloned) and a dilution of SNK6 in BJAB (an EBV negative cell line) - to result in approximately 15% EBV positive NK cells - as comparators. In the absence of doxycycline LMP1 expression is stringently, though not absolutely, repressed. Low concentrations of doxycycline are seen to induce LMP1 expression in a dose-dependent manner. LMP1 expression levels essentially reached a plateau at concentrations greater than 10ng/ml (data not shown).

Figure 37B demonstrates characteristic membrane staining using MAbs to LMP1 in an immunofluorescence assay. The focal, polarised morphology of the protein expression in these LMP1-expressing primary NK cells is akin to the SNK6 cell line data shown in Figure 29 (page169).

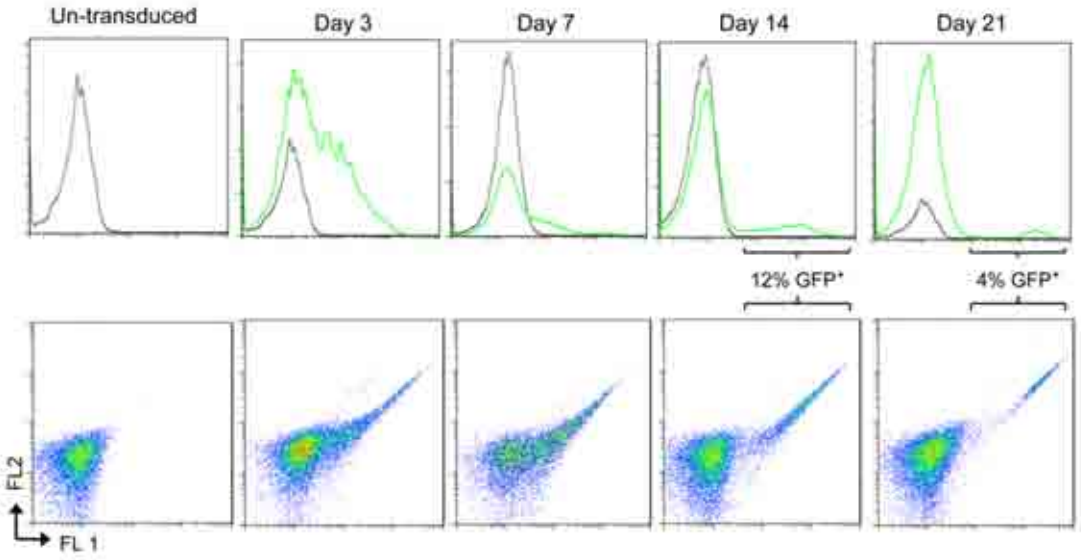


Figure 36 Stable transduction of primary NK cells with a LMP1-expressing lentivirus

Flow cytometric plots representing a time-course following infection of cultured primary NK cells with a GFP^+ ($LMP1^+$) lentivirus, compared to an untransduced control. The top panels show data presented in histogram form whilst lower panels show GFP^+ cells detected by fluorescence channels 1 and 2 (uncompensated data) as applied for gating of subsequent cell sorting.

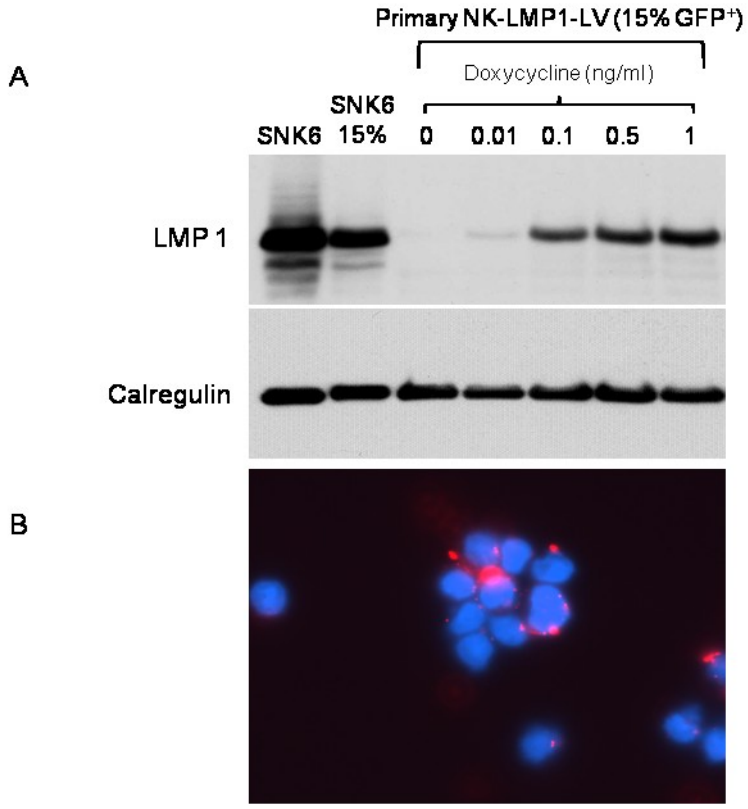


Figure 37 Inducible expression of LMP1 in primary NK cells via a lentiviral vector.
Panel A is a Western blot probed with antibodies to LMP1. Expression of LMP1 protein in LMP1-lentiviral-infected primary NK cells, un-induced and induced (at indicated doxycycline concentrations) is compared to the SNK6 cell line and a 15% dilution of SNK6 protein in BJAB (EBV-negative BL). Calregulin serves as a loading control.
Panel B shows LMP1 expression in primary NK cells at the single-cell level, by phase-contrast immnuofluorescence microscopy. DAPI is the blue nuclear stain and the red fluorochrome represents LMP1 expression in the NK cell cytoplasm/membrane.

Purification of lentiviral-transduced NK cells and LMP1 transcript quantitation

As shown in Figure 36, a small but distinct population of GFP^+ NK cells are readily identified by flow cytometry. In order to obtain a purified population of $LMP1^+$ primary NK cells for RNA extraction and subsequent analyses of transcriptional changes, we used the MoFlo cell sorter to separate GFP^+ from GFP^- cells, 48 hours following LMP1-induction with 10ng/ml doxycycline. Figure 38A shows a typical fluorescence plot for such a cell-sort, where the lentiviral-transduced cells are gated on the basis of GFP as detected by FL1 and FL2 fluorescence channels. GFP-negative cells were collected as a control. Figure 38B represents a RT-Q-PCR analysis of LMP1 transcripts in RNA extracted from one such cell sort, compared to the SNK 6 ENKTL line and a LCL. In this instance the GFP^+ NK cells were 97% pure, whilst the GFP- population was 99.5% pure on reanalysis. Expression of LMP1 mRNA in the transduced primary NK cells is comparable to that of the SNK6 line, whilst transcripts can also be detected in the GFP- population at a level in excess of that explicable by the negligible GFP^+ contamination. The reason for this is unclear but may represent LMP1 transcription from a population of transduced cells with insufficient GFP expression for fluorescent detection.

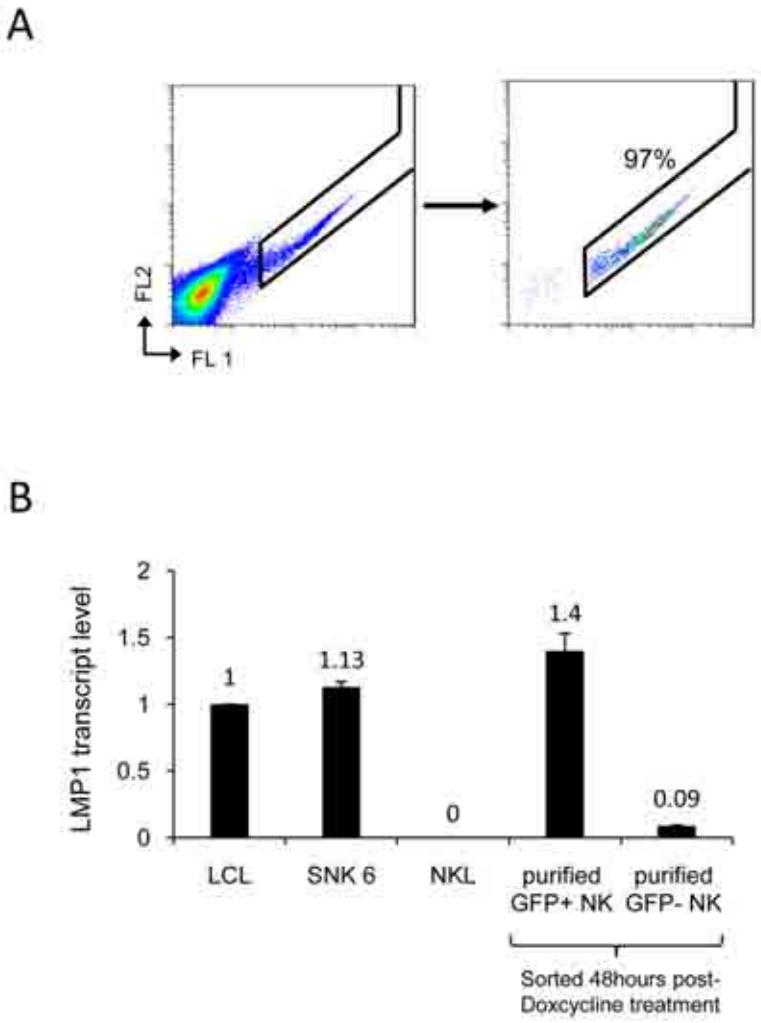


Figure 38 Purification of lentiviral-transduced NK cells and LMP1 mRNA quantitation

Panel A: Gating strategy (MoFlo cell-sorter) for isolation of purified GFP-expressing lentiviral-infected NK cells. GFP positive cells were isolated as indicated via fluorescence detection in channels 1 and 2 (uncompensated data).

Panel B: RT-Q-PCR data of LMP1 transcript expression in purified GFP^{+}/- NK cells from the cell-sort shown in panel A. Cells were treated with 10ng/ml of doxycycline 48 hours previously to induce LMP1 expression; RNA was extracted immediately following cell-sorting. Transcript levels were normalised to GAPDH and compared to those seen in the SNK6 line and a LCL (ascribed a value of 1) assayed in parallel.

Discussion (II): EBV gene expression in NK and T cell malignancies and studies with LMP1

Latent EBV gene expression in NK and T cell lymphoproliferative disease has been generally regarded as a Latency II pattern, although this description has been largely based on a small number of semi-quantitative studies[73,564-565,610]. Comprehensive, quantitative analyses have been hindered by the rarity of these malignancies and the related challenge of obtaining good quality viable tissue for study; particularly as ENKTL tissue is frequently necrotic[474]. Based on available data it appears that a majority of ENKTL tumour cells within a lesion are of latency I type whilst, at a given point in time, only a subpopulation of cells additionally express LMP1[73] - the number of which can vary substantially between tumours[614]. Expression of LMP2 has been largely unresolved and is investigated further in results Part III. In the context of CAEBV, initial non-quantitative analyses of PBMC detected Qp, LMP1 and LMP2A transcripts in some donors, although LMP1 was seen in less than half the samples analysed and, when expressed, often at apparently low levels[564]. A follow-up, quantitative study[563] of CAEBV PBMC, has confirmed heterogeneity of LMP1 expression between donors; undetectable in one-third of cases, whilst often low in others. However, single-cell analyses for LMP1 were not performed.

Our quantitative analyses of T/NK cell lines, derived from Japanese patients with ENKTL and CAEBV, support the existence of a latency II pattern of viral gene expression (Figure 28, page 169) but, by contrast to the situation apparent in primary tissue, the majority of tumour cells express LMP1 (Figure 29, page 170). These observations are pertinent both for the use of these cell lines as representative *in vitro* tumour models and for understanding the regulation of LMP1 expression *in vitro* and *in vivo*.

It is conceivable that LMP1 expression independently provides a survival advantage amongst T/NK tumour cells, promoting outgrowth of this subpopulation *in vitro* and thus the establishment of a cell line representative of the parent. However, because corresponding data is not available on LMP1 expression at the single-cell level for the specific tumours from which these cell lines

were established[562], it remains difficult to be certain how representative the observed LMP1 expression is. Our own analysis of LMP1 expression in primary tissue at the mRNA level (Figure 39 and discussed further in Results Part III) illustrates substantial heterogeneity between tumours – consistent with previous immunohistochemical studies[614]. Interestingly, a recently established ENKTL cell line (derived from a Caucasian patient with disseminated ENKTL) kindly donated by a French group[716], has dramatically lower LMP1 mRNA levels than SNK6 and SNT8 (data not shown). The significance of this is unclear but cautions against the interpretation that all ENKTL lines express higher LMP1 than CAEBV lines.

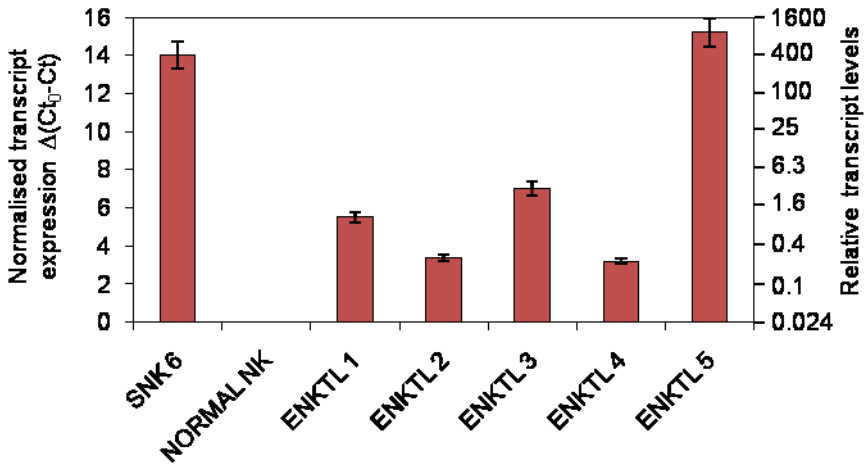


Figure 39 Inter-tumour heterogeneity of LMP1 mRNA expression in ENKTL biopsies
Quantitative RT-PCR results of LMP1 transcripts. LMP1 Ct values are adjusted for GAPDH Ct values. Ct_0 is the assay threshold of detection. The left y-axis represents (Ct_0-Ct), while the right-hand y-axis shows the equivalent fold difference in transcript levels. RNA was originally extracted from frozen ENKTL biopsy sections (1-5) used in a previous study[713] and high-quality, random-primed cDNA was kindly donated by Professor WC Chan, Omaha, Nebraska. SNK6 cell line and cultured primary NK cells served as positive and negative controls respectively.

A further level of complexity is that considerable heterogeneity of LMP1 is seen *within* tumour lines; at the threshold of immunofluorescence detection in some cells whilst highly expressed in adjacent cells. In fact similar observations have been made in LCLs, where LMP1 levels vary among individual cells such that the difference between the highest and lowest 5% of cells may be as great as 100-1000fold[717-718]. Using an RT-Q-PCR assay that detects 'total' LMP1 mRNA (i.e. does not discriminate between ED-L1 or TR-L1 promoter usage), we noted a marked discordance between levels of protein and trancript in SNK6 and SNT8. This was seen both in standard culture conditions (Figure 28, page 167) and following IL-2 withdrawal (Figure 31, page 173), where protein expression is substantially higher than may be expected for the transcript level (as compared to a LCL within the same assays). This observation was unexpected as LMP1 is a relatively short-lived protein with a half-life of 2[719] to 5[720] hours in B cells, but the situation in T and NK cells could be quite different; a less labile protein may not be so susceptible to temporal changes in rates of new protein synthesis. Interestingly, a recent study reported a phenomenon of temporal fluctuations in LMP1 levels within individual cells, observed by isolating $LMP1^{HIGH}$ and $LMP1^{LOW}$ subpopulations from a LCL[721]. To explain the rapid fluctuation, the authors propose a model in which LMP1 drives its own synthesis through an autocatalytic route until a threshold is reached at which the protein induces autophagy and, thereby, its own degradation. On a similar note, fluctuating LMP1 levels are seen to demonstrably impact on the level of specific T cell recognition - mediated through LMP1's dose-dependent activation of HLA class I expression[711].

It is also likely that regulatory factors governing LMP1 expression *in vitro* and *in vivo* differ in the two environments; either the existence of potent *in vitro* signals augmenting expression and/or, loss of repressive factors present *in vivo*. On this point, the influence of specific cytokines on LMP1 expression in ENKTL cells *in vitro* had been described[115] and we confirmed these findings, showing that the effect is transcriptionally mediated (Figure 31, page 173). Relevant to this observation is that IL-2 is a potent activator of the JAK/STAT pathway in NK cells[712] and that both known LMP1 promoters (page 11-12 and figure 3, page 13) respond to activation of the JAK/STAT pathway, with evidence of functional STAT-binding motifs at both sites[108,110]. Although

it is reasonable to assume that the EBNA2-independent, L1-TR promoter (pages 11-12 and figure 3, page 13) is the principal driver of LMP1 transcription in T/NK cells, this remains to be formally shown. pL1-TR is transcriptionally active in the other latency II tumours, NPC[68,81,108] and HL[108] but, as non-quantitative assays have been applied, there remains some doubt as to which promoter dominates in these tumours[124,108]. STAT3 was specifically implicated in activation of L1-TR, via the kinase v-Src[108]. Interestingly, STAT3 has been recently implicated in survival and proliferation of ENKTL cells *in vitro*, alongside evidence of constitutive STAT3 activation in ENKTL tissue biopsies[716].

Appreciation of the influences of such regulatory factors allowed important methodological lessons to be learnt during our investigations. Experimental culture conditions, including IL2 concentration and type/batch of serum, were seen to substantially influence LMP1 protein expression. Faced with these confounding factors careful control of culture conditions within experiments was required, but to meaningfully address the function of LMP1 we needed to establish a system of inducible LMP1 expression impervious to the influence of cytokines.

We encountered considerable difficulties in efficiently transfecting and maintaining plasmids within T/NK lines, but ultimately established a lentiviral system that can demonstrably deliver individual EBV genes into a normal NK background. Furthermore, effective transcriptional repression in the absence of doxycycline and dose-dependent inducibility (Figure 37A, page 183) of LMP1 will allow both genotypic and phenotypic analyses of its role in the initiation and/or potentiation of T/NK lymphomagenesis. The $CD56^{BRIGHT}$ NK population is most amenable to *in vitro* studies given its proliferative response to IL-2 and indeed the available data suggests this may be the apposite NK population to study in the context of ENKTL[473,716]. Immunofluorescent analysis of $LMP1^{+}$ primary NK cells (Figure 37B, page 183) showed that the membrane localisation and apparent focal aggregation of the protein to be similar to the pattern observed in SNK6 cells (Figure 29, page 169). Indeed, in B cells, the anatomy of LMP1 localisation in the cell membrane is thought to be critical for its effective function[722-723] . Interestingly, a study that transfected a LMP1 plasmid into EBV-negative T cell lymphoma lines (including CEM[117]) showed

that expression of the viral protein was exclusively restricted to the nucleus and, accordingly, appeared to lack associated signalling functions seen in B cells[724]. Time considerations did not permit a formal assessment of the functions of LMP1 expressed in primary NK cells, but the pattern of expression at the single-cell level infers that the viral protein will maintain signalling capabilities described in B cells.

If time had permitted, planned experiments included a global transcriptional mRNA array, using an Affymetrix U133 platform. RNA from IL2-stimulated primary NK cells from the same donor, transduced with a similar lentivirus (made by ligation of the FTGW vector without insertion of LMP1) (Figure 35, page 180), have been generated in parallel as a control for this array. Comparison of this transcriptional profile with existing data on resting, IL2-stimulated and malignant NK cells may allow some insight into the respective roles of IL2, LMP1 and other EBV-encoded transcripts. Figure 40 is a schematic outlining this proposal, hypothesising that expression of a distinct group of genes, common to $LMP1^+$ NK cells and ENKTL tumour tissue, may exist: either within a subpopulation of malignant cells or more widely expressed at the transcript level in primary tumours. Differentially expressed genes could be subsequently validated both by RT-Q-PCR and protein analyses in the same LMP1-expressing NK cells and in primary ENKTL/CAEBV tissue/cells. On this note, during the course of these investigations we have established collaboration with the ongoing International T cell project (CI Professor Massimo Federico, Modena, Italy). This prospective study encompasses collection of clinical data and diagnostic biopsy material of all mature T and NK cell neoplasms – of which 50-100 ENKTL biopsies are anticipated. This affords a unique opportunity to examine viral and cellular gene expression in a large, pathologically and clinically well-defined, cohort (from patients of various ethnicities) and correlate with response to treatment and clinical outcome. Genes of interest identified in the LMP1-array and validation studies can be addressed in this group. These studies would be complemented by characterising the apoptotic and proliferative phenotype of $LMP1^+$ NK cells, where the effect of cytokines including IL-2 can also be evaluated.

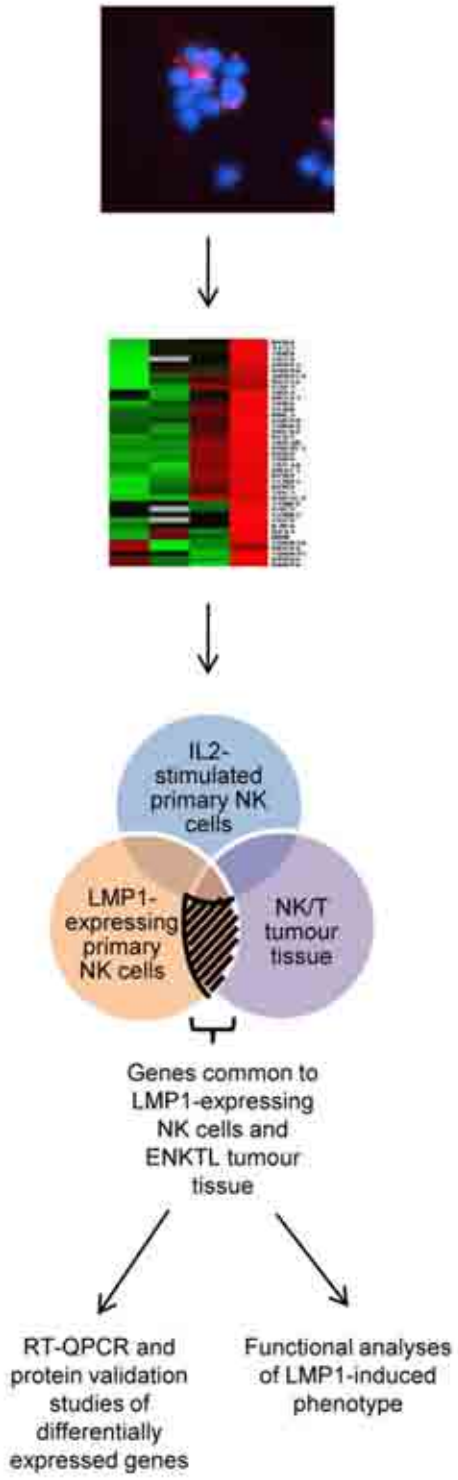


Figure 40 Schematic outlining experimental plan for global gene expression analysis of LMP1-expressing primary NK cells.

Results (III): Mystery of the missing target[§§§]

Introduction

As discussed in the introduction (pages 68-70 and 76-79), a majority of patients with ENKTL and CAEBV will fail to respond to, or relapse following, conventional chemo- and/or radiotherapy[473,561,623,628]. Novel therapeutic approaches are therefore urgently required for the spectrum of EBV-associated NK- and T-cell malignancies. In the context of PTLD, expression of the full complement of EBV proteins has permitted successful therapeutic targeting of this disease with EBV-specific cytotoxic T cells (CTLs) with the resultant clinical outcome data serving as a proof-of-principle for such an approach[416,421]. For EBV^+ malignancies with a more restricted pattern of gene expression, in the absence of the highly immunogenic EBNA3 family of proteins, such an approach has been more challenging[434]. However, more recent experience with EBV^+ HL has shown that infusion of polyclonal autologous CTLs containing augmented LMP2-specificities, achieved via an *in vitro* protocol of LMP2-overexpression in antigen-presenting cells[396], can result in meaningful disease responses including attainment of CR[395]. These encouraging data open up the possibility of adopting a similar approach in T/NK lymphoproliferations and indeed preliminary data have suggested responses in this disease group[395]. However, a fundamental prerequisite to any CTL-based therapy is that an appropriate cognate antigen is present within the tumour cell; in T/NK lymphoproliferations the expression of LMP2 has been far from clear.

In the context of CAEBV, non-quantitative PCR studies of PBMC and cell lines demonstrated expression of LMP2 mRNA, but did not distinguish between the LMP2 species[564-565] and no analyses of protein expression have been available.

[§§§] **Chapter title originally used for an *Inside Blood* commentary by Richard Frederick Ambinder, Johns Hopkins School of Medicine (reference 725. Ambinder RF. Mystery of the missing target. *Blood*. 2010;116(19):3691-3692.) in relation to the Plenary Paper (reference 726. Fox CP, Haigh TA, Taylor GS, et al. A novel latent membrane 2 transcript expressed in Epstein-Barr virus-positive NK and T cell lymphoproliferative disease encodes a target for cellular immunotherapy. *Blood*. 2010.) containing data presented within this chapter of the thesis.**

For ENKTL, no convincing evidence of LMP2 protein expression has been presented, whilst non-quantitative PCR studies suggest low or absent levels of both LMP2A and 2B in primary tissue[73,610]. Against this background, we decided to investigate the expression of LMP2 in T/NK malignancies and its utility as a CTL-target.

Antigen expression and presentation in ENKTL and CAEBV cell lines

Before investigating LMP2 as a putative immunologic target in ENKTL and CAEBV, we first asked whether the target malignant cells have retained the capability to process and present antigen, noting that defects in the MHC class I/transporter associated with antigen processing (TAP) pathway have been described in EBV^+ BL[458]. Expression of surface HLA class I and II complexes was confirmed in all 4 EBV-positive NK/T tumour cell lines analysed by flow cytometry. Figure 41A shows SNT 16 and SNK 6 as representative examples demonstrating moderate to high expression of surface HLA class I, while HLA class II expression was equivalent to levels in an LCL. Western blot analysis of cell lysates (Figure 41B) shows total MHC class I and TAP levels to be broadly equivalent across cell lines, and comparable with an LCL.

The original descriptions of the SNK and SNT cell lines, demonstrating presence of LMP1 and absence of EBNA 2 protein[562,709], suggested a Latency II pattern of viral gene expression but did not address LMP2 expression. We initially probed a Western blot with the 14B7 MAb[131] and detected LMP2A protein in the LCL control but not in any of the ENKTL or CAEBV cell lines (Figure 42A). However, because the available monoclonal antibodies for detection of LMP2 protein all recognise defined epitopes within the LMP2A-unique N terminus[130], the LMP2B protein cannot be detected with this technique. A schematic of the exon structure and conventional splicing pattern of LMP2A and LMP2B mRNAs, traversing the terminal repeat (TR) region, is shown in Figure 42B. Consistent with the immunoblotting data, LMP2A transcripts were virtually undetectable in all cases by RT-Q-PCR Figure 42C.

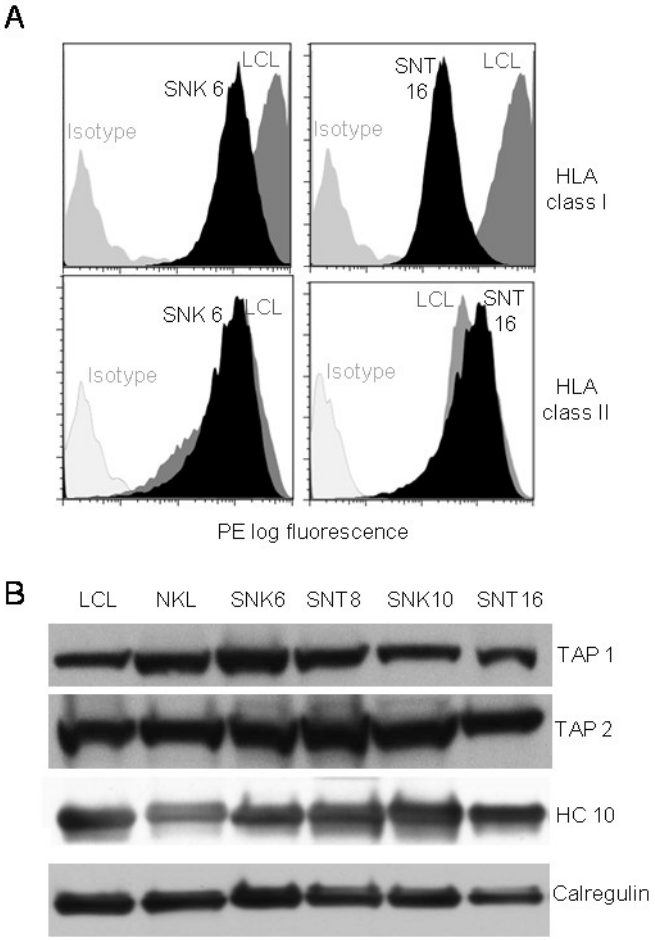


Figure 41 Antigen processing and presentation capability of EBV$^+$ T and NK tumour lines.

Panel A: Flow cytometric analysis of cell surface HLA class I and II. Representative data from 2 lines (SNT 16 and SNK 6, black shading) compared with a LCL (dark grey shading) and isotype controls (light grey) are shown.

Panel B: Western blot of whole cell lysates probed with antibodies to transporter associated with antigen processing (TAP) 1 and 2 and total cellular class I heavy chains (HC 10) in NK- and T-cell lines, shown relative to levels in a LCL. Calregulin serves as the loading control.

We then used a combination of primers to amplify across the exon 1B-exon 2 junction by RT-Q-PCR and found that LMP2B transcripts were also expressed at extremely low levels in all 4 NK- and T-cell lines (Figure 42C).

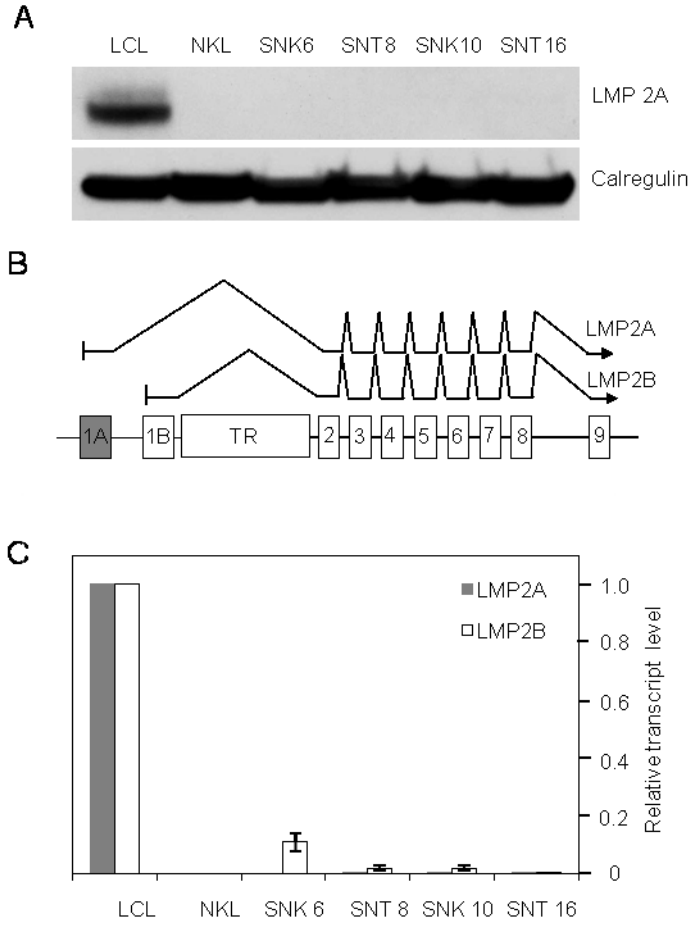


Figure 42 Expression of LMP2 in the context of 'Latency II' T and NK tumour lines

A. Western blot of whole cell lysates of EBV-positive NK and T cell tumour lines probed with 14B7 MAb to LMP2A alongside a LCL and an EBV-negative NK line (NKL).

B**.** Schematic indicating the exon structure of the LMP2 gene and splicing patterns of conventional LMP2A and LMP2B transcripts, across the terminal repeat region of the genome. Each transcript contains a unique 5' first exon ('1A' for LMP2A, shaded grey and '1B' for LMP2B, shaded white).

C. RT-Q-PCR for LMP2A and LMP2B mRNA. Grey and white shaded bars represent levels of LMP2A and LM2B transcripts respectively, relative to a LCL. Each data point was assayed in triplicate and error bars indicate standard deviations from the mean value.

To exclude the possibility that the apparent lack of LMP2 mRNA expression was due to sequence variation within the LMP2 gene, we performed genomic sequencing of LMP2 exons 1 and 2 in all 4 cell lines. Figure 43 shows the exon sequences corresponding to the PCR primers (yellow shading) and probe (red shading), aligned against the prototype B95-8[21] and Chinese GD1[25] strains and highlights the base substitutions (both coding and silent). This confirmed that the LMP2 gene was intact in these cells and aside from previously described[156] single-base polymorphisms detected in SNT16 compared with the prototype B95-8 EBV, corresponding to the common reverse LMP2 primer[133], there were no other sequence variations to explain the low transcript levels detected by PCR. The RTQ-PCR data for SNT16 shown in Figure 42C was obtained using an alternate reverse primer, accordingly designed to account for the polymorphisms shown.

Thus, the finding of extremely low (100-1000 fold lower than a LCL in most cases) LMP2A and LMP2B mRNA levels in the T/NK lines appeared to be a genuine result. However, it was difficult to reconcile these findings with emerging clinical evidence suggested of LMP2-CTL-therapy-mediated responses in ENKTL and CAEBV *in vivo* (Dr C. Bollard, Houston, personal communication January 2009 and reference[651]). Noting recent evidence suggesting that MHC class I epitopes are predominantly generated from rapid degradation of newly-translated (defective) ribosomal products[727], we questioned whether these low LMP2 transcript levels were sufficient for CTL recognition *in vitro.* Consequently, we set-out to investigate CTL recognition of LMP2 in these T/NK lines.

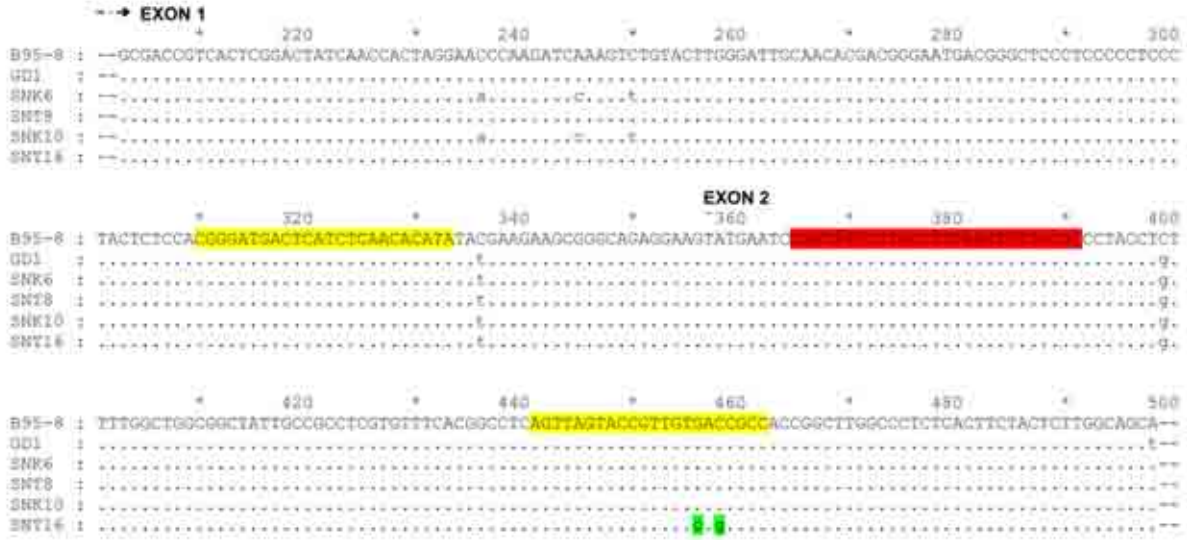


Figure 43 DNA sequence alignment of LMP2A exon 1 and 2 coding sequences relevant to RT-Q-PCR primers/probe

Segments of LMP2A coding sequences of all four SNT and SNK cell lines relevant to the RT-Q-PCR primers/probe and aligned against the prototype B95-8 and Chinese GD1 strain sequences. Each dot represents a base unchanged from the prototype sequence whilst base substitutions are specified; lower case synonymous, upper case replacement. The highlighted regions in yellow and red correspond to the sequence targets of the 5′ and 3′ PCR primers and Taqman probe respectively. The bases highlighted in green are polymorphisms identified in the SNT 16 sequence corresponding to the reverse LMP2 PCR primer.

LMP2-specific T cell recognition of EBV$^+$ T/NK tumour lines

Polyclonal LMP2 T-cell effectors recognise and kill ENKTL and CAEBV cell lines

We first asked whether the polyclonal CTL preparations generated from CAEBV and ENKTL patients[395,651] for clinical use (containing expanded LMP2-specificities), could recognise and kill our NK- and T-cell lines in spite of the very low transcript levels. These initial studies were performed by Dr Catherine Bollard, Houston, Texas, following collaborative discussions initiated by Dr C Fox. Polyclonal CTLs were produced as per Dr Bollard's clinical trial protocol (ClinicalTrials.gov identifier: NCT00062868) by stimulation of PBMCs from ENKTL patients with autologous antigen-presenting cells transduced with a recombinant adenovirus expressing an LMP1/LMP2A fusion protein[395-396,728].The epitope specificities of the CTLs were analysed by peptide-loaded pentamer staining[395-396] and their ability to kill HLA-compatible ENKTL and CAEBV tumour lines tested in standard 5-hour ^{51}Chromium release assays. Figure 44 comprises data from two such analyses: CTLs from patient 1 contained 6.9% and 2.3% CD8$^+$ cells specific for HLA-A*0201-CLGGLLTMV and HLA-A24-TYGPVFMSL, respectively (Figure 44A top plots), while CTLs from patient 2 contained 4.75% and 4.79% CD8$^+$ cells specific for HLA-A*0201-CLGGLLTMV and HLA-A*0201-FLYALALLL, respectively (Figure 44A bottom plots). In cytotoxicity assays, CTLs from both patients killed SNT 16 and SNK 6 at significant levels compared with an autologous LCL (Figure 44B).

Recognition and killing of ENKTL and CAEBV lines by LMP2-specific CD8$^+$ T-cell clones

The observation that these polyclonal CTLs contained considerable numbers of LMP2-specific T cells suggested that these cells were the dominant effectors in the cytotoxicity assays, but the presence of T cells with other specificities could not be excluded. Thus, faced with paradoxically low, or absent, levels of LMP2A and LMP2B transcripts in the T and NK

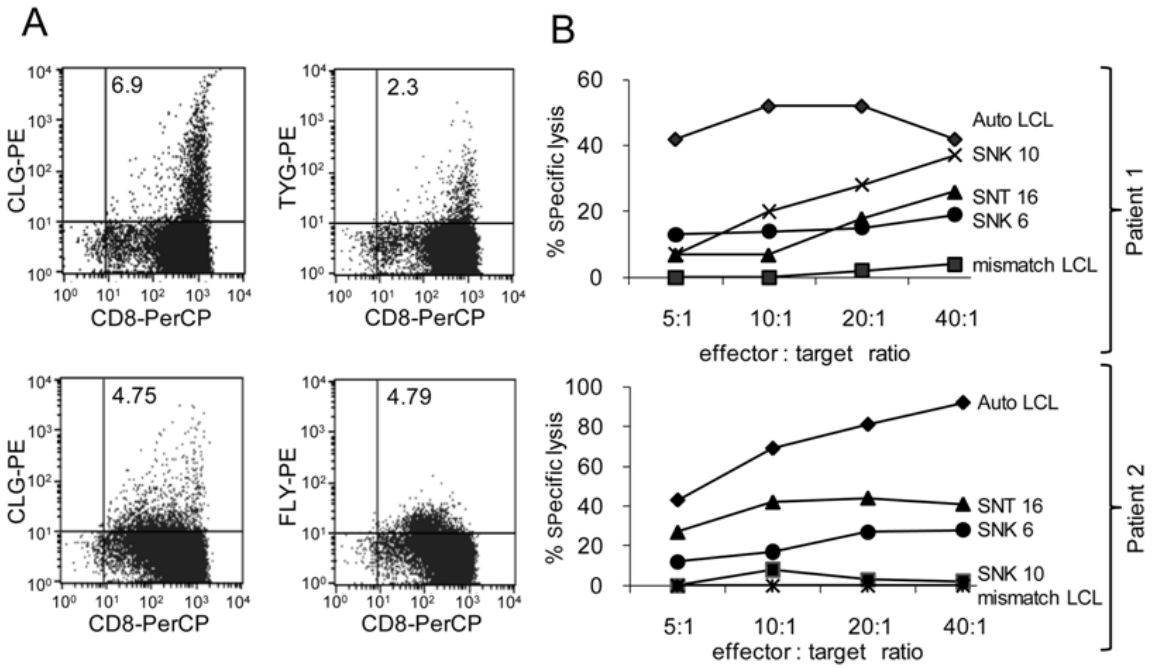


Figure 44 Specific killing of EBV$^+$ NK and T cells by polyclonal CTL lines, containing LMP2-specificities, from ENKTL patients.

Panel A. Dual-colour flow cytometry of CTL lines derived from two ENKTL patients prepared by *ex vivo* stimulation with LMP2/LMP1-transfected antigen-presenting cells. CTLs were stained with a PerCP (Peridinin-chlorophyll protein) conjugated anti-CD8 antibody and with the PE-conjugated HLA-peptide pentamers: HLA-A*0201-CLGGLLTMV, HLA-A*0201-FLYALALLL, HLA-A24-TYGPVFMSL as described in reference[395-396]). Numbers within the right upper quadrant of each plot indicate the percentage of viable peptide/pentamer-specific CD8$^+$ cells.

Panel B. The CTL lines shown in panel A were tested in standard 51Chromium-release assays for their ability to kill EBV$^+$ NK and T cell tumor lines. Results are expressed as the mean percentage of specific chromium release from the target cells at effector: target ratios titrated from 5:1 to 40:1, tested in triplicate. Autologous and HLA-mismatched LCLs were positive and negative controls respectively, for each assay.

lines, we further examined the specificity of this recognition in our laboratory. Two previously characterised $CD8^+$ effector clones with different LMP2-peptide specificities (FLYALALLLL, TYGPVFMCL)[390,729] restricted through HLA-A2 (FLY) and -A24 (TYG) respectively, were initially tested in standard 18-hour IFNγ–release assays. Figure 45A shows specific recognition of SNT 16 by both $CD8^+$ clones, albeit at lower levels compared with HLA-matched LCL targets within the same assay. For these 2 effector clones, the immune recognition translated into specific killing, as demonstrated using a standard 5-hour ^{51}Cr-release assay (Figure 45B). Augmentation of the recognition by preloading target cells with excess exogenous cognate peptide indicated that the maximum killing of the SNT 16 targets was similar to that of HLA-matched targets, suggesting that the weaker recognition of the endogenously LMP2 antigen might reflect differences in levels of LMP2 expression or efficiency of FLY- and TYG-peptide processing. Recognition of SNT16 by $CD8^+$ clones with specificities against LLWTLVVL[390] and, to a lesser extent CLGGLLTMV[390], was also observed (data not shown). Experimental blocking of surface HLA class I by pre-incubation with the W6-32 MAb, substantially reduced recognition of SNT16 by the TYG clone - as determined by a >90% reduction in IFNγ-release (Figure 46).

In view of these data with the CAEBV-derived SNT16 line, we sought to extend our investigations of LMP2-recognition to encompass other EBV^+ NK and T cell lines. Methodologically, there were 2 obstacles to overcome: first, to broaden the HLA repertoire of potential LMP2 epitope presentation and, second, to resolve the problem of how to measure cytokine production by the effector clones in the face of substantial levels of spontaneous cytokine release (including IFN-γ, TNFα and GMCSF) from the other 3 tumour cell targets. To address the first issue, expression of a recombinant HLA-A11 molecule in SNK6 and an LCL control was achieved by infection with a retroviral construct, allowing us to test for recognition by a HLA-A*1101-restricted $CD8^+$ LMP2 clone with specificity for SSCSSCPLSK[390]. Expression of an irrelevant HLA allele, HLA-DR1, in the target cells using the same retroviral construct served as a negative control. To delineate between spontaneously produced IFN-γ from the SNK 6 target with that from the effector T cells, we pre-stained the effectors with CFSE, enabling quantitation of IFN-γ production in $CFSE^+$

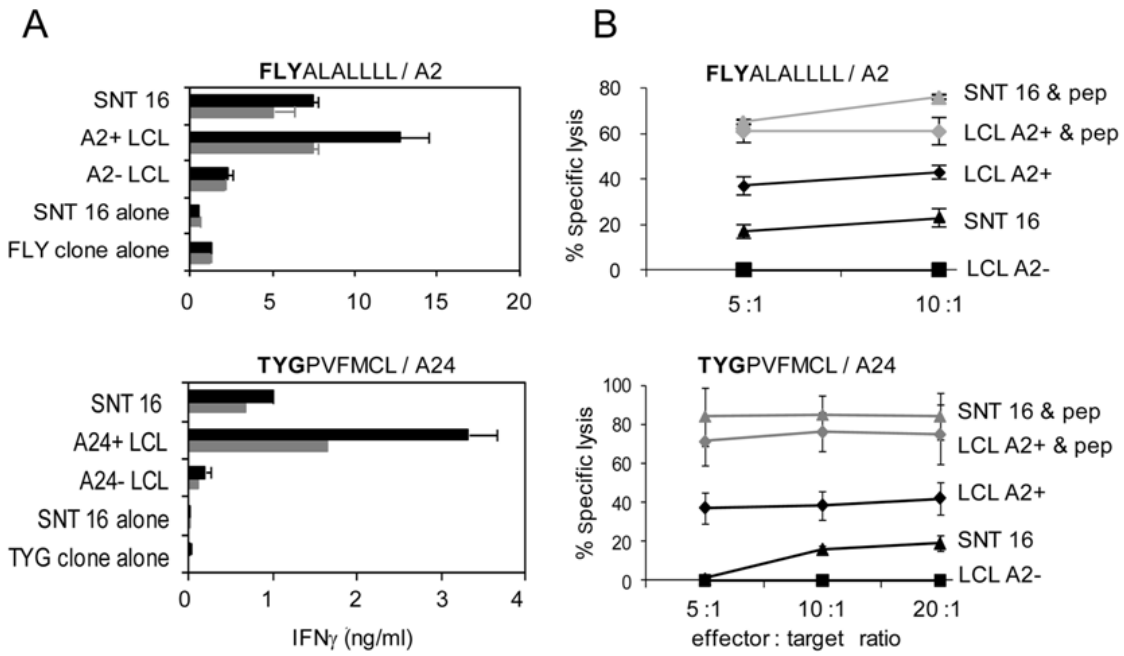


Figure 45 Recognition and killing of SNT 16 by LMP2-specific CD8$^+$ T cell clones through HLA A2 and A24.

Panel A. CD8$^+$ clones specific for endogenously processed LMP2-derived peptides FLYALALLLL and TYGPVFMCL were co-cultured for 18 hours with 10^5 target cells (gray and black bars indicate 5000 and 10,000 CD8$^+$effectors per well, respectively). HLA-matched and -mismatched LCLs were positive and negative controls respectively. Other controls included SNT 16 and CD8$^+$ effectors cultured alone to assess spontaneous IFN-γ release. Error bars indicate one standard deviation from the mean. Results are representative of those seen in 3 separate experiments. Relative to the maximal values seen using the same target cells pre-loaded with 5μM cognate peptide, recognition of the unmanipulated cells were as follows: FLY (SNT16=14%, LCL=26%), TYG (SNT16=5%, LCL=15%).

Panel B. Lysis of SNT 16 and LCL targets by the LMP2-specific FLY and TYG CD8$^+$ clones shown in A. Five hour ^{51}Chromium-release assays were conducted with SNT 16, HLA class I–matched and mismatched LCL targets. Spontaneous release of ^{51}Cr was <30% of maximum release. Results are expressed as the percentage of specific chromium release from the target cells at the indicated effector-to-target ratios. Grey lines indicate results following pre-incubation for one hour with 5μM of cognate peptide. Results are representative of those seen in 3 different experiments. Error bars indicate one standard deviation from the mean.

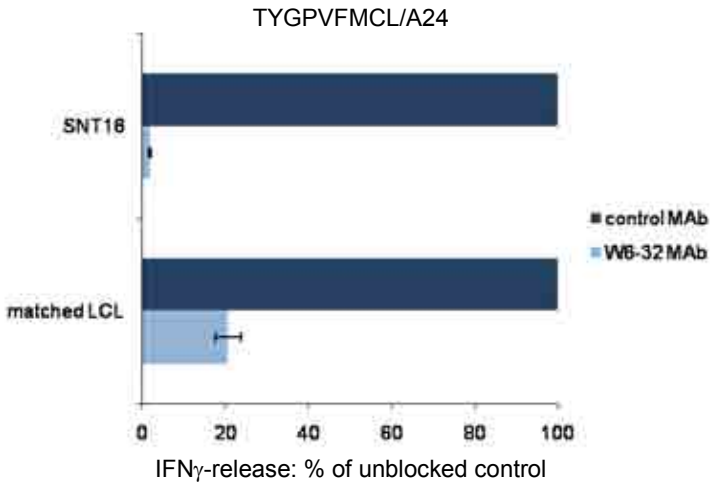


Figure 46 LMP2-specific recognition of SNT16 is restricted through HLA class I

$CD8^+$ clones specific for TYGPVFMCL (5000 per well) were co-cultured for 18 hours with 10^5 target cells (SNT16 and HLA-matched LCL). Targets were pre-incubated with mAbs specific for HLA class I (clone W6-32[683]) or controls (YE2/36-HLK antibodies[685]) for 1 hr before introducing effector T cells to the assay. IFN-γ release (ng/ml) is represented as a percentage of the control (class II MAb) result.

T cells by intracellular cytokine staining. These additional, labour-intensive experiments and retroviral cloning were performed by Miss Tracey Haigh, to complement a parallel project within the immunology group and allowed Dr C Fox to focus on the subsequent molecular characterisation work. A representative example of data, derived from Tracey Haigh's experiments is illustrated in Figure 47A and clearly demonstrates A11-restricted recognition of SNK6-A11 by the SSC clone. Notably, recognition of SNK6-A11 cells exceeded the recognition of an A11-matched LCL. Finally, to further substantiate the LMP2 specificity of SNK6-A11 recognition, a recombinant TCR, recently developed in our laboratory (S.P. Lee et al, manuscript in preparation) and specific for HLA-A*1101/SSCSSCPLSK was employed. The A11-SSC TCR, expressed in a non-SSC specific $CD8^+$ clone derived from a healthy donor, was tested against SNK6-A11 in a CFSE/IFN-γ–release assay as before. The results (Figure 47B) confirmed the specificity and magnitude of LMP2-recognition of the ENKTL-derived cell line. Taken together, the data in figures 45 and 47 clearly demonstrate LMP2-specific T-cell recognition and killing of

tumour cell lines derived from both CAEBV and ENKTL patients, through a number of epitopes in the context of 3 different HLA restrictions. The magnitude of T-cell recognition was comparable with an LCL in some instances, and was always greatly in excess of what might be expected from the apparent expression of LMP2A and LMP2B transcripts in the tumour lines (Fig 41C, Pg 195).

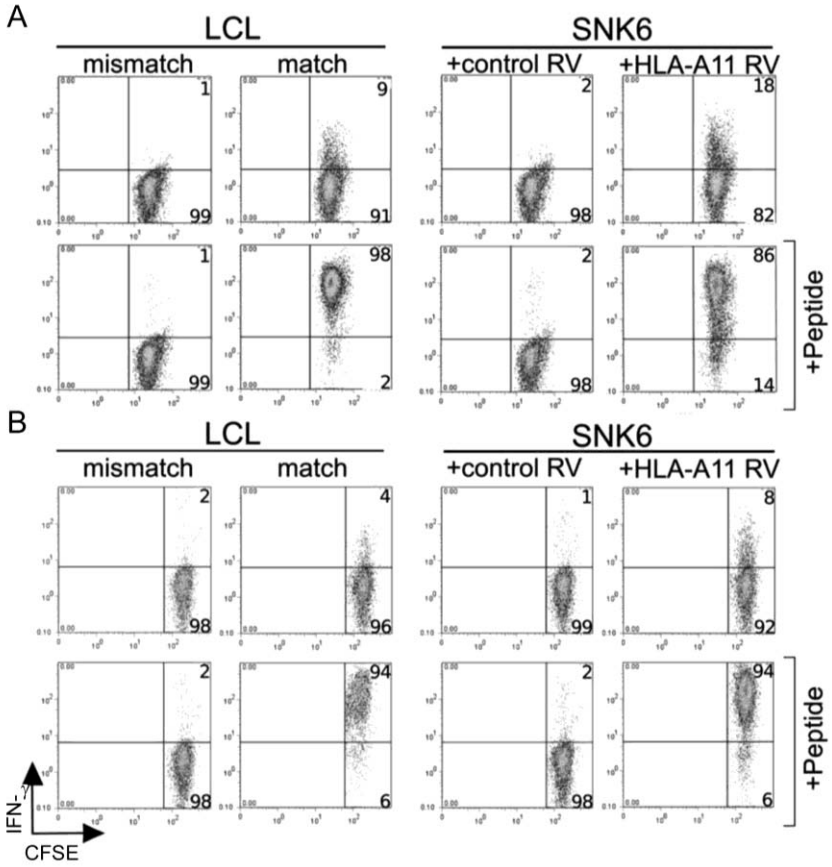


Figure 47 LMP2-specific T cell recognition of SNK 6 exceeds that of a LCL

Dual-colour flow cytometry: the x axis represents fluorescence from CFSE-stained $CD8^+$ effectors and the y axis denotes IFN-γ production as determined by intracellular staining. CFSE-negative cells (i.e. LCL or SNK6 targets) have been gated out of this analysis. HLA class I mismatched LCLs and SNK6 transduced with a control retrovirus (+control RV) are negative controls. The upper plots within panel A and B indicate T cells expressing IFN-γ in response to endogenously-processed peptide, whilst the lower plots of each panel show IFN-γ expression by T cells following pre-incubation of targets with exogenous cognate peptide. Numbers in the right quadrants indicate the percent of cells positive (upper quadrant) or negative (lower quadrant) for IFN-γ production. **Panel A** shows SSC-specific $CD8^+$ clones tested against HLA-matched LCLs and SNK6-A11. **Panel B** shows effector T cells expressing a recombinant SSC/A11 TCR tested against $A11^+$ LCL and SNK6-A11.

LMP2 epitope sequences in SNT16

Previous work analysing LMP2 sequences of virus derived from both healthy donors and EBV-associated tumours, across a range of ethnicities, had identified that LMP2 epitope-encoding sequences were largely conserved amongst isolates[390]. However, to be certain that no significant sequence changes were present in the SNT16 line that would preclude the observed T cell recognition, we sequenced the full–length LMP2 gene from DNA extracted from these cells. Figure 48 is an amino acid alignment of SNT16 LMP2A compared against the B95-8 and Chinese GD1 strains. The majority of defined $CD8^+$ epitopes are located in the transmembrane domains of the protein[391] (including the five epitopes shown). Aside from a single amino-acid substitution corresponding to a non-anchoring amino-acid in TYG and CLG there were no epitope sequence changes in SNT 16, reinforcing its legitimacy as a target for specific $CD8^+$ effectors.

We therefore resolved to further investigate expression of LMP2 in these cells to reconcile the immunologic data with the paradoxically low LMP2A and LMP2B transcripts.

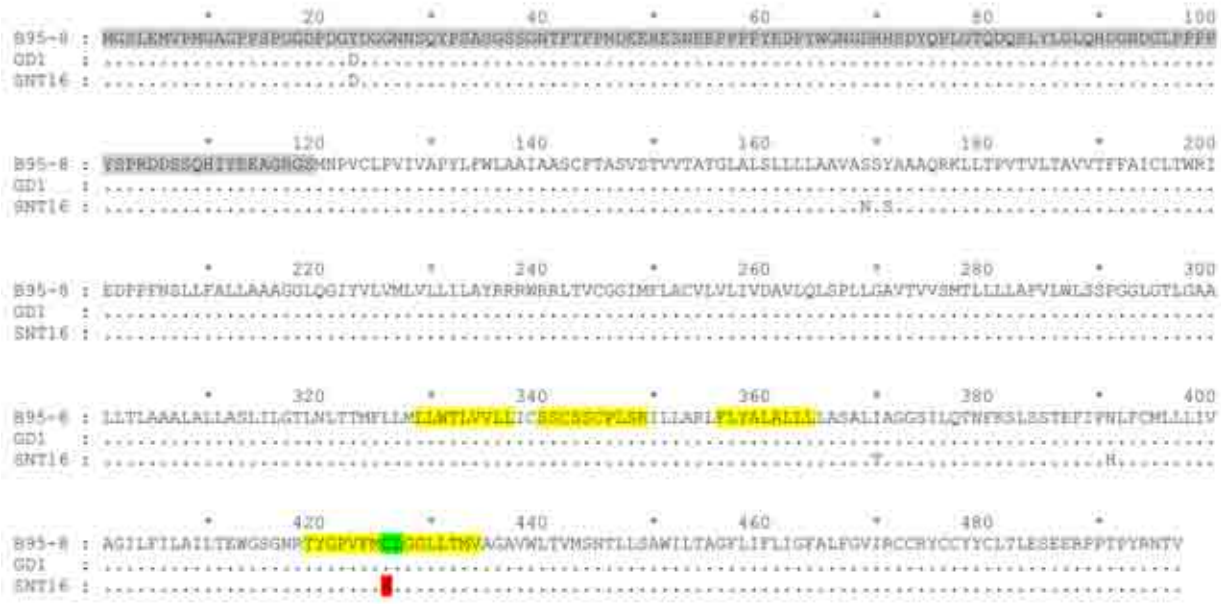


Figure 48 Epitope sequence conservation of LMP2 epitopes in SNT 16: legitimate CD8$^+$ T cell targets

Predicted amino acid sequence of the full-length LMP2A protein, derived from the coding exon sequences of SNT16, compared against the prototype B95-8 and Chinese GD-1 sequences. The LMP2B protein starts at amino-acid position 121. Each dot represents an amino-acid unchanged from the prototype sequence, whilst sequence changes resulting in amino-acid replacement are shown. The key for highlighted regions is as follows:

Grey: LMP2A N-terminus

Yellow: Epitopes for CD8$^+$ LMP2-specific T cells

Green: Amino-acids common to two epitopes

Red: Amino acid polymorphism

Identification of an alternative LMP2 transcript

The schematic shown in Figure 41C, (page 195) indicates the known exon structure and splicing pattern of conventional LMP2A and LMP2B mRNAs as seen in LCLs. To address whether alternatively spliced or variant LMP2 transcripts may be expressed in our NK- and T-cell lines, we initially used conventional end-point PCR to simply ask whether mRNA transcripts comprising exons 1-3 (straddling the variably sized terminal repeat region) and/or exons 2-6, (located 3′ to the terminal repeat region) were expressed in the tumour lines. While an exon 1-3 product was clearly present in the LCL control, such spliced LMP2 transcripts were undetectable in all 4 NK- and T-cell lines (Figure 49A top gel). By contrast, using a forward primer located in exon 2 and a reverse primer within exon 6 resulted in visible PCR products of the expected size in all 4 cases (Figure 49A bottom gel). Interestingly, similarly to LMP1 (Figure 28) the 2 ENKTL lines (SNK 6 and SNT 8) apparently expressed these LMP2 transcripts at levels comparable with an LCL, while the 2 CAEBV lines expressed much lower levels of LMP2 exons 2-6 (Figure 49B).

In light of this finding, we designed two new RT-Q-PCR assays that amplified LMP2 sequences within exon 2 and exon 6 and quantitated their relative expression compared with that seen in a LCL. We focused on exon 2 as it represents the first transcribed exon 3′ to the TR region and exon 6 as this encodes peptides recognised in the T-cell assays described earlier. We designed these assays against sequences within the respective exons to avoid exon-exon junctions in case of unidentified splice variants. We found that LMP2 transcripts containing exon 2 and exon 6 were highly expressed in SNK 6 and SNT 8, exceeding LCL levels, whereas lower levels corresponding to 5%-20% of LCL levels, were detected in SNK 10 and SNT 16 (Figure 5B). These results indicated expression of an alternative LMP2 transcript initiated upstream of exon 2 and pointed to an explanation for the initially paradoxical T-cell recognition data. To eliminate the unlikely possibility that these transcripts arose in the conventional LMP2B first exon (‘1B’) - thereafter ‘running through’ the TR region, un-spliced, to exon 2 – we designed a further quantitative RTPCR assay. Using a forward primer within exon 1B and a reverse primer in the 3′

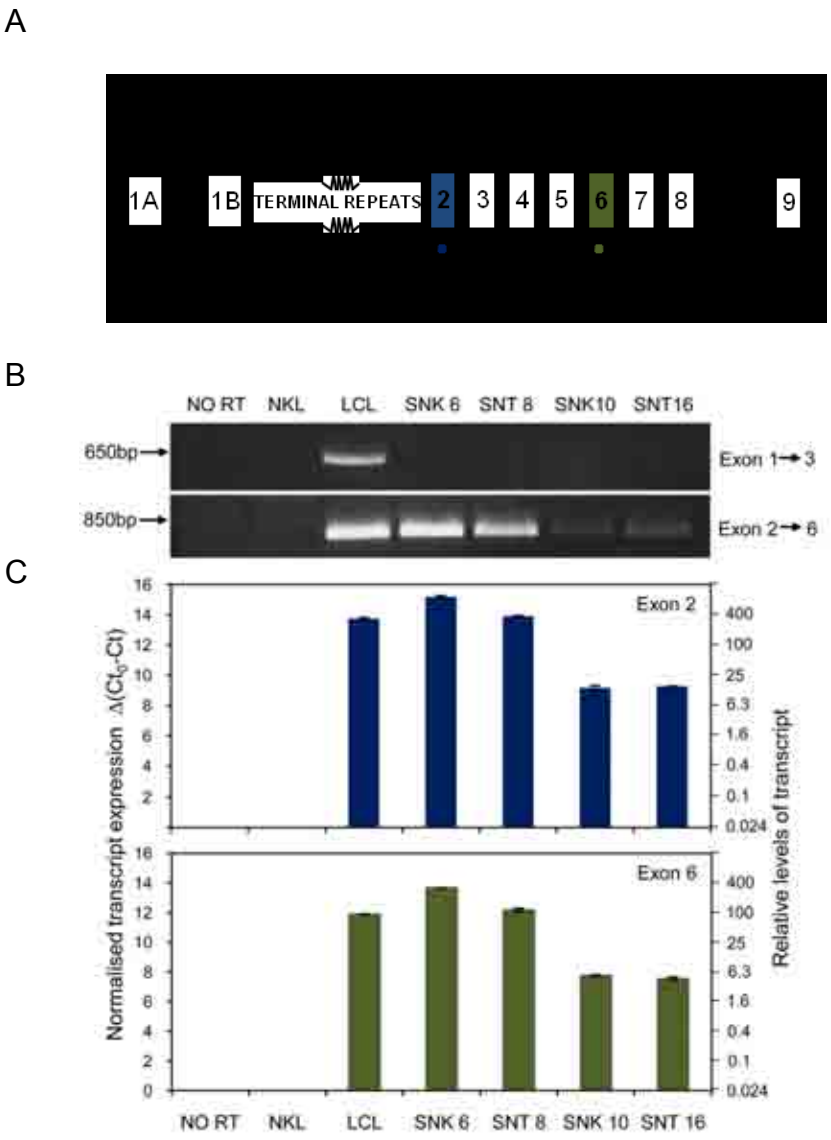


Figure 49 Expression of a LMP2 transcript containing exons 2 to 6 in EBV positive NK and T cell tumour lines

A: Schematic of LMP2 gene structure and location of RT-PCR primers. **B:** Products of conventional RTPCR (35 cycles) separated and visualised on a 1% Agarose gel. The upper panel shows products of RTPCR amplification using a forward primer in exon 1 and reverse primer within exon 3 (F1 and R3 – see supplementary table 1), whilst the lower panel shows results using a forward primer in exon 2 and a reverse primer within exon 6 (F2 and R6). **C:** Quantitative RT-PCR assays– using primers and probes designed within exons 2 and 6 respectively. Data is expressed using cycle threshold (Ct) values, where 'Ct^{0}' = assay threshold of detection. LMP2 transcript Ct values are normalised to GAPDH Ct values. The left-hand y axis represents $\Delta(Ct\text{-}Ct^{0})$, whilst the right hand axis shows the equivalent, relative levels of transcript.

non-coding region prior to the TR, we excluded the existence of significant levels of such an mRNA (data not shown).

Identification of 5′ sequences of the novel LMP2 transcript in ENKTL and CAEBV lines

To further investigate the origin of LMP2 transcripts in the NK and T tumour cells, we subjected SNK6 RNA to 5′ rapid amplification of cDNA ends (RACE). 5' RACE, or “anchored” PCR, is a technique that facilitates the isolation and characterisation of an unknown 5' origin of an mRNA template, starting downstream from a defined internal sequence within the transcript[688]. A schematic of the procedure is shown in Figure 9 (page 104).

We initially used a reverse primer within exon 6 (see table 5) for synthesis of first-strand cDNA, followed by nested gene-specific primers within LMP2 exons 5 and 4. The final nested PCR product was gel-extracted and cloned into TA vectors for sequencing of individual cDNAs (page 103). The sequences of two novel LMP2 cDNAs (identified in 4 separate cDNA clones obtained from SNK6) are shown in Figure 50, initiating upstream of the start codon of exon 2. For cDNA clone SNKR2B1d, the 5′ end was identified within the non-coding region upstream of exon 2, but not quite within the TR, whilst the longer SNKR2B1j sequence initiated within the last repeat of the TR region; the 5′ end is located 84 bases upstream of exon 2. Interestingly, the SNKR2B1j cDNA appeared to be ‘missing’ a segment compared to the genomic sequence but realigned at the start codon of exon 2. The significance of this observation was not further investigated at this stage. Importantly, when the same RACE experiment was performed on LCL RNA (analysed in parallel), we identified 5′ cDNA ends containing exon 1A or exon 1B upstream of the TR, indicating the expected pattern of mRNA splicing (Figure 51).

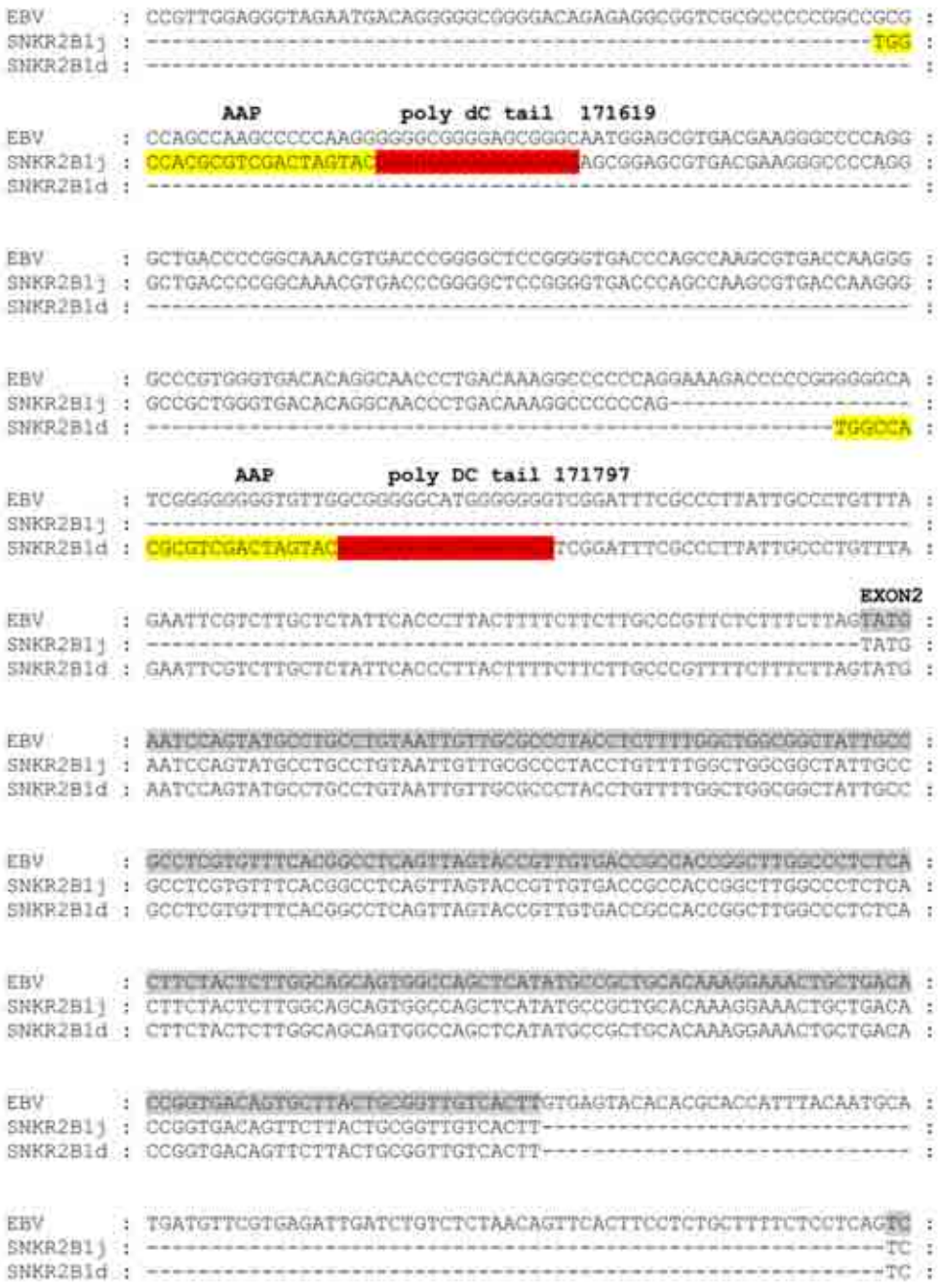


Figure 50 Identification of 5' ends of LMP2 transcripts by 5′ RACE of SNK 6 cDNA

5' RACE products from SNK 6 LMP2 cDNA. cDNA strands amplified from 2 of 7 isolated clones (SNKR2B1j and SNKR2B1d) are shown aligned to the B95-8 genome sequence. Grey shading indicates LMP2 coding sequence. Runs of G bases (shaded red) represent complementary sequence to the annealed poly-DC primer and correspond to the 5′ end of the isolated cDNA. The abridged anchor primer (AAP) sequence is highlighted yellow.

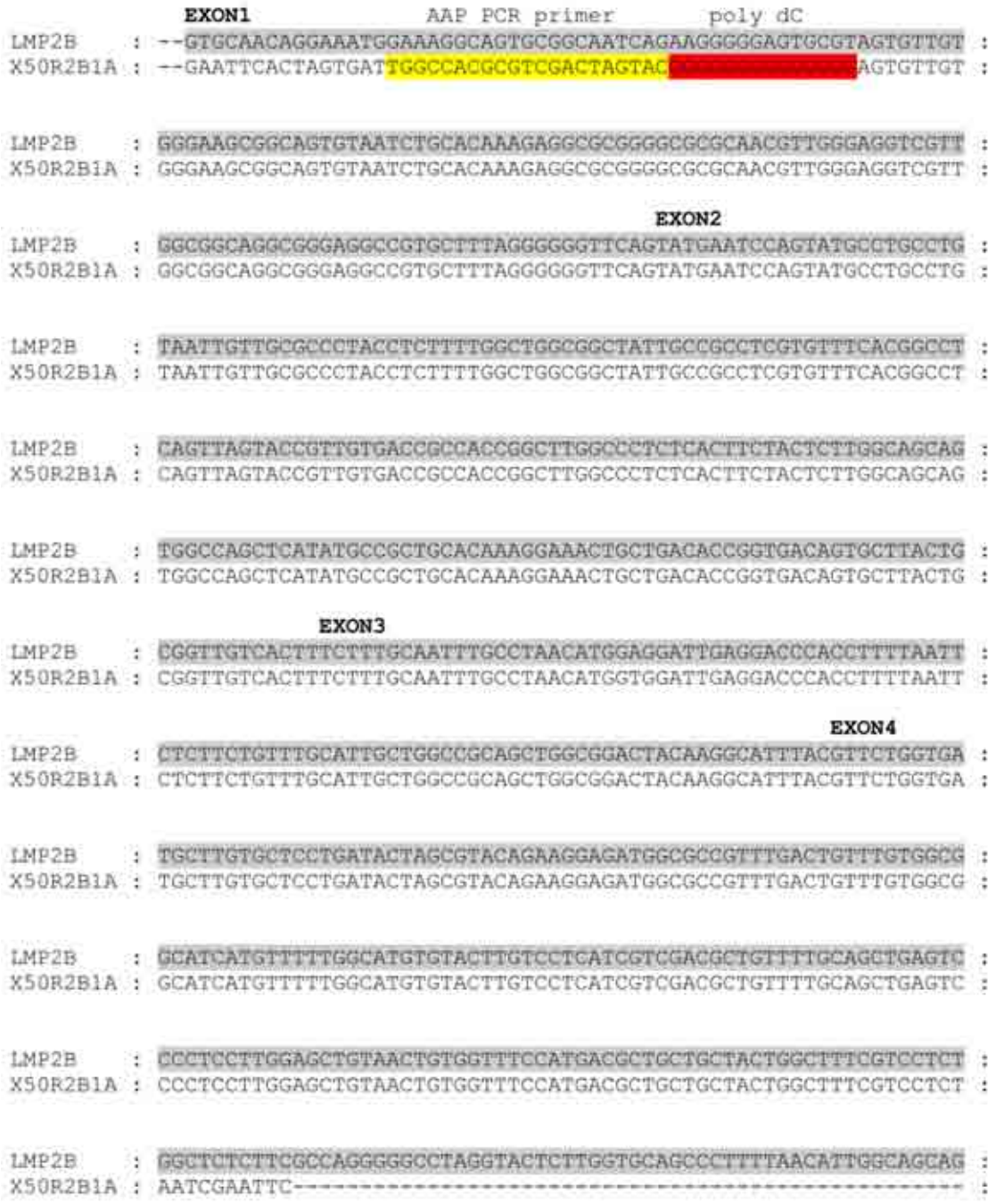


Figure 51 LCL 5′ RACE sequence: confirmation of LMP2 transcript initiation upstream of the TR from a conventional LMP2 first exon

5' RACE products from LMP2 cDNA of a LCL control. A representative cDNA clone is shown (X50R2B1A) aligned against B95-8 RNA sequence. The run of G bases (shaded red) represent complementary sequence to the annealed poly-DC primer and correspond to the 5′ end of the isolated cDNA. The abridged anchor primer (AAP) sequence is highlighted yellow.

Quantitation of novel LMP2 transcripts in T/NK lymphoproliferative disease

These results suggested that a previously uncharacterised LMP2 transcript may initiate from a hitherto unidentified promoter within the TR of the EBV genome and that this transcript (termed LMP2-TR), rather than the conventional LMP2A/2B mRNAs, is preferentially expressed in these NK- and T-cell lines. Figure 52A is a schematic showing the location of the 5′ ends of the novel cDNAs in the context of the LMP2 gene structure. To confirm that the LMP2-TR mRNA is the dominant LMP2 transcript in these cells, we designed a further quantitative RT-PCR assay using a forward primer in the intervening sequence between the TR region (see table 5, page 102, for primer sequences and co-ordinates) and exon 2; note this assay does not detect conventionally spliced LMP2A or LMP2B transcripts, both of which splice directly into the first ATG of exon 2. As this assay would also detect residual DNA, all RNA samples were treated with DNASE I and, moreover, parallel RNA samples without reverse transcriptase (no RT controls) were included to exclude the possibility of contaminating DNA being detected. The quantitative data from this new assay are presented alongside levels of conventionally spliced LMP2A and LMP2B transcripts in Figure 52B. The novel LMP2-TR mRNA was by far the dominant LMP2 transcript in all 4 ENKTL and CAEBV cell lines.

We next analysed cDNA derived from an additional series of ENKTL and CAEBV cell lines (YT, NKYS, HANK1, KA13, SNK1, SNT13[713]). For these cell lines and the subsequent PCR analyses of primary tissue (see below) the exon 6 forward primer was substituted for a primer spanning the exon 5-6 junction, as the RNA from these samples had not been not treated with DNAse I, and included a no RT control as before. In this extended panel of cell lines we found a similar pattern of LMP2 transcript expression, in which LMP2-TR dominated in all cases (Figure 53). Consistent with previous results, where seen in occasional T/NK cell lines, expression of conventional LMP2A and/or LMP2B transcripts was substantially lower (≥100-1000 fold). Notably, the LMP2-TR mRNA was also transcribed in EBV-transformed B cells, albeit at 30 to 60-fold lower levels than in SNK 6 and SNT 8 (Figure 52B).

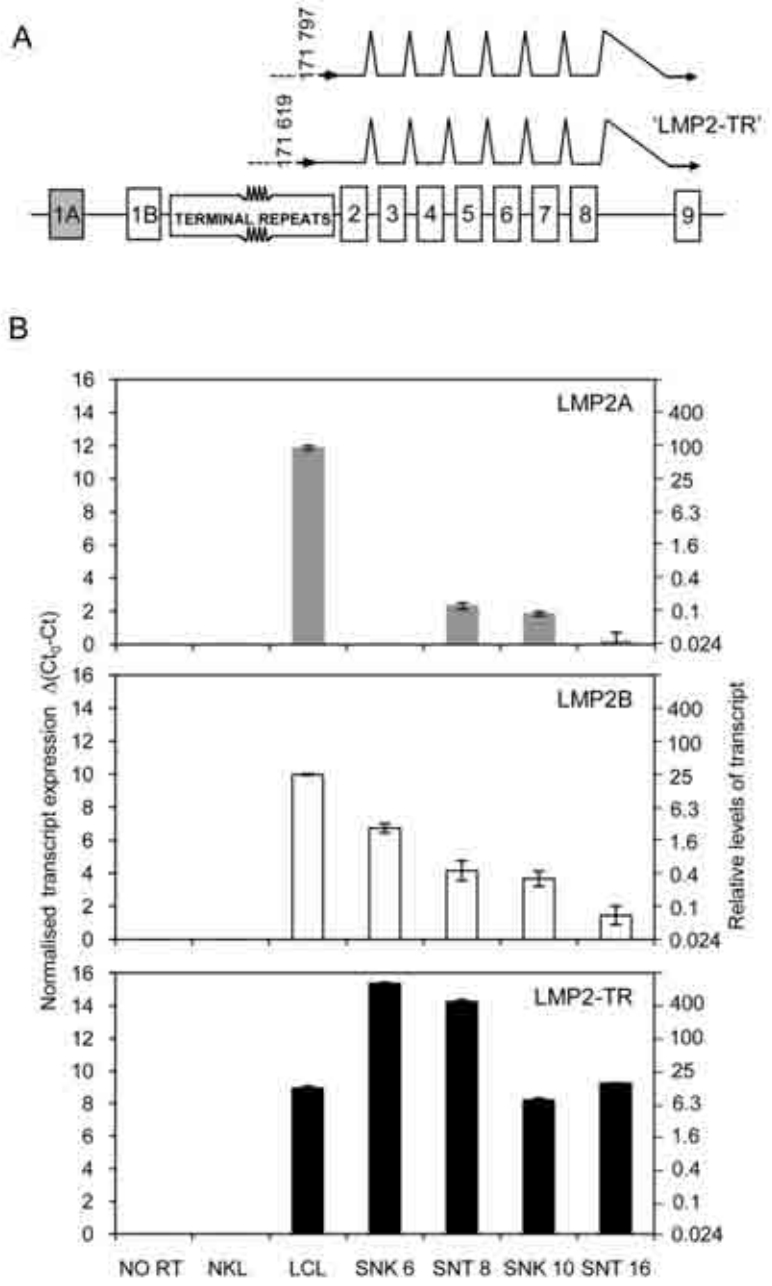


Figure 52 Identification of 5 ends by RACE and quantitation of novel LMP2-TR transcripts in NK and T-cell tumour lines.

(A) LMP2 schematic: numbers indicate the exact 5 ends of 4 of 7 cDNAs identified by RACE (EBV genome coordinates: NC007605). Three other 5 cDNA ends identified in SNK 6 (coordinates 19, 80, and 83) correspond to the intervening intron and beginning of exon 2.

(B) Quantitative RT-PCR results of conventional LMP2A (grey), LMP2B (white) and the novel LMP2-TR (black) transcripts. LMP2 Ct values are adjusted for GAPDH Ct values. Ct_0 is the assay threshold of detection. The left y-axis represents (Ct_0-Ct), while the right-hand y-axis shows the equivalent fold difference in transcript levels.

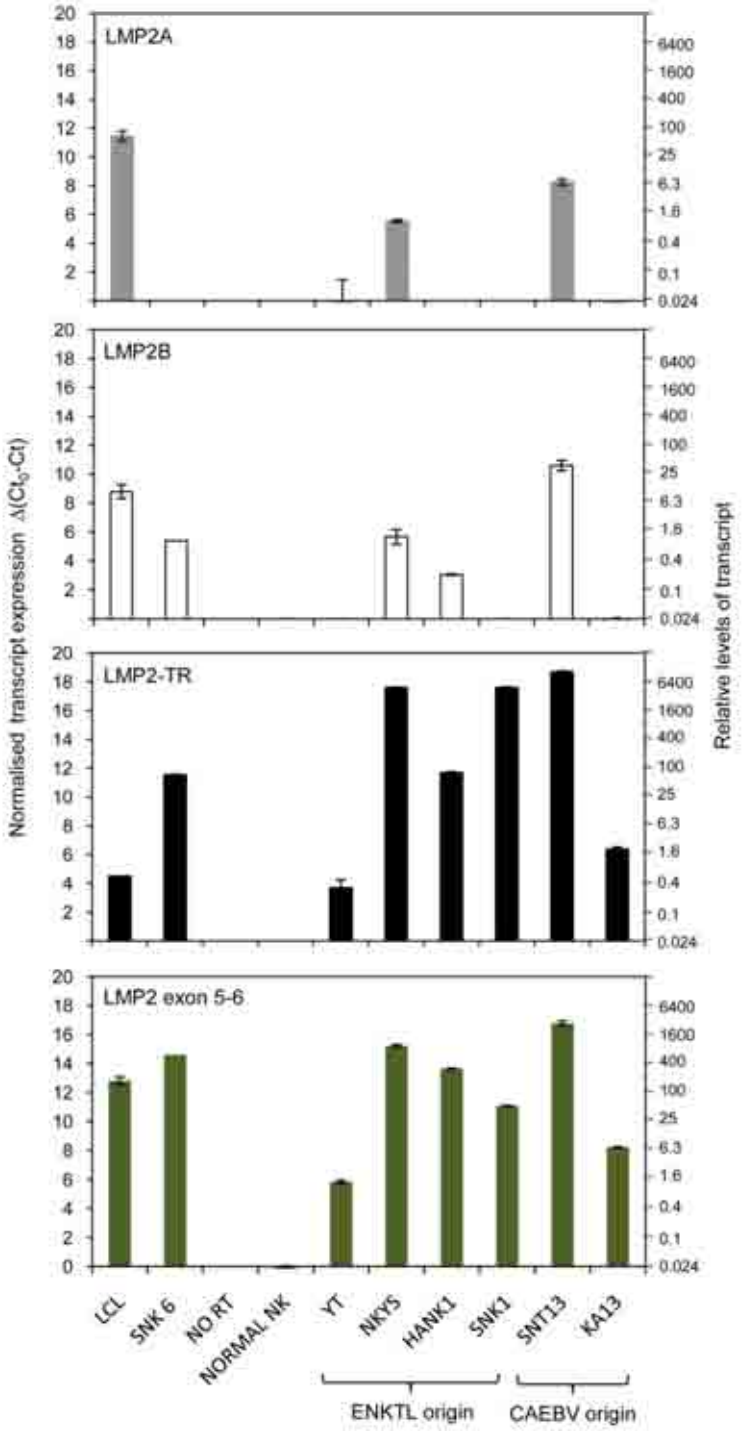


Figure 53 LMP2-TR is the dominant LMP2 transcript in an extended panel of ENKTL and CAEBV cell lines

Quantitative RT-PCR results of conventional LMP2A (grey), LMP2B (white), novel LMP2-TR (black) and 'total' LMP2 transcripts (green) as judged by the exon5/6 assay. Note that for these PCR assays, input cDNA was 2µl in a reaction volume of 20µl, compared to 5µl in 25µl for the cell line PCR studies for Figures 47 and 50.)

Recognising that observed patterns of gene expression in cell lines *in vitro* may not necessarily be representative of the situation in primary tissue, we turned to biopsies from patients with ENKTL. By means of immunohistochemical staining with the 15F9[131] LMP2A MAb, we found no evidence of LMP2A protein expression on formalin fixed biopsy sections from a total of 7 ENKTL patients (3 such examples shown in Figure 54A), while an EBV^+ polymorphic PTLD biopsy included as a positive control demonstrated characteristic membrane/cytoplasmic staining in a subpopulation of cells (Figure 54A). Because the LMP2 MAbs engage with the N-terminus of LMP2A, detection of LMP2B (and LMP2-TR) is only possible by RT-PCR. We therefore examined a second set of biopsies from which good quality RNA, extracted from frozen material, was available[713]. While conventional LMP2A and LMP2B transcripts were absent or virtually undetectable, LMP2 transcripts were readily detected in 7/7 ENKTL biopsies using RT-Q-PCR assays that amplified LMP2 exons 5-6 and TR-initiated transcripts. No signals were seen in (EBV-negative) normal NK cells, or in a single case of EBV^+ aggressive NK leukaemia (Figure 54B). For the latter ANKL case, we confirmed the EBV positivity of this tumour by demonstrating high levels of EBER expression by RT-Q-PCR (data not shown).

Although the exact transcription initiation site for the novel LMP2-TR transcript in ENKTL and CAEBV cells was not precisely mapped, the results of the RT-PCR and RACE experiments infer that the promoter lies within the TR region. This situation is somewhat analogous to LMP1 expression in Latency II NPC cells where transcription is initiated from a promoter within the terminal repeats[109] and raised the possibility that, similarly to the bidirectional LMP1/LMP2B promoter active in LCLs[126], another bidirectional promoter in the terminal repeats might give rise to both LMP1 and LMP2 transcripts. On this point, we considered the transcriptional regulation of LMP1 mediated by IL2 and other cytokines in the SNK 6 cell line (as presented in Results part II, Figure 31, page 173) and asked whether LMP2-TR levels were similarly influenced. Using the same RNA samples as analysed in the assay of Figure 31, whereby SNK6 cells were deprived of IL-2 stimulation over several days followed by re-stimulation with IL2 or IL15, we also quantitated LMP2 transcripts by RT-Q-PCR.

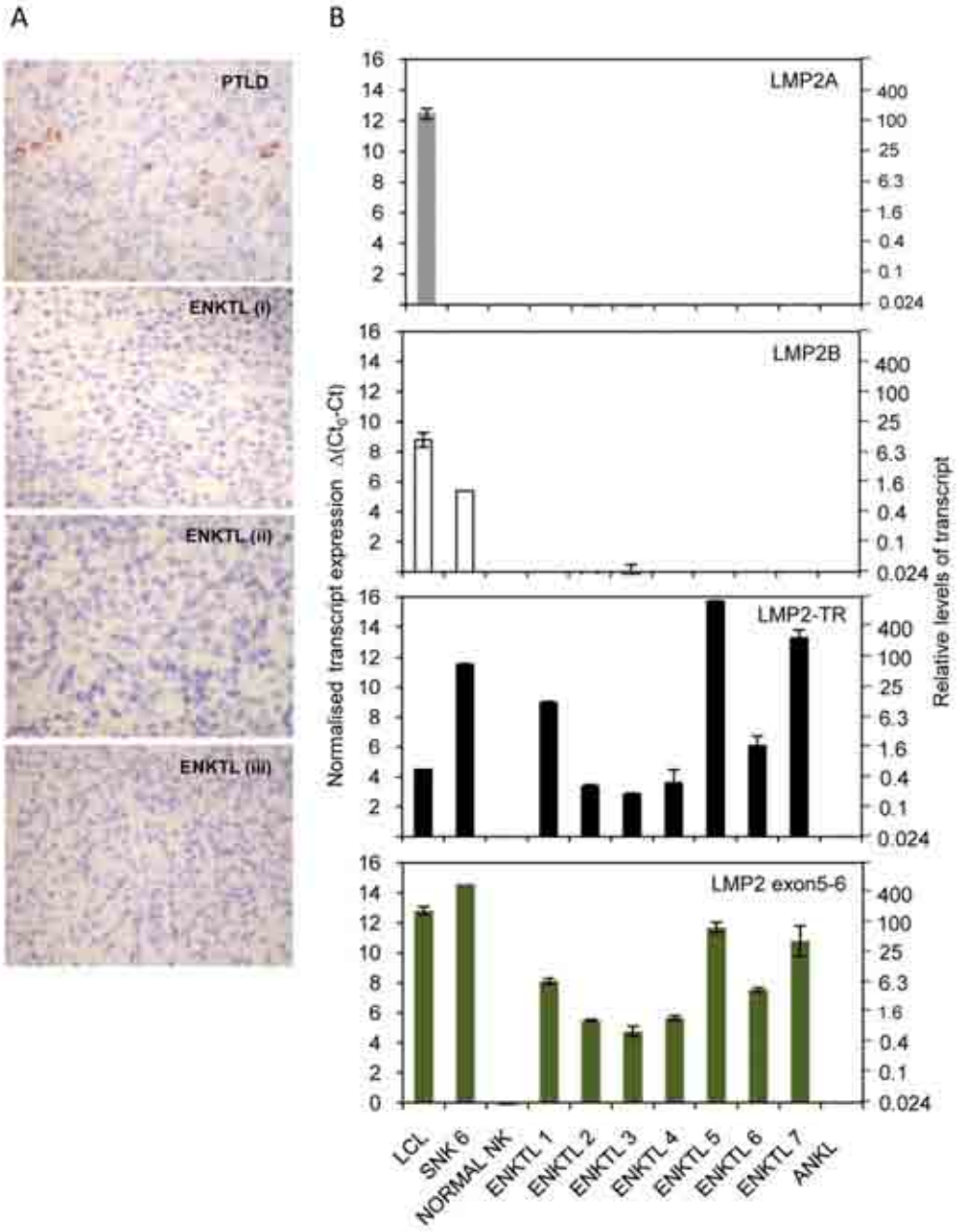


Figure 54 LMP2-TR, but not LMP2A or LMP2B, are expressed in primary ENKTL tissue

A Immunohistochemistry of FFPE tissue sections with antibody clone15F9, specific for LMP2A. Images were captured with a Nikon Coolpix E995 digital camera, via a Nikon Eclipse E400 microscope (optical magnification x400). A case of EBV^+ B cell PTLD is a positive control. Sections from 3 representative cases of ENKTL (i-iii of seven) shown.

B. Quantitative RT-PCR data using cDNA derived from 7 primary ENKTL frozen tissue biopsies and 1 EBV^+ aggressive NK leukaemia (ANKL) sample. Note that for these PCR assays, input cDNA was 2µl in a reaction volume of 20µl, compared to 5µl in 25µl for the cell line PCR studies for Figures 47and 50.

Figure 55 shows RT-Q-PCR data from LMP2 exon 6 and LMP2-TR assays performed on these samples and compared against LMP1 mRNA levels. For each assay, normalised transcript values are expressed relative to day 0 which was assigned a value of 1. Mirroring the pattern seen with LMP1 transcripts, the LMP2-TR and exon 6 transcript levels fall rapidly in the absence of IL-2 and do not recover spontaneously in its absence. Similarly to LMP1, LMP2-TR transcript levels are re-established with IL-2 re-treatment and, to a lesser extent, with IL-15 treatment. With these dynamic expression data in mind, we quantitated LMP1 transcripts in a number of T/NK cell lines and the same primary ENKTL tissue samples as analysed in Figure 54B. Figure 56 compares the Δct values (adjusted for GAPDH levels) of LMP1 with LMP2-TR and shows that in a majority of both cell lines (panel A) and primary tissue (panel B), a clear correlation of the two transcripts is apparent. The notable exceptions were primary biopsy cases ENKTL 6 and ENKTL 7, with low and high levels of LMP2-TR respectively, where no LMP1 transcripts were detectable.

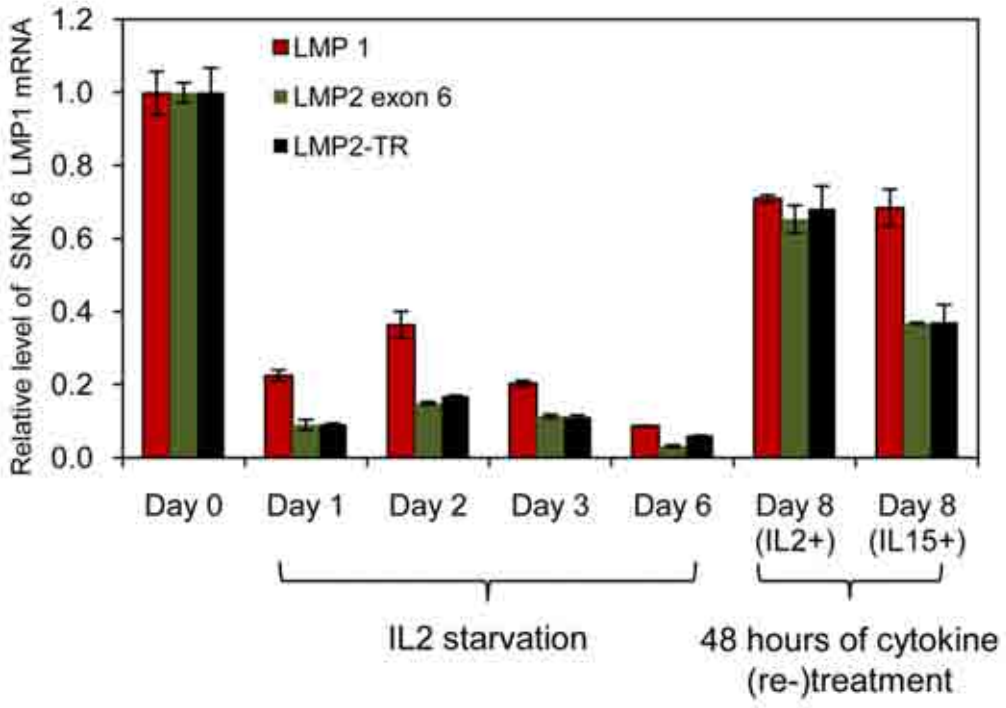


Figure 55 Expression of LMP2-TR is dependent on exogenous cytokines and mirrors expression of LMP1 in the SNK6 cell line

RT-Q-PCR data of LMP1 and LMP2 mRNA levels in the SNK6 cell line. Transcript levels were normalised to GAPDH and represented relative to a nominal day 0 value of 1.

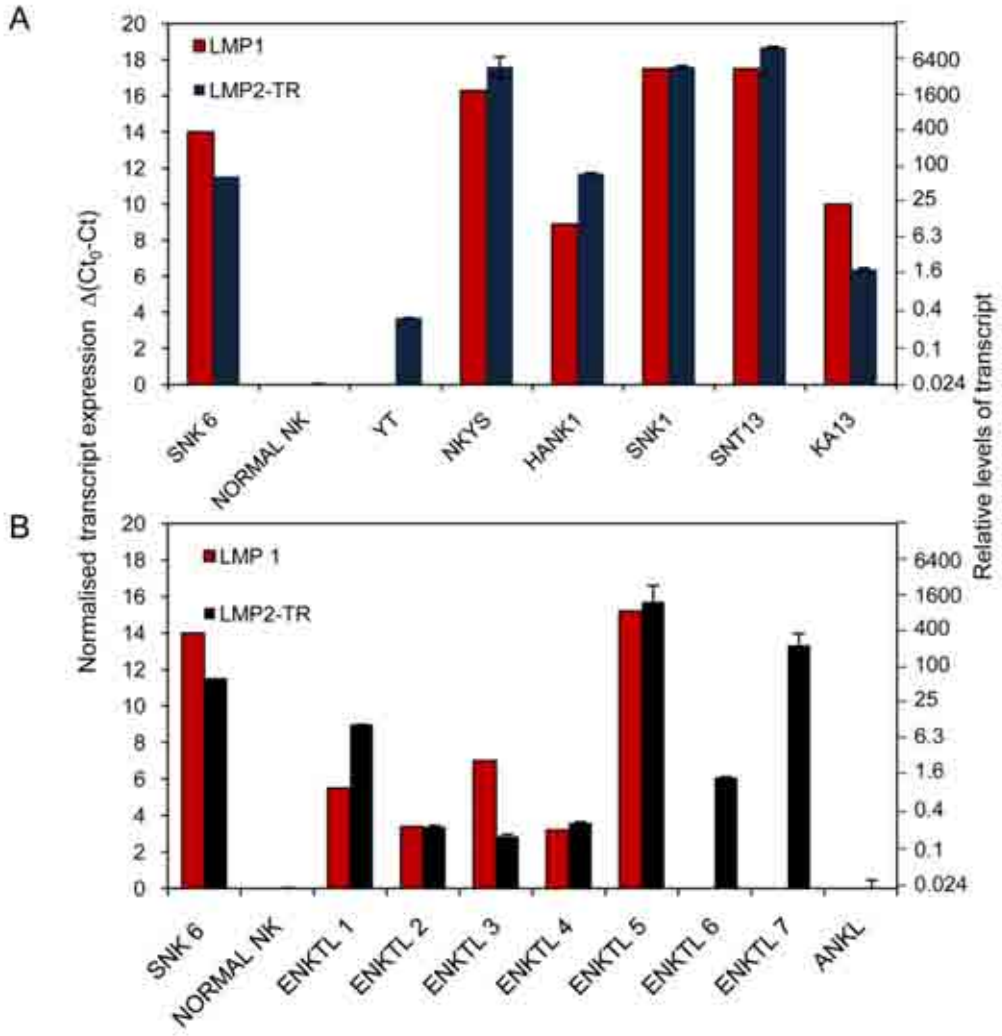


Figure 56 LMP2-TR expression closely correlates with LMP1 expression in a panel of ENKTL and CAEBV cell lines and primary ENKTL tissue

RT-Q-PCR data of LMP1 and LMP2-TR mRNA levels in ENKTL and CAEBV cell lines (panel A) and primary ENKTL biopsies (panel B). LMP transcript Ct values were normalised to cellular GAPDH Ct values. The left hand y axis represents $\Delta(Ct\text{-}Ct_0)$, whilst the right-hand axis shows the equivalent fold difference in transcript levels. Ct_0 represents the assay threshold of detection. (Note that for these PCR assays, input cDNA was 2µl in a reaction volume of 20µl, compared to 5µl in 25µl for the cell line PCR studies for Figures 47 and 50.)

Discussion (III)

Conventional LMP2 expression and T cell recognition of T/NK tumour lines

Results from ongoing clinical studies targeting EBV-encoded proteins in 'Latency II' malignancies have shown promising results[395,651]. However, in contrast to HL[131] and NPC[132], where the most immunogenic of the available target proteins, LMP2, is demonstrably expressed, the situation in NK and T lymphoproliferations remained to be clarified. A single study previously reported the presence of conventional LMP2A and LMP2B mRNAs by non-quantitative PCR in some ENKTL biopsies, although in the majority of their cases the expression of these transcripts appeared low or absent[73].

Our results showed that ENKTL and CAEBV cell lines had the machinery for antigen-processing and presentation but, interestingly, evidence of LMP2A protein expression was not apparent. Moreover, conventionally spliced LMP2A and LMP2B transcripts were virtually undetectable using sensitive reverse transcriptase (RT)-Q-PCR assays[37,133]. Although substantial variations in LMP2 sequences are uncommon[390-391], exon 2 polymorphisms (relative to B95-8) have been described in NPC[156] and we therefore applied an alternative RT-Q-PCR reverse primer to address these sequence changes. Additionally, we undertook sequencing of the exon 1 and exon 2 segments in the 4 NK and T cell lines to encompass the regions corresponding to the PCR primers and probe. In three cases, no relevant polymorphisms were seen, whilst the single-base changes SNT16 were accounted for by the alternate reverse primer[156].

The apparent absence of LMP2 expression in these cells raised serious concerns about targeting of this viral antigen *in vivo* with cellular immunotherapies. However, our unexpected *in vitro* observation was difficult to reconcile with preliminary data from trials of adoptive transfer of LMP2-containing CTL preparations in ENKTL patients where frequent evidence of clinical response, including sustained complete responses in some cases, was emerging[651]. Given the

anticipated prognosis of patients with relapsed/refractory ENKTL, whereby very few patients are salvaged even with intensive chemotherapy[473], these clinical outcome data were compelling.

We thus pursued our interest in LMP2 as an immunotherapeutic target by initially asking whether the same therapeutic polyclonal CTL products, generated by *in vitro* LMP2-stimulation[396] of PBMC from ENKTL and CAEBV patients, could recognise and kill class I-matched T/NK lines. Specific tumour cell lysis by such CTL products containing LMP2-specificities was indeed observed. We could not be certain however, that such killing was LMP2-specific given the nature of the *in vitro* stimulation protocol; the contribution of minor LMP1 specificities, for example, could not be excluded. Moreover, some of these polyclonal lines contained appreciable numbers of CD4 effectors which could have contributed to the observed killing. On this note, HLA class I mismatched CTLs from two donors were seen to kill SNT16, whilst a class II mis-matched LCL in the same assay was not killed (Data not shown; Dr C Bollard, personal communication June 2010); raising the possibility of CD4-mediated killing of SNT16.

Encouraged by these suggestive data, we confirmed the LMP2-specificity by showing clonal $CD8^+$ LMP2-specific T cell recognition and killing of SNT16 and SNK6 via a number of distinct transmembrane epitopes, across three HLA restriction elements, in a range of T cell assays. The class I specificity of the interactions was assured by HLA class I blocking experiments and, critically, by employing a recombinant A11/SSC TCR.

These persuasive immunologic data, particularly in the case of SNK6 where the magnitude of T cell recognition was comparable to or exceeded that of an LCL, strongly implied that LMP2 was indeed expressed in the T and NK tumour lines, but a paradox was presented by the mRNA and protein data. In common with the vast majority (>90%) of known LMP2 class I epitopes, those tested in our work correspond to transmembrane regions of the LMP2 protein[391] - common to both LMP2A and LMP2B - and thus could not help distinguish between the two forms. Although the majority of LMP2 epitopes appear to be conserved among different isolates we confirmed the

sequence conservation of SNT 16 LMP2 (figure 48, page 205), underscoring its validity as a target for the clones tested.

Investigating expression of an alternate LMP2 transcript

Given the convincing immunologic evidence, we were impelled to more critically examine the expression of LMP2 in the T and NK tumour cells. It became apparent that a LMP2 transcript, originating upstream of exon 2, was indeed highly expressed in the ENKTL cell lines (SNK6 and SNT8) and at lower levels in the CAEBV lines (SNK10 and SNT16). Notably, the level of expression in SNK6 and SNT16 appeared to correlate with the magnitude of T cell recognition in the assays described. These novel LMP2 transcripts appeared to initiate within the TR region of the viral genome, although the alternate LMP2 promoter was not precisely mapped.

The longest cDNA isolated from the 5′ RACE procedure initiated >80 bases upstream of exon 2, but curiously appeared to be missing a segment of intervening, non-coding sequence (figure 50, page 209). This was not investigated at this time but could be potentially explained by alternative splicing into the first ATG of exon 2. Targeted quantitation of LMP2 transcripts arising from within the TR confirmed that LMP2-TR was clearly the dominant form expressed in a total of 10 ENKTL and CAEBV lines examined, where levels of conventional LMP2A and 2B were very low or absent.

Crucially, this pattern of expression was mirrored in ENKTL primary tissue biopsies; an absence of LMP2A protein and LMP2A/2B mRNA, whilst TR-initiated LMP2 was appreciable in all cases examined. We found substantial variations in the LMP2-TR transcript levels and, although we did not have access to matching tissue sections to appraise the extent of tumour infiltrate in each case, the implication is that significant heterogeneity of expression exists between biopsies. Of note, in some cases (ENKTL 5 and ENKTL 7, Figure 54, page 215) LMP2-TR expression exceeded that of the SNK6 line, where the cell population is demonstrably 100% EBV^{+}. We did not have the opportunity to examine primary cells from CAEBV cases, but note with interest a recent quantitative study that examined viral gene expression in PBMC from Japanese CAEBV

patients[563]. In contrast to the majority of published reports, the authors used a LMP2 PCR assay that targeted sequences in exon 6 and would therefore have detected all forms of LMP2 including, albeit inadvertently, the novel LMP2-TR transcript. However, due to differences in methodology and representation of their data, direct comparison with our results is difficult.

The LMP2-TR mRNA is predicted to encode a LMP2B-like protein - the N-terminus of LMP2A will certainly be absent from the translated product - and thus cannot be detected by currently available antibodies[130-131]. Consequently, at the present time, we are unable to determine the pattern of LMP2-TR expression at the single cell level. It would be particularly interesting to determine whether expression is uniform throughout the tumour cell population or, similarly to LMP1 protein expression in ENKTL, is restricted to a sub-population of malignant T/NK cells. On this point we considered the L1-TR promoter identified in NPC[109] that, if bidirectional, could give rise to both LMP1 and LMP2-TR transcripts – akin to the LMP1/LMP2B promoter in LCLs[126]. Consistent with this possibility, we observed an intimate association of LMP1 and LMP2-TR mRNA levels in cytokine-manipulated SNK6 cells and, moreover, a clear relationship between LMP2-TR and LMP1 mRNA levels in primary ENKTL tissue was apparent. Although these observations require careful investigation and further characterisation, they suggest the existence of a co-regulated LMP1/LMP2 promoter within the TR region. The two observed exceptions to this were ENKTL cases 6 and 7, where LMP1 mRNA appeared absent despite substantial levels of LMP2-TR. We did not have matched biopsy material to address LMP1 expression at the protein level in these cases, but our inability to detect transcripts may relate to sequence variation; LMP1 is recognised to be highly polymorphic gene[730-731]. Primer sequences for the LMP1 PCR assay were originally chosen in conserved regions spanning the exon 2–exon 3 junction, identified by sequence analysis of a panel of virus isolates from different geographical regions[133]. These primers were used in conjunction with two alternative probes specific for either the B95-8 or CAO LMP1 alleles, but in neither case were LMP1 transcripts amplified in ENKTL 6 and 7 (Figure 56, page 218). However, other unknown sequence changes cannot be excluded as an explanation for these data.

We cannot be certain the putative LMP2-TR promoter is present in each of the 538bp TR sequences, although this may be anticipated from their repetitive nature[27]. The alternate LMP1 promoter (L1-TR) identified in a study of NPC[109] was not found beyond the first TR, but the nature of the RACE technique – whereby the smaller of the isolated transcripts are likely to be preferentially amplified – cautions against this interpretation and is also relevant to our findings. If multiple copies of the LMP2-TR promoter, corresponding to the variable number of TRs, do indeed exist then it would be interesting to examine the impact of TR number on transcript expression. Within individual donors, different B-cell clones can differ by as many as 15 to 20 TR sequence reiterations[732]. For reasons that are not yet clear, a recent study of epithelial cells unexpectedly determined an inverse relationship between LMP1 levels and TR number; increased activity of the L1-TR promoter was seen at lower TR reiterations. On this point it is interesting to recall the original descriptions of the SNK and SNT lines[562] whereby SNT8 and SNK6 (the ENKTL lines with high LMP1 levels) appear to have a fewer TRs (by Southern blot analysis) than the CAEBV lines SNK10 and SNT16 which express lower levels of LMP1 (and LMP2-TR). These observations add a further level of complexity to regulation of both LMP1 and LMP2-TR expression and require further study.

Although the L1-TR promoter is demonstrably transcriptionally active in NPC[68,81,108] and HL[108], quantitative data is lacking[124,108] and it thus remains somewhat uncertain which LMP1 promoter dominates in these cell types. Indeed the same question can be applied to LCLs, where the relative contribution of the L1-TR promoter to LMP1 mRNA expression is unclear. Although some caution should be applied when directly comparing RTQ-PCR data from different assays (due to variations in, for example, PCR efficiency), our analyses of LMP2 expression in LCLs suggest that the putative LMP2-TR promoter generates a small but significant contribution to the 'total' LMP2 levels within a LCL (Figure 52, page 212). It remains to be clarified why the virus has evolved to have two different promoters to drive expression of LMP2B-like and LMP1 proteins. Expression of conventional LMP2A and 2B cannot occur without circularisation of the viral DNA[125], but our data suggests that LMP2-TR could be transcribed from the linear viral genome

immediately following infection. Studies in B cells have shown that viral episomes are detectable in the nucleus after 16-24 hours following *in vitro* infection[41,53], raising the question as to what benefit the virus would derive from a more rapid production of latent membrane transcripts immediately following cell entry.

We are not aware of previous reports describing naturally high levels of LMP2B in the absence of LMP2A. Although the function of LMP2B in B cell and epithelial malignancies is ill-understood (page 16), evidence of a negative regulatory effect upon LMP2A signalling[151] has been presented. The high level of LMP2-TR transcripts (and LMP2B protein as detected by LMP2-specific effector T cells) in the absence of detectable LMP2A, in both primary ENKTL tissue and derived cell lines, suggests that the LMP2B protein is likely to have another hitherto unidentified function that might be involved in lymphomagenesis. Studies in epithelial cells have suggested that, rather than simply modulating the activity of LMP2A, LMP2B may directly contribute to the attenuation of cellular IFN responses[155]. The function of LMP2A has been well characterised in B cells where a major function - mimicry of BCR signalling - appears to confer a survival advantage[137]. However for cells that lack a BCR - such as T and NK cells - such functions may be dispensable. Moreover, as the experimental data examining LMP2B function are derived from studies of B cells (and epithelial cells), these findings cannot necessarily be extrapolated to the T/NK setting where LMP2A is absent.

Our preliminary observations indicating co-regulation of LMP1 and LMP2-TR expression in T/NK cells (Figures 55 and 56, pages 217-8) could indicate functional complementarity of the two proteins or participation in a feedback mechanism to regulate the putative bidirectional promoter. Further work is clearly required to ascertain the function of the translation product of LMP2-TR (predicted to be LMP2B or LMP2B-like) in NK and T cells and to delineate its phenotypic impact from that of LMP1. Notwithstanding this, the consistent expression of LMP2-TR as the dominant LMP2 mRNA in a range of cell lines and primary tissue, in some cases exceeding cell line levels, implicates this truncated protein in the initiation and/or potentiation of T/NK lymphomagenesis.

As an immunotherapeutic target, our data support the current clinical approaches of LMP2-directed targeting of T/NK lymphoproliferations and, potentially, provide a basis for 'screening' patients (via PCR analyses on biopsy material) prior to embarking on such therapies. However, the lack of a suitable antibody to allow routine detection of LMP2B in tissue sections currently limits the applicability of such an approach.

Final conclusions

Factors contributing to the pathogenesis of T and NK lymphoproliferations

By comparison to EBV-associated lymphomas of B cell origin such as BL and HL, a substantially smaller body of literature has reported on investigations into the pathogenesis of EBV-associated T and NK lymphoproliferations. This may be partly accounted for by their relatively recent characterisation and incorporation into international lymphoma classification systems[474,569], compounded by their comparative rarity[250-252,374,473,561,615-618], particularly outside East Asia[473]. However the importance of these diseases, both biologically and clinically, can be appreciated by three essential observations. First, the strength of the association with EBV; virtually 100% of ENKTL and ANKL cases contain clonal EBV[460-462,242-243,245,463-471], irrespective of geographic origin, such that the absence of virus would cast doubt on the accuracy of diagnosis of these entities[474]. Indeed, such an association is more consistent than the much-studied EBV^+ HL and ('non-endemic') BL. Second is the broad clinical spectrum of disease, manifest across a wide range of age groups: EBV-HLH, often associated with primary infection, that can present within the first few years of life[374]; CAEBV that presents most commonly in children at a mean age of approximately 11 years[561]; and ANKL and ENKTL that most commonly present in the 5^{th} and 6^{th} decades of life. Third is the poor prognosis associated with all these disease entities; a feature common to this range of lymphoproliferations is an inherent resistance to conventional cytotoxic agents. Interestingly, this latter point contrasts strikingly with HL and BL which are amongst the most chemosensitive of all malignancies.

Of further interest is why infection of similar or identical cell types is associated with such a diverse spectrum of clinical illnesses, occurring both in previously EBV-naive and ostensibly EBV-immune individuals. For example, NK cell infection may give rise to CAEBV, ENKTL or HLH. Although a relationship between the phenotype of the infected cell and the nature of the clinical presentation (at least for the systemic childhood lymphoproliferations) has been suggested[471-472],

such delineation may not be absolute. Our investigation of EBV-HLH in adult patients{Fox, 2010 #2146} suggests that more detailed phenotypic analyses of EBV-infected cells are required before definitive conclusions can be drawn. The importance of these analyses relates to understanding the biology of the normal cell counterpart of the disease in question as well as diagnostic and classification considerations. Initial misapprehensions regarding the T cell origin of ENKTL, due in part to diagnostic technicalities, have turned out to be only partly incorrect. It is now understood that although the majority of tumours are of NK origin, a significant minority are derived from cytotoxic T cells. Work in this area is still active: a very recent study has, by gene expression profiling, further defined a functionally distinct subset of γδ T cells as giving rise to a minority of cases classified as ENKTL[733].

Irrespective of the cell phenotype, cellular genetic abnormalities in CAEBV and ENKTL are a consistent finding. Chromosomal aberrations, identified by both conventional and high-resolution methodology, are frequent and often complex in these diseases[556,713]. Although not well characterised, recent studies have highlighted the potential contribution of some of these abnormalities to disease pathogenesis[713,733-734]. Furthermore, such observations on cellular abnormalities raise fundamental questions relating to early events in pathogenesis: is the cell genetically unscathed at the time of viral entry, or are pre-existing abnormalities a requisite for viral entry and/or establishment of latency?

Other non-viral contributors to pathogenesis have not been clear but the distinct geographical distributions of these diseases, clustered in East Asia and Central/South America, may suggest a host genetic or environmental predisposition. Studies examining associations of viral strain with EBV^+ malignancies in different geographical regions have mostly concluded that the tumour-associated virus strains are simply representative of that seen in the corresponding healthy control population[33], although ENKTL and CAEBV have not been comprehensively examined in this regard.

For the majority of patients with EBV-associated T and NK lymphoproliferations, the illness is not anteceded by clinical evidence of immune-compromise; these patients are considered to be immunocompetent by conventional criteria. However, the quality and quantity of EBV-specific immune responses in affected patients has not been examined and it thus remains unclear whether subtle defects in viral-specific cellular or humoral responses may contribute to pathogenesis. Further considerations relate to the complexity and influence of immune cells in the tumour microenvironment[735].

The contribution of Epstein-Barr virus to the pathogenesis of T and NK lymphoproliferations

The unanticipated association of EBV with rare but characteristic T- and NK cell lymphoproliferations was substantiated by studies throughout the 1980s and 1990s that consistently identified monoclonal viral genomes in lymphoma tissue[460-462] and circulating NK cells, $CD8^+$ and $CD4^+$ lymphocytes [242-243,245,463-471] from these patients. Although clinically heterogeneous, the principal unifying feature of such illnesses appeared to be the clonal expansion of EBV-infected T and/or NK cells associated with a restricted pattern of viral gene expression[73,563,610,614].

Whilst EBV-associated B cell malignancies can be viewed as rare accidents of the virus' natural lifestyle within the B cell system of the host, its association with T and NK disease raises many new questions about the viral entry route into these cell types and the viral contribution to lymphomagenesis.

In addition to the challenges posed by the rarity of the illnesses, a major hindrance to addressing these questions has been the lack of a suitable *in vitro* model of infection of T and NK cells. An isolated report[301] of efficient NK infection has, to-date, not been reproduced by other laboratories and whilst virus-binding (and infrequent entry) to T cells can be achieved[289,292], the establishment of a latently–infected cell has proved experimentally very challenging.

Consequently we began our studies by addressing a relatively straightforward, but as yet unanswered, question: What is the frequency T and NK cell infection *in vivo* and under what circumstances may this occur? By sensitively quantitating EBV genomes in highly purified peripheral blood and tonsil lymphocyte populations from a range of donors, we were able to draw some conclusions. Firstly, instances of tonsillar (but apparently not peripheral blood) $CD4^+$ and $CD8^+$ T cell infection are occasionally appreciable in the context of primary infection and healthy persistence although the fate of these cells in this context remains unclear. Secondly, the interesting and unexpected finding of high viral genome numbers in tonsillar $CD56^+$ cells *ex-vivo* from immunosuppressed persons and (from healthy donors) following *in vitro* culture was resolved, at least in part, by delineating these cells as of B cell and not NK origin. These findings have highlighted new questions about the phenotype of B cell subsets *in vivo* and *in vitro* and their relationship to EBV. Moreover, these observations served to underscore our findings - in a range of donors - that infection of normal NK cells in health and immune deregulation is a very rare event.

The evident limitations of our experimental approach were the lack of data at the single-cell level (i.e. numbers of genomes per infected cell), interpretative difficulties posed by small numbers of contaminating populations and the inability of the PCR assay to distinguish between surface and intracellular virions/viral DNA. The recent description of a combined 'immuno-FISH' technique[708] to identify the phenotype of EBV-harbouring cells (by virtue of EBER expression) has merit in this respect. Moreover, recent technological advances in flow cytometry permit high-throughput, high resolution images of individual cells directly in flow, alongside high fluorescence sensitivity[736-737]. In principle this would allow assessment of the intensity and location of fluorescent probes (to EBV DNA or RNA) on or within cells, in rare sub-populations within heterogeneous samples.

Our *ex-vivo* findings need to be reconciled with the consistent finding of clonal forms of EBV in patients with T/NK disease; providing evidence that an infection event occurs early in pathogenesis. Taken together with our data, in contrast to the situation in B cells, a valid hypothesis may be that in the rare instances when EBV erroneously infects a T or NK cell *in vivo*,

a relatively high chance of developing disease ensues. It is possible that the frequently observed cellular chromosomal aberrations result from EBV-mediated genomic injury (for example via expression of EBNA1[738] or LMP1[739]) or, alternatively, they pre-date and predispose to viral infection. However, it cannot be assumed that such a target cell is a normal and fully differentiated T or NK cell at the time of initial infection. Considering this latter point alongside the marked inefficiency of *in vitro* infection of these lineages, it remains possible that T, NK or common precursors are at enhanced susceptibility of infection. Relevant to this point are findings of CAEBV PBMC, where co-existing infection of $CD4^+$ and/or $CD8^+$ and/or $CD16^+$ cells[564] with monoclonal EBV has been described. Interpretative caution should be applied however, as the clonality analyses in this study were performed on total PBMC, rather than individual T and NK populations. Attempts at *in vitro* infection of NK and/or T cells at distinct stages along their differentiation pathway from a common lymphoid progenitor may be a worthwhile next step. An alternative approach could take advantage of recent developments in humanised mouse models[740], to provide an *in vivo* environment in which T or NK cell infection may be more readily manipulable. The current lack of meaningful *in vitro* models of infecting these cell types remains a major barrier for biological progress in this area.

The absence of an experimental model of infection of T and NK cells presents challenges in determining both the pattern of viral gene expression and the function of expressed transcripts and proteins in these cell types. However from the available molecular pathological evidence obtained from studies of primary ENKTL and CAEBV tissues, a limited number of gene products can be implicated in pathogenesis. We therefore sought to establish a method of expressing individual viral genes in primary NK cells, such that their expression would be both stable and inducible, to allow delineation of their impact upon genotype and phenotype of this cell lineage. LMP1 was, initially at least, the most obvious candidate to investigate given its presence in ENKTL tumour cells and its well characterised oncogenic role in B cells. Unfortunately there was insufficient time to characterise the effects of LMP1 expression in $CD56^{BRIGHT}$ primary NK cells. Nevertheless, the cell model we established, with levels of mRNA and topography of protein

expression at the single-cell level similar to ENKTL tumour lines, provides opportunities to directly examine the effects of LMP1 in a normal NK cell background. However, to implicate LMP1 in tumour pathogenesis we need to reconcile the findings in biopsy material from T/NK diseases where the LMP1 protein is frequently absent from some tumour cells within an ENKTL biopsy and is apparently not expressed in some cases of CAEBV PBMC. Three potential explanations follow: LMP1 expression is cyclical within individual cells in order to achieve an optimum equilibrium of its phenotypic effects, perhaps to balance proliferative and anti-apoptotic functions; or that LMP1 is truly restricted to a sub-population of cells and invokes expression of soluble factors that promote growth/survival of LMP1-negative cells in a paracrine manner; or that other EBV-encoded gene products are widely expressed within a tumour and are more essential for pathogenesis whilst LMP1 serves a minor, complementary role.

In the interest of tumour cell survival, any benefit that is derived from the expression of viral antigens is offset by their liability as targets for host effector immune cells. Unlike PTLD, occurring in the T cell compromised host, T/NK lymphoproliferations do not express the immunogenic EBNA3 family of proteins and of the notionally available Latency II proteins, LMP2 is the most immunogenic. However, expression of LMP2 had not been well characterised across the spectrum of T/NK diseases, although early clinical results indicated convincing responses to polyclonal LMP2-specific CTLs in the context of poor-prognosis ENKTL and CAEBV. Against this background, our investigations of LMP2 unexpectedly led to the finding of a hitherto unidentified LMP2 transcript; initiated from within the TR region of the viral genome, encoding downstream epitope-encoding exons serving as targets for LMP2-specific $CD8^+$ CTLs. The results of this chapter of work can be viewed as having four principal implications: first, that LMP2 is indeed a valid immunotherapeutic target in T/NK disease as LMP2-TR appears to be widely expressed and contains cognate epitopes for CTL recognition; second that the heterogeneous and often high levels of expression of LMP2-TR in cell lines and primary tissue implicate this truncated protein in initiation or potentiation of T/NK lymphomagenesis; third, that any future analyses of LMP2 expression in EBV-infected cells (of all lineages) should take account of LMP2-TR when

assessing expression at the mRNA and protein level; and fourth, that the identification of a novel LMP2B-like protein from a putatively bidirectional TR promoter has connotations for the range of EBV-associated tumours and raises new questions about the complementarity of LMP1 and LMP2B in EBV-infected cells.

Subsequent investigations may be best focused on the function of these latent viral proteins by individual and co-expression in a normal cell background (via inducible lentiviral expression systems) and complemented by gene silencing (via inducible lentiviral shRNA systems[714]) in T/NK tumour cell lines *in vitro*. The recent description of a SCID-mouse xenograft model of ENKTL may offer opportunities for related *in-vivo* studies of gene expression and function[741]. As discussed, perhaps the most significant hindrance to biological progress is the lack of an efficient model of T/NK infection; this remains a priority area to study. Critically, the findings from such investigations should be directly related to, and in some cases may inform, studies of pathological and clinical data derived from international collaborative studies. This approach offers an opportunity to maximise the acquisition of clinically and biologically meaningful data, which may ultimately lead to improved diagnostic and therapeutic tools for these poor prognosis diseases.

Looking forwards, the study of these fascinating lymphoproliferations of T and NK origin, strongly associated with EBV, not only provides an opportunity for progress in our understanding of pathogenesis of these rare diseases but may also allow broader virological, immunological and pathological lessons, relevant to the relationship of EBV and its human host, to be learned.

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