Studies on Immune Regulation of Epstein-Barr Virus

Karen A. McAulay

Doctor of Philosophy
2008

Declaration of authorship

This thesis was composed by the undersigned candidate. The work included is the candidate's own, unless otherwise stated.
No part of this work has been, or will be submitted, for any other degree or qualification.

Karen A. McAulay

Laboratory for Clinical and Molecular Virology
School of Biomedical Sciences
University of Edinburgh
1 Summerhall Square

Abstract

Epstein-Barr virus (EBV) is a gammaherpes virus that infects >90% of the adult population worldwide. During childhood infection is generally sub-clinical, however if delayed until adolescence infectious mononucleosis (IM) may develop. The virus has also been aetiologically linked with a number of tumours including B-cell lymphoma following organ transplantation: post-transplant lymphoproliferative disease (PTLD). The symptoms of IM are caused by an expansion of immune cells in response to infection whilst in the transplant situation immunosuppressive drug therapy allows the outgrowth of the tumour. Understanding the immuno-regulatory mechanisms involved in such EBV-associated diseases is crucial for devising new treatment strategies.

We undertook 3 separate studies (1-3) investigating different aspects of the immune response to EBV. In a recently reported phase II trial using allogenic, EBV-specific cytotoxic T-cell (CTL) to treat PTLD, tumour response was significantly increased with a high degree of donor/recipient HLA-allele matching suggesting that further refinement of the matching procedure may be important. In study 1 we investigated the epitope specificity and T-cell receptor (TCR) clonality of the infused CTL to identify potential areas for refinement. We found the protein specificity of the CTL to be polyclonal with dominant recognition of Epstein-Barr nuclear antigen-3 proteins and sub-dominant recognition of Latent membrane protein (LMP)-1 and LMP-2 proteins. Where possible, specificity was confirmed at the peptide level. No single TCR family was preferentially used by CTLs. The CTL epitope specificity did not differ between treatment responders and non-responders however the response was improved in those with several CTL HLA-restricted epitope matches and those infused with CTL containing polyclonal TCR families as opposed to monoclonal. CTL/recipient matching based on HLA matching alone was improved when also matched via HLA- restricted epitiope specificity. Therefore mapping CTL peptide epitope specificity prior to CTL infusions may enhance patient responses.

In recent years, interest has developed in genetic variation within components of the immune system. Of particular interest are cytokine/cytokine receptor genes

and genes of the human leukocyte antigen (HLA), both of which act to regulate the immune response. Variation within these genes could potentially alter the immune response leading to disease. In study 2 we investigated single nucleotide polymorphisms (SNPs) in several cytokine genes (TNF, IL-1, -6, -10) in both IM and PTLD cases and compared with relevant control groups. We found that the frequency of two TNF promoter alleles was significantly increased in PTLD patients compared to controls whilst the frequency of a TNF receptor II allele was increased in IM and EBV seropositive individuals, suggesting a role for this allele in susceptibility to EBV infection. The frequency of a second TNF receptor II allele was increased in both PTLD and IM subjects compared to controls highlighting the possible significance of TNF and its receptor in the development of EBV associated disease.

In study 3 we analyzed two microsatellite markers and two SNPs located near the HLA class I locus in IM, PTLD and control subjects to further determine whether the HLA genes may affect development of EBV-associated diseases. Alleles of both microsatellite markers were significantly associated with development of IM. Specific alleles of the two SNPs were also more frequent in IM patients. Moreover IM cases possessing the associated microsatellite allele had significantly fewer lymphocytes, increased neutrophils, and displayed higher EBV titres and milder IM symptoms relative to IM cases lacking the allele. The results indicate that HLA class I polymorphisms may predispose patients to development of IM upon primary EBV infection and suggest that genetic variation in T cell responses can influence the course of EBV infection.

Acknowledgements

This thesis would not have been possible without the help and support of a number of individuals. Firstly, I would like to thank my supervisor and mentor Professor Dorothy H. Crawford for allowing me to undertake this work within her lab, for sharing her extensive knowledge and thoughts, and for encouraging me along the long, hard road to submission. My co-supervisor Dr. Simon Talbot also deserves thanks for critical reading of this thesis.

I am indebted to many past and present members of our research laboratory who over the years have helped to provide me with the clinical samples used in this thesis. Special thanks to Dr. Karen Macsween (for chasing IM cases around Edinburgh!), the staff from the Edinburgh University Health Service, Dr. Tanzina Haque and other CTL/PTLD trial researchers, and of course all of the students and patients who have taken the time to provide the samples.

Thanks also go to Professor Ruth J. Jarrett and Annette Lake for their contribution to the SNP analysis in chapter 5, to Shonna Johnston for help with FACS and to Jill Lovell for help with sequencing/GeneMapper analysis. I am also extremely grateful to Craig D. Higgins - my statistician guru - for providing statistical analysis and guidance.

Scientific research can sometimes be a demoralising endeavour however I have been lucky to have made some wonderful friends who always seem to provide the coffee and chocolate at just the right time! Therefore, thanks to Jill, Lara, Phoebe, Anna, Gillian, Maggie, Victoria and the numerous others that are too many to mention.

Finally, I would like to thank my family for their support, especially my parents for providing me with perhaps the greatest gift of all: an education! A special thank you - and a great big kiss - goes to my husband Vincent for putting up with my many moods and for always believing in me.

Karen

Table of Contents

5. Results III: Analysis of HLA microsatellite markers and single nucleotide polymorphisms in acute infectious mononucleosis (IM) and post-transplant lymphoproliferative disease (PTLD)

Index of Tables

Index of Figures

Abbreviations and Symbols

α	Alpha
β	Beta
γ	Gamma
µ	Micro
%	Percentage
~	Approximately
℃	Degrees centigrade
1°	Primary
2°	Secondary
A	Absorbance
BARTs	Bam H rightward transcripts
BMT	Bone marrow transplant
Bp	Base pair
BSA	Bovine serum albumin
BL	Burkitt's lymphoma
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
CDR	Complementarity determining region
CO_2	Carbon dioxide
Cr	Chromium
CAEBV	Chronic active Epstein-Barr virus
CTL	Cytotoxic T-cell line
DC	Dendritic cell
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5' triphosphate
DRiP	Defective ribosomal product
EA	Early antigen
ELISA	Enzyme linked immunosorption assay
EBV	Epstein-Barr virus
EBER	Epstein-Barr virus encoded small RNAs
EBNA	Epstein-Barr nuclear antigen
FACS	Fluorescence activated cell scanning
FAM	Carboxyfluorescein
FBC	Full blood count
FBS	Foetal bovine serum
FITC	Fluorescein isothiocynate
g	gram
GC	Germinal centre
gp	Glycoprotein
HWE	Hardy Weinberg equilibrium
HL	Hodgkin's lymphoma
HHV	Human herpes virus
HLA	Human leukocyte antigen
IE	Immediate early
IFN	Interferon
Ig	Immunoglobulin
IM	Infectious mononucleosis
IL	Interleukin
IR1	Internal repeat 1
ITAM	Immunoreceptor tyrosine activation motif

LMP	Latent membrane protein
LP	Leader protein
LCL	Lymphoblastoid cell line
LT	Lymphotoxin
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
ml	Millilitre
min	Minute
MOI	Multiplicity of infection
NPC	Nasopharangeal carcinoma
NK	Natural killer
NF	Nuclease free
OHL	Oral hairy leukoplakia
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
pfu	Plaque forming units
PKR	RNA activated protein kinase
PTLD	Post transplant lymphoproliferative disease
R	Receptor
RBP	Ribonucleoprotein
Rvacc	Recombinant vaccinia virus
RT	Reverse transcriptase
RNA	Ribonucleic acid
SAP	Signalling lymphocytic-activation molecule (SLAM) associated protein
SCID	Severe combined immunodeficiency
SLAM	Signalling lymphocytic-activation molecule
SNP	Single nucleotide polymorphism
SOT	Solid organ transplantation
SSP	Sequence specific primer
TARC	Thymus and activated regulated cytokine
TCR	T cell receptor
Th	T helper
TR	Terminal repeat
TK	Thymidine kinase
TNF	Tumour necrosis factor
U_L	Unique long
U_s	Unique short
UV	Ultraviolet
Vβ	Variable β chain
VCA	Viral capsid antigen

Chapter 1: Introduction

1. Introduction

1.1 The Herpes Family

The herpesvirus family is one of the most widely disseminated, infecting mammals and birds as well as a range of lower vertebrate (reptilian, amphibian and fish) and invertebrate (bivalve) hosts. Since 1971 herpesvirus taxonomy has been addressed by the International Committee on Taxonomy of Viruses (ICTV) and over the years a consensus for herpes virus classification has been achieved based upon morphological, biological and molecular attributes. In the most recent ICTV report the family Herpesviridae is shown to consist of 3 sub-families: *Alphaherpesvirinae* (containing the *Simplexvirus*, *Varicellovirus*, *Mardivirus* and *Iltovirus* genera), *Betaherpesvirinae* (containing the *Cytomegalovirus*, *Muromegalovirus* and *Roseolovirus* genera) and *Gammaherpesvirinae* (containing *Lymphocryptovirus* and *Rhadinovirus* genera). The 3 sub-families are estimated to have arisen approximately 400 million years ago with subsequent division (genera) occurring around 80 million years ago (McGeoch, Rixon, and Davison, 2006;McGeoch *et al.*, 1995).

The primary criterion for inclusion in the herpes family is that of virion morphology. The virion is spherical in shape, and comprises 4 major components: the core, capsid, tegument and the envelope (Figure 1.1). The virion size differs between species but is approximately 200nm in diameter. The core consists of a single copy of a linear, double-stranded DNA molecule, ranging in size from 125-240 kilobase pairs (kbp) and encoding between 70 and 200 genes (Pellet and Roizman, 2006). The icosahedral shaped capsid (diameter 100-110nm) consists of 162 capsomeres each containing 5 or 6 copies of the major capsid protein and is surrounded by the tegument which contains 30 or more viral proteins and is poorly defined structurally. The tegument is further surround by a lipid envelope containing viral membrane glycoproteins (Kieff and

Rickinson, 2006). The protein composition of the tegument and envelope components varies across the herpes family.

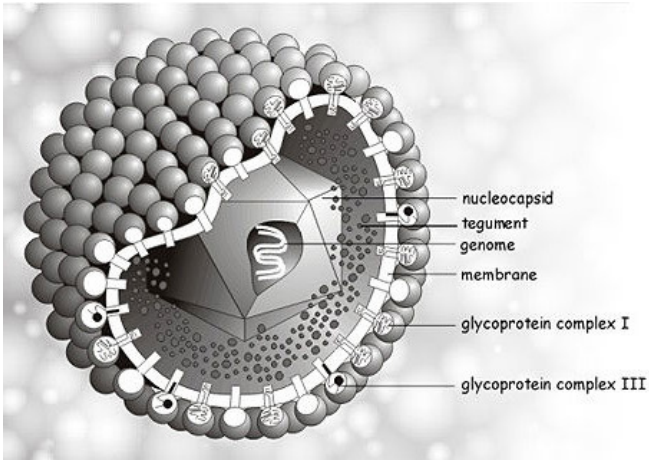


Figure 1.1 Herpes virus virion structure
Herpes virus virion detailing spherical shape consisting of genome, nucleocapsid, tegument and membrane components. Reproduced with permission from Dr. Marko Reschke, www.biografix.de.

The second main criterion for inclusion within the family is that of biological attributes. There are four principal biological characteristics attributed to Herpes viruses:

1. Herpes viruses express a large number of enzymes involved in the metabolism of nucleic acid, DNA synthesis and processing of proteins.
2. The synthesis of viral genomes and assembly of capsids occurs in the nucleus. The capsid is then enveloped when budding from the nuclear membrane.
3. Productive viral infection is accompanied by cell destruction.
4. Herpes viruses are able to establish and maintain a latent state in their host. Latency involves stable maintenance of the viral genome in the nucleus with limited expression of a subset of viral genes.

Due to advances in molecular technology a third criterion of genomic characterisation is now also used to define family members. Nucleotide and amino acid sequence analysis

allow genomic comparisons to be made across the various viruses and the construction of phylogenetic trees. Figure 1.2 shows an example of such a tree for the herpes virus family.

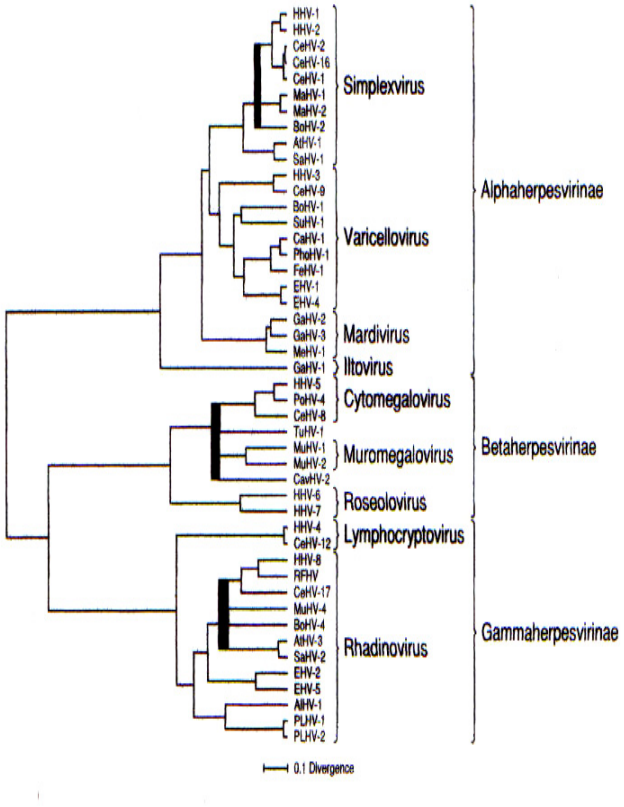


Figure 1.2 Composite phylogenetic tree of the herpes viruses.
The tree is base upon amino acid sequence alignments of 8 sets of homologous genes. Thick lines designate areas of uncertain branching. From 'Overview of classification' by A.J.Davison (2007); Human herpesviruses: Biology, Therapy, and Immunoprophylaxis (Arvin, A. et al eds), by permission of Cambridge University Press (Davison, 2007).

1.1.2 Human Herpes Viruses

Eight human herpes viruses (HHV) have so far been identified with HHV-8 the most recent inclusion in 1994 (Chang *et al.*, 1994). All 3 herpes virus sub-families contain at least one human representative, summarised in Table 1.1.

Similar to the herpes family in general, the 8 HHV are categorised according to their morphological, biological and molecular characteristics. The biological properties of the HHV are shown in Table 1.2.

Table 1.1 Human herpes virus classification

Human Herpes Virus	Sub-family	Genus	Alternative name
1	Alpha-	Simplexvirus	Herpes-Simplex 1 (HSV-1)
2	Alpha-	Simplexvirus	Herpes-Simplex 2 (HSV-2)
3	Alpha-	Varicellovirus	Varicella Zoster virus (VSV)
4	Gamma-	Lymphocrytovirus	Epstein-Barr virus (EBV)
5	Beta-	Cytomegalovirus	Human Cytomegalovirus (HCMV)
6	Beta-	Roseolovirus	-
7	Beta-	Roseolovirus	-
8	Gamma-	Rhadinovirus	Kaposi's Sarcoma-associated Herpes Virus (KSHV)

Table 1.2 Biological properties of human herpes viruses

Herpes Sub-family	Genus	Biological Properties		
		Growth Cycle	Cytopathology	Site of Latency
Alpha	Simplexvirus	Short (< 24hrs)	Cytolytic	Sensory ganglia and neurons
	Varicellovirus			
Beta	Cytomegalovirus	Long (> 24hrs)	Cytomegalic	Glands Kidney
	Roseolovirus		Lymphoproliferative	Lymphoid tisssue
Gamma	Lymphocrytovirus	Variable	Lymphoproliferative	Lymphocytes
	Rhadinovirus			

1.2 Epstein Barr Virus (EBV)

In 1957 Denis Burkitt, a British surgeon, working in Mulago Hospital in Kampala, Uganda, was consulted about a child with unusual swellings in the jaw. Whilst visiting a second hospital sometime later he came across another child with similar swellings. He later wrote 'a curiosity can occur once, but two cases indicated something more than a curiosity' (Burkitt, 1983). His subsequent investigation of the swellings revealed that the jaw tumours were common, sometimes associated with tumours at other anatomical sites (adrenal glands, kidney and liver) and usually presented within the first decade of life (Burkitt, 1958). Following collaboration with a pathologist colleague the tumour

was designated a lymphoma and subsequently became known as Burkitt's lymphoma (Burkitt and O'Conor, 1961).

In 1961, Denis Burkitt presented his findings to the Academic Surgical Unit at The Middlesex Hospital, London. In the audience was M.Anthony Epstein, an experimental pathologist with an interest in the oncogeneicity of Rous sarcoma virus. On hearing Burkitt's description of the tumour, and the distribution patterns associated with climatic factors, Epstein postulated that a biological agent may play a role in the aetiology of the lymphoma. A collaboration between the two was established, with Burkitt sending biopsy material from his lymphoma patients to Epstein's laboratory in London. For 2 years Epstein and colleagues investigated the biopsy material, specifically looking for a viral agent, but with no success. In 1963, a fortuitous transport delay in biopsy material resulted in the formation of a single cell suspension of tumour cells, from which the first BL-derived continuous cell line (EB-1) was established (Epstein and Barr, 1964). This finding was quickly followed by the identification of a virus-like particle in the BL line using electron microscopy (Epstein, Achong, and Barr, 1964). Confirmation of the finding was performed in the laboratory of Werner and Gertrude Henle in Philadelphia where the virus was referred to as 'herpes-type' and subsequently renamed Epstein-Barr (EB) virus after the original BL-cell line (Epstein *et al.*, 1965).

The past 40 years have seen advances in our understanding of the molecular characteristics of the virus and of the various interactions of the virus with the host immune system. EBV has been shown to infect a number of cell types, including B-cells, epithelial cells and T-cells, both *in vitro* and *in vivo*. It is now associated with several other diseases including infectious mononucleosis (IM), Hodgkin's lymphoma

(HL), nasopharangeal carcinoma (NPC) and more recently some forms of gastric cancer.

1.2.1 EBV epidemiology and transmission

EBV is one of the more successful herpes viruses infecting over 90% of the adult population globally. Recent epidemiological studies suggest that in western populations approximately 75% are infected with the virus by early adulthood (Crawford *et al.*, 2002;Crawford *et al.*, 2006) whilst in non-westernised countries this percentage is increased with almost all children over the age of two infected (de-The *et al.*, 1978). If infection is delayed until adolescence then infectious mononucleosis (IM), a self-limiting lymphoproliferative disease, may result (Niederman *et al.*, 1970). Although two peaks of sero-conversion occur (Pereira, Blake, and Macrae.A.D, 1969) only a few studies have attempted to identify the factors involved in sero-conversion. Early studies associated high socio-economic status with the level of EBV sero-negativity (Hesse *et al.*, 1983;Hallee *et al.*, 1974) however, more recently EBV sero-positivity has also been associated with female gender, age and the number of siblings (Higgins *et al.*, 2007). EBV is routinely detected in saliva of infected individuals and transmission is thought to predominately occur through salivary contact, however the virus has also been detected in both male and female genital secretions (Israele, Shirley, and Sixbey, 1991;Sixbey, Lemon, and Pagano, 1986). In a recent sero-epidemiological study of university students sexual contact was identified as a significant risk factor for EBV sero-conversion (Crawford *et al.*, 2002).

Two sub-types of EBV have been identified since its discovery. EBV sub-type 1 (or A) and EBV sub-type 2 (or B) share between 70 and 85% sequence homology (Sample *et al.*, 1990) and studies suggest that type 1 is more prevalent than type 2, although type 2 is more prominent in Africa (Gratama and Ernberg, 1995). Indeed recent investigation

of young adults suggests that 77% of infected individuals carry type 1 EBV, 17% type 2 and around 5% carry both types (Higgins *et al.*, 2007). The same study also found type 1 infected subjects to have similar risk factors for sero-conversion to that of EBV overall whilst sexual activity was the only risk factor for type 2 infected subjects (Higgins *et al.*, 2007). Type I EBV was also identified as a risk factor in the development of IM (Crawford *et al.*, 2006).

1.2.2 The EBV genome

EBV (the B95-8 strain) was the first of the herpes virus family to be completely sequenced (Baer *et al.*, 1984). More recently, the sequences of the B95-8 and Raji strains have been used to create a composite reference sequence- EBVwt (de Jesus *et al.*, 2003). Its genome is composed of a linear, double stranded DNA approximately 170kb in length and contains 84 open reading frames (ORFs). A repeated DNA sequence, the terminal repeat (TR), present at either end of the linear form mediates circularisation upon infection of a cell. EBV also contains tandemly repeated DNA sequences, one of which is the major internal repeat (IR1), which contains the latency promoter Wp. The IR1 divides the genome into the long (U_L) and short (U_S) unique sequences where the majority of the genes are clustered (Figure 1.3).

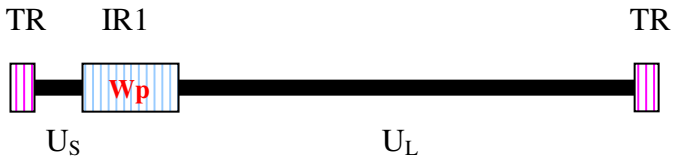


Figure 1.3 Schematic diagram of the EBV linear genome.

TR: terminal repeat; U_L: unique long; U_S: unique short; IR1: internal repeat 1. The linear form is circularised via the terminal repeats.

In other herpes virus genomes the genes are numbered according to their position within the unique sequences, however in EBV they are named according to the *Bam*H1 restriction fragment in which they start. Conventionally the fragments are named according to size, with A the largest. For example BARF1 is the first right forward reading frame in *Bam*H1A. Figure 1.4 depicts the *Bam*H1 restriction endonuclease map of the B95-8 genome.

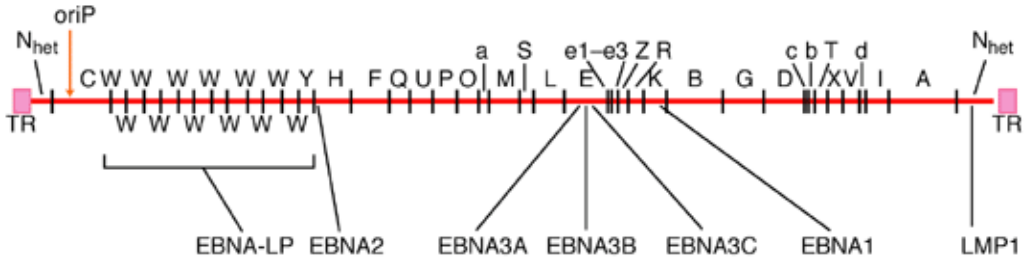


Figure 1.4 B*am*H1 restriction endonuclease map of B95-8 genome.
The map shows the location of the ORFs for the latent proteins. The largest fragment is A with lowercase letters referring to the smallest fragments. TR: terminal repeats; N_{het}: heterogenic region. Reproduced by kind permission of Cambridge University Press: Epstein-Barr virus infection: basis of malignancy and potential for therapy, Murray,PG and Young,L. Exp Rev Mol Med; 2001: 3(28):1-20.

The EB virus expresses different gene combinations and proteins depending on the type of infection: latent (types I, II and III) or lytic.

1.2.2.1 EBV latency patterns

EBV proteins are differentially expressed in a variety of EBV-associated diseases, more commonly known as EBV latency patterns or programmes. There are 3 latency patterns observed in EBV-associated disease (I, II and III) with the highest number of antigens expressed found in latency type III and the lowest with latency type I (summarised in Table 1.3). In normal healthy individuals resting EBV-infected B-cells display a fourth latency pattern, type 0, where only Epstein-Barr encoded small RNA (EBERs) are detected.

Table 1.3: EBV latency patterns and associated disease

Latency Pattern	Antigen expressed	Disease association
Latency I/ latency programme	EBERs* EBNA-1 BARTs*	Burkitt's Lymphoma
Latency II/ default programme	EBERs* EBNA-1 LMP-1 and or LMP-2A BARTs*	Hodgkin's Lymphoma Nasopharangeal carcinoma Undifferentiated nasopharangeal carcinoma T-cell Lymphoma
Latency III/ growth programme	EBERs* EBNA-1, -2, -3A, -3B, -3C, -LP LMP-1, -2A, -2B BARTs*	Lymphoblastoid cell lines in vitro Infectious Mononucleosis Post-transplant Lymphoproliferative Disease

EBERs: Epstein-Barr encoded small RNA, EBNA: Epstein-Barr nuclear antigen, LMP: latent membrane protein, LP: leader protein, BARTs: *Bam*H1 A rightward transcripts
*Detection of RNA only

1.2.2.2 Latent gene expression

Latent cycle genes were discovered through a mixture of RNA and protein analysis. There are a total of 9 latent viral antigens, 6 of which are nuclear antigens (EBNAs) and 3 which are membrane proteins (LMPs). Epstein-Barr encoded small RNAs (EBERs) are also transcribed but not translated during latency. Transcripts from the *Bam*H1A region (BARTs) can also be detected (Table 1.4).

Within 24 hours of infection the linear genome circularises to form an episome within the infected cell (Hurley and Thorley-Lawson, 1988;Alfieri, Birkenbach, and Kieff, 1991) (Figure 1.5). In latency type III all the EBNAs are expressed from a single mRNA transcript that undergoes alternate splicing to produce mRNAs for the individual EBNAs. During initial infection transcription is initiated from the Wp promoter in the IR1. As the cells immortalise and expand the Wp promoter activity is reduced and transcription is then initiated from a second promoter, Cp (Woisetschlaeger, Strominger, and Speck, 1989;Woisetschlaeger *et al.*, 1990). This switch in promoter usage is regulated by expression of the EBNA-2 protein. In latency type I only EBNA 1 is

expressed and the mRNA is transcribed from a third promoter, Qp (Schaefer, Strominger, and Speck, 1995). EBNA-2 and EBNA-LP are the first viral genes expressed followed by EBNA-1 (Allday, Crawford, and Griffin, 1989;Rooney *et al.*, 1989). Expression of the membrane proteins is controlled by the expression of EBNA-2. Both LMP-1 and LMP-2B are transcribed from a bi-directional promoter that responds to EBNA-2 (Johannsen *et al.*, 1995) while LMP-2A is controlled by a different promoter also controlled by EBNA-2 (Zimber-Strobl *et al.*, 1991;Zimber-Strobl *et al.*, 1993). Both promoters are located within the B*am*H1N fragment (Young and Murray, 2003). The coding exons for both LMP-2A and -2B are spliced across the terminal repeat region with LMP-1 expressed subsequent to EBNA-1 expression, as EBNA-1 acts as a transcriptional transactivator, up-regulating the LMP-1 promoter (Kieff and Rickinson, 2006).

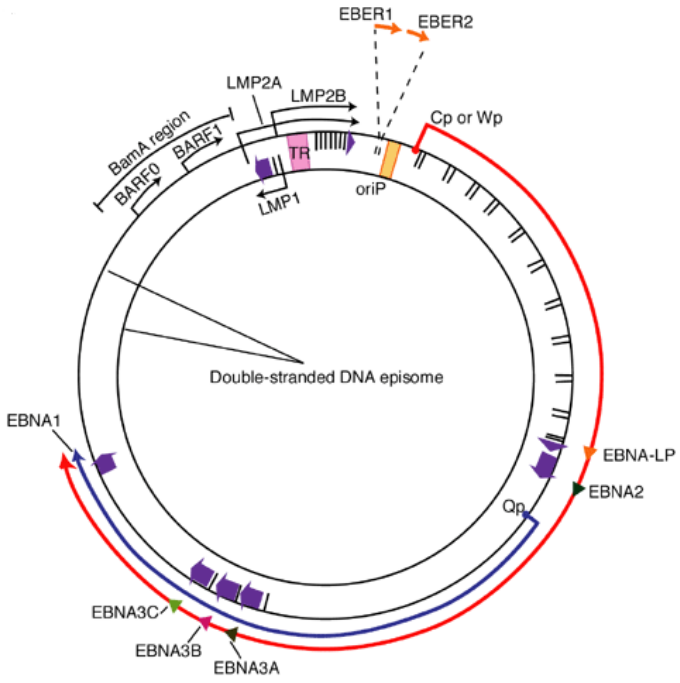


Figure 1.5 Location and transcription of EBV latent genes on the episome
Purple solid arrows represent the exons encoding for the latent EBNA and LMP proteins. LMP-2A and -2B are composed of multiple exons located on either side of the terminal repeat. Orange arrows represent transcription of EBERS. The red arrow is the pattern followed during type III latency while the blue arrow is that followed by type I latency. Reproduced by kind permission of Cambridge University Press: Epstein-Barr virus infection: basis of malignancy and potential for therapy, Murray,PG and Young,L. Exp Rev Mol Med; 2001: 3(28):1-20.

Combined with EBV antigen expression, there is up-regulation of various cellular genes including interleukins (ILs)-5, -6 and -10, tumour necrosis factors (TNF), receptors CD21, CD23 and CCR7, as well as adhesion molecules CD11a, CD58 and CD54. It is postulated that these molecules induce and maintain proliferation by EBV through autocrine routes and cell-cell contact. Indeed EBV drives the infected cell from G0 to G1-phase and then into S-phase of the cell cycle, replicating the viral genome alongside the cell genome (Adams, 1987;Murray and Young, 2001). These regulatory events are primarily controlled by the various latent genes and summarised in table 1.4.

Table 1.4 Latent genes and their function

Latent Gene	Gene Function
EBNA-1	Genome maintenance (binds origin of replication)
EBNA-2	Transcription factor Activates cellular and viral genes
EBNA-3A	Regulates EBNA-2
EBNA-3B	Transcriptional regulator
EBNA-3C	Overcomes cell cycle check points
EBNA-LP	Co-activates EBNA-2 responsive genes and increases efficiency of immortilisation
LMP-1	Activates NFκB, a homologue to CD40 and prevents apoptosis
LMP-2A	Inhibitor of B cell receptor signalling and provides survival signals
LMP-2B	Function unclear – may regulate LMP-2A
EBER RNAs	Possible role in tumourigenicity
BARTs	Function unclear

EBNA: Epstein-Barr nuclear antigen; LP: leader protein; LMP: latent membrane protein;
EBER: Epstein-Barr virus-encoded small RNA; BARTs: Bam H1A rightward transcripts.
NFκB: nuclear factor kappa B

EBNA-1

EBNA-1 is a phosphoprotein, coded for by the BKRF1 open reading frame (Figure 1.4, pg.8). It is the only viral protein that binds directly to DNA in a sequence specific manner (Rawlins *et al.*, 1985) with DNA binding mediated through the carboxy-terminal domain which is rich in acidic and basic residues. Multiple EBNA-1 binding sites, termed the family of repeats and the dyad symmetry element, occur in the latent

viral origin of replication, *Ori P*, where the binding of EBNA-1 is the only viral factor required for viral episome maintenance (Rawlins *et al.*, 1985;Yates, Warren, and Sugden, 1985). The only other EBNA-1 binding sites occur in the Qp promoter where EBNA-1 binding negatively regulates the promoter ensuring that the gene is only transcribed when required (Davenport and Pagano, 1999).

EBNA-1 also contains a glycine-alanine repeat region within the N-terminal domain that has been shown to prevent proteasomal degradation and peptide loading to major histocompatability complex (MHC) class I molecules (Levitskaya *et al.*, 1995;Levitskaya *et al.*, 1997). This allows infected cells a mechanism to bypass recognition by circulating cytotoxic T-cells. More recently the glycine- alanine repeat region has also been implicated in prohibiting the translation of EBNA-1 mRNA (Yin, Manoury, and Fahraeus, 2003).

EBNA-2

The EBNA-2 open reading frame, BYRF1 is located near the genome dividing IR1 region (Figure 1.4, pg.8). Deletion of this open reading frame results in virus unable to immortalise B-cells suggesting that EBNA-2 is essential for B-cell immortalisation (Miller *et al.*, 1974). EBNA-2 is a phosphoprotein that localises to various compartments of the nucleus, including the nucleoplasm, the chromatin fraction and the nuclear matrix (Petti, Sample, and Kieff, 1990;Sauter and Mueller-Lantzsch, 1987) where it plays a crucial role in activating the transcription of other viral and cellular genes summarised in Table 1.5. Unlike EBNA-1, EBNA-2 lacks any sequence specific DNA binding activity and therefore requires alternative DNA binding factors to activate promoters of viral and cellular genes. A cellular protein, RBP-Jκ, mediating binding of EBNA-2 to a response element was first described for the LMP-2A promoter and then

subsequently for other EBNA-2 responsive promoters (Ling, Rawlins, and Hayward, 1993;Grossman *et al.*, 1994;Zimber-Strobl *et al.*, 1993;Laux *et al.*, 1994a).

Table 1.5: EBNA-2 responsive viral and cellular genes and possible functions

Gene	Function	EBNA-2 effect
LMP-1	Viral oncogene. CD40 homologue that induces B-cell proliferation and protects from apoptosis	Up-regulation
LMP-2	Inhibits virus lytic cycle. B-cell receptor homologue that protects from apoptosis	Up-regulation
EBNAs	Maintenance of viral genome and transactivation of viral and cellular genes involved in lymphoproliferation	Up-regulation
CD21	C3d receptor that binds EBV	Up-regulation
CD23	B-cell activation marker implicated in cell cycle progression and IgE receptor	Up-regulation
BLR2/EB 11	G-protein coupled receptor with role in lymphocyte trafficking	Up-regulation
BATF	Negative regulator of AP-1. Inhibits lytic cycle	Up-regulation
c-fgr	Cellular proto-oncogene. Encodes a tyrosine kinase that promotes cell proliferation	Up-regulation
c-myc	Cellular proto-oncogene and transcription factor. Activates genes involved in cell cycle progression	Up-regulation
Ig-μ	Encodes IgM	Down-regulation

Table adapted from 'EBNA2 transcription regulation in EBV latency' by Zetterberg H. and Rymo L.; Chapter 22, Epstein-Barr Virus, edited by Robertson ES. By permission of Caister Academic Press.

Indeed EBV carrying a mutated EBNA-2 unable to bind RBP-Jκ cannot immortalise B-cells (Yalamanchili *et al.*, 1994). RBP-Jκ binds to a conserved sequence and is involved in both activation and repression of genes depending on other recruited complexes. EBNA-2 can also activate viral and cellular genes in a RBP-Jκ-independent manner. A number of transcription factors including PU.1, POU domain protein, AUF1 and DP103 have been associated with EBNA-2 (Sjoblom *et al.*, 1998;Johannsen *et al.*, 1995;Voss *et al.*, 2001). The transcription activity of EBNA-2 is potentiated by interaction with EBNA-LP (Peng *et al.*, 2004;Peng, Zhao, and Kieff, 2004). This interaction is important in the transition from G0 to G1-phase of the cell cycle (Sinclair *et al.*, 1994). Also EBNA-3C has been associated with regulation of EBNA-2 mediated gene activation (Rosendorff *et al.*, 2004).

The EBNA-2 gene shows extensive DNA sequence diversity between type-1 and type-2 EBV with homology of approximately 55%. The most conserved regions include those regions essential for immortalisation.

EBNA-3 (3A, 3B and 3C)

The EBNA 3 (A, B and C) proteins, also known as EBNA 3, 4 and 6, are coded for by the BERF open reading frames and are thought to have arisen from a common point of origin (Figure 1.4; pg.8). They are encoded by alternatively spliced transcripts initiating from the Cp promoter. EBNA-3A and -3C are required for immortalisation of B-cells whereas EBNA-3B is not (Tomkinson, Robertson, and Kieff, 1993). All 3 proteins show sequence divergence between EBV types-1 and -2 but these differences do not affect their immortalisation potential (Sample *et al.*, 1990;Tomkinson and Kieff, 1992). The EBNA-3B protein has been associated with transcriptional regulation of CD40, CD77 and vimentin genes whilst the EBNA-3C protein has been associated with the LMP-1 and CD21 genes (Allday, Crawford, and Thomas, 1993;Silins and Sculley, 1994;Wang *et al.*, 1990). All 3 EBNA 3 proteins also inhibit activation of EBNA-2 responsive promoters by preventing RBP-Jκ and associated complexes from binding to their RBP-Jκ binding sites thereby counterbalancing the action of EBNA-2 (discussed above) (Bain *et al.*, 1996;Johannsen *et al.*, 1996;Radkov *et al.*, 1997;Radkov *et al.*, 1999). EBNA-3C has also been shown to cooperate with EBNA-2 in the activation of the LMP-1 promoter through the transcription factor PU.1 (Zhao and Sample, 2000) and to over-ride a number of cell cycle check points at G1 and G2/M-phases (Wade and Allday, 2000;Parker *et al.*, 1996;Parker, Touitou, and Allday, 2000).

EBNA-LP

EBNA-LP, also known as EBNA-5, is encoded by the BWRF1 (Figure 1.4; pg.8) open reading frame and forms either the leader sequence of the EBNA mRNAs or, via alternative splicing, EBNA-LP mRNA. EBNA-LP is one of the first viral genes expressed after infection (Allday, Crawford, and Griffin, 1989;Rooney *et al.*, 1989) and although it is not strictly essential for B-cell immortalisation it does promote efficient outgrowth of lymphobalstoid cells *in vitro* (Allan *et al.*, 1992;Hammerschmidt and Sugden, 1989). The exact function of EBNA-LP is still unknown. It is thought to interact with EBNA-2 in transcriptional regulation and potentiate the transctivating functions of EBNA-2, such as the activation of LMP-1(Nitsche, Bell, and Rickinson, 1997;Peng, Tan, and Ling, 2000). The protein has been reported to co-localise in the nucleus with the retinoblastoma gene product Rb (Jiang *et al.*, 1991) and to bind to both Rb and the tumour suppressor gene product p53 *in vitro* (Szekely *et al.*, 1993). EBNA-LP phosphorylation has also been found to be dependent on cell cycle stage (Kitay and Rowe, 1996) and EBNA-LP, in collaboration with EBNA-2, can induce cyclin D2 and cell cycle activation (Sinclair *et al.*, 1994). These findings together would suggest that EBNA-LP is involved in the control of cellular proliferation.

LMP-1

The LMP-1 gene is located adjacent to the EBV genome terminal repeat and 3 alternative promoters can regulate LMP-1 gene transcription. In latency type III LMP-1 transcription is regulated by EBNA-2 and EBNA-LP (Peng *et al.*, 2004;Peng, Zhao, and Kieff, 2004;Nitsche, Bell, and Rickinson, 1997) whilst in latency type II LMP-1 transcription initiates from a signal transducer and activator of transcription (STAT) regulated upstream promoter in the terminal repeat region (Sadler and Raab-Traub, 1995). A third promoter located in the first intron is activated late in lytic replication

and transcribes a truncated form of LMP-1 (Fennewald, van Santen, and Kieff, 1984). LMP-1 expression is required for *in vitro* proliferation and immortalisation of B-cells, thereby acting as an oncogene (Dirmeier *et al.*, 2005). Expression at low levels has been shown to induce a number of adhesion molecules and activation markers including CD23, CD39, CD40, CD54 and CD58 (Wang *et al.*, 1990). LMP-1 can induce up-regulation of the anti-apoptotic molecule Bcl-2 as well as down-regulate pro-apoptotic molecules such as Bax (Cahir-McFarland *et al.*, 2004;Dirmeier *et al.*, 2005). Bcl-6, a gene physiologically involved in the formation of germinal centres, is also down-regulated by LMP-1 (Carbone *et al.*, 1998). Several of these functions have been attributed to the ability to induce nuclear factor kappa B (NF-κB) (Cahir-McFarland *et al.*, 2000;Feuillard *et al.*, 2000). LMP-1 is now known to be a member of the tumour necrosis factor (TNF) receptor family, activating several downstream cellular pathways, including the NF-κB, the JNK/AP-1, the p38/MAPK and JAK/Stat pathways (reviewed in (Cahir-McFarland and Kieff, 2005). The functional properties of LMP-1, such as activation of NF-κB, JNK and p38 cellular pathways, are similar to that the B-cell activator CD40 (Gires *et al.*, 1997;Hatzivassiliou *et al.*, 1998). Indeed, CD40 ligand can maintain lymphoblastoid cell line (LCL) proliferation in the absence of LMP-1 (Kilger *et al.*, 1998) and LMP-1/CD40 fusion proteins can replace LMP-1 in LCL outgrowth assays (Dirmeier *et al.*, 2003). Another shared function between LMP-1 and CD40 is the ability to bind the lytic transactivator BZLF1, thereby contributing to the maintenance of the latent state (Adler *et al.*, 2002). However the signalling methods of LMP-1 and CD40 are quite distinct. LMP-1 signals through the TNF receptor-associated death domain (TRADD) whilst CD40 signals through the associated factor-6 (TRAF6) (Bornkamm, 2001).

LMP-2

The LMP-2 genes are encoded near the termini of the EBV genome and upon genome circularisation the 2 LMP-2 genes (2A and 2B) are alternatively transcribed from 2 different promoters. Both promoters are regulated by EBNA-2 through the transcription factors RBP-Jκ and PU.1 (Laux *et al.*, 1994b;Meitinger *et al.*, 1994). LMP-2B is a truncated form of LMP-2A and lacks the cytosolic amino-terminus of LMP-2A. Neither LMP-2A nor LMP-2B is essential for B-cell immortalisation *in vitro* (Kim and Yates, 1993) however LMP-2A shares several properties with molecules involved in B-cell receptor signalling. It undergoes tyrosine phosphorylation, is associated with several phosphotyrosine kinases (PTK) and has an immunoreceptor tyrosine based activation motif (ITAM) (Burkhardt *et al.*, 1992;Beaufils *et al.*, 1993). Studies have shown that LMP-2A disrupts B-cell signalling through association with PTKs and acts as a constitutively activated B-cell receptor homologue (Caldwell *et al.*, 1998). Disruption of B-cell signalling allows LMP-2A to prevent EBV infected cells from entering the lytic replication cycle, thereby contributing to maintenance of latency (Miller *et al.*, 1994). The role of LMP-2B is unclear; however, the conservation of both LMP-2B expression and the EBNA-2 responsive element of its promoter would suggest that LMP-2B plays an important role (Rivailler, Quink, and Wang, 1999). One suggested role is that it down-regulates the actions of LMP-2A.

EBERS

Epstein-Barr virus encoded small nonpolyadenylated RNAs (EBERS) -1 and -2 are the most abundant viral transcripts in latently infected cells and are coded for by the BCRF1 open reading frame. EBERs are largely located in the nucleus however they have also been detected in the cytoplasm and in the nuclei of interphase cells (Schwemmle *et al.*, 1992). They have a similar secondary structure and genomic

organisation to the Adenovirus-associated RNAs (VA1 and VA2), although little sequence homology (Bhat and Thimmappaya, 1983), and like VAs bind and inactivate RNA activated protein kinase (PKR); a mediator of the antiviral effect of interferon (Sharp *et al.*, 1993). EBERs exist in nuclear ribonucleoprotein (RBP) complexes that bind to the La protein and the large ribosomal subunit L22 (Toczyski *et al.*, 1994;Toczyski and Steitz, 1991) however the significance of this aggregation is still unclear. It has been suggested that L22 levels may be a determinant in cell immortalisation and that L22 interferes with EBER induced inhibition of PKR (Elia *et al.*, 2004). EBERs are not required for B-cell immortalisation however they may have a role to play in oncogenesis. The re-introduction of EBERs into EBV-negative Akata B2 cell line clones restored tumourigenicity in SCID mice, resistance to apoptotic inducers and up-regulation of Bcl-2 (Komano *et al.*, 1999;Ruf *et al.*, 2000;Maruo, Nanbo, and Takada, 2001). More recently, EBERs have also been shown to induce human IL-10 expression in BL cells (Kitagawa *et al.*, 2000), IL-9 expression in EBV infected T-cells (Yang *et al.*, 2004) and expression of insulin-like growth factor in EBV-associated gastric carcinoma (Iwakiri *et al.*, 2003). In all 3 cases EBERs are thought to act as an autocrine growth factor.

BamH1 A rightward transcripts (BARTs)

Similar to EBERs, BARTs are highly spliced polyadenylated RNAs (Brooks *et al.*, 1993), the function of which remains unclear. BARTs are expressed in number of EBV-associated malignanacies including NPC, BL and HL (Tao *et al.*, 1998;Chiang *et al.*, 1996;Deacon *et al.*, 1993) and are thought to encode proteins that may potentially interact with the Notch pathway and the subsequent expression of LMP-1. As the Notch pathway is primarily involved in cellular differentiation and proliferation, protein

interactions with this pathway could affect the development of EBV malignancies (Hayward, 2004).

1.2.2.3 Lytic gene expression

The herpesviruses follow a strategy of life-long latency in the host that on occasion is interrupted by lytic reactivation in a small subset of infected cells thereby allowing the production of free virions for shedding and transmission between hosts. In the case of EBV latency a number of established cell lines are available for investigation however there is no definitive culture system for investigating lytic replication. Instead our understanding of lytic replication is derived from latently infected cell lines that are induced to reactivate using a number of methods including B cell receptor cross-linking via anti-immunoglobulin antibody (anti-IgG) (Shimizu and Takada, 1993;Takada, 1984), transforming growth factor-β (TGF-β) activation (Fahmi *et al.*, 2000) and CD4+ T-cell activation (Fu and Cannon, 2000). From such studies we now know that during lytic EBV infection a cascade of viral genes is activated that functions to produce the free virion – the immediate early genes followed by the early and then the late genes (Figure 1.6).

The viral immediate early genes are induced via signal transduction after initial activation (anti-IgG, TGF-β, CD4+). Messenger RNAs encoding two immediate early proteins BZLF1 and BRLF1 are expressed within 30 minutes of activation (Flemington, Goldfeld, and Speck, 1991), and function as transcription factors. BZLF1 appears to be the dominant transactivator as expression of BZLF1 alone is sufficient to trigger the expression cascade (Rooney *et al.*, 1989). BZLF1 has binding sites in several viral early gene promoters, the lytic origin of replication (*oriLyt*), its own promoter (Zp) and it also activates the adjacent BRLF1 gene (Speck, Chatila, and Flemington, 1997). Both

BZLF1 and BRLF1 are required for subsequent activation of the early genes (Cox, Leahy, and Hardwick, 1990;Feederle *et al.*, 2000).

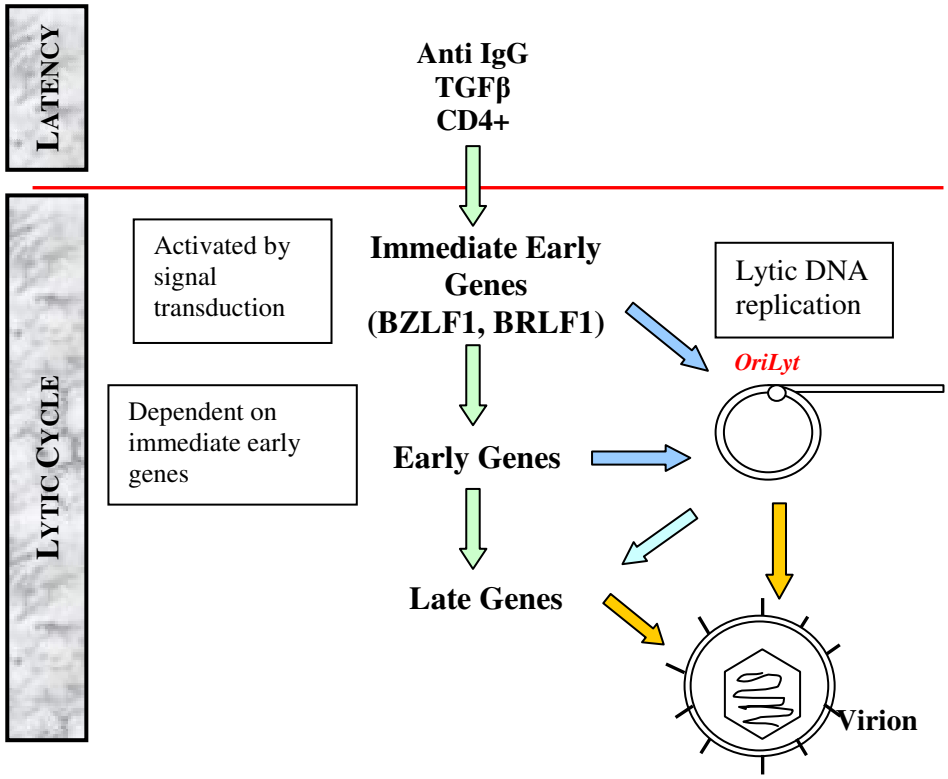


Figure 1.6:EBV lytic cycle
Immediate early, early and late genes are expressed in sequential order. (Figure adapted from 'Reactivation of Epstein-Barr virus from latency' by Amon W. and Farrel P (2005). Rev Med Virol: 15: 149-156. By permission of John Wiley & Sons, Ltd)

Early lytic gene products function to regulate transcription, RNA transport and to inhibit cellular apoptosis. For example, the early gene product BMRF1 activates the *oriLyt* promoter and the BHLF1 gene (Holley-Guthrie *et al.*, 2005). BMLF1 promotes the transport of viral transcripts from the nucleus to the cytoplasm (Boyer, Swaminathan, and Silverstein, 2002); BHRF1, a homologue of Bcl-2, prevents apoptosis mediated by a number of different agents (Henderson *et al.*, 1993;Tarodi, Subramanian, and Chinnadurai, 1994). Regulation of the viral late genes is less well understood. EBV late

promoters are thought to be activated upon viral replication however some evidence exists in support of late gene activation by the immediate early gene product BRLF1 (Ragoczy and Miller, 1999). Nucleocapsid proteins, glycoproteins, anti-apoptotic proteins and viral IL-10 are all encoded for by late proteins and function to package the virus, minimise detection by the host immune system and prevent cellular apoptosis (Salek-Ardakani, Arrand, and Mackett, 2002;Inman *et al.*, 2001).

1.2.3 EBV attachment and fusion

EBV was originally recognised for its ability to infect and immortalise B-cells; however, soon after initial discovery it was also shown to infect epithelial cells in NPC. Epithelial cell infection was subsequently confirmed in a number of studies (Shibata and Weiss, 1992;Sixbey *et al.*, 1984). Under some circumstances the virus will also infect T-cells, natural killer cells and smooth muscle cells (Rickinson and Kieff, 2006), and more recently EBV has also been shown to infect monocytes and neutrophils (Savard and Gosselin, 2006;Tugizov *et al.*, 2007). Our understanding of how EBV enters each of these different cell types is limited however intense investigation of B-cell and epithelial cell entry highlights some of the mechanisms that may be involved.

1.2.3.1 EBV infection of B-cells

Eight virus glycoproteins have been implicated in EBV entry into either B-cells or epithelial cells (Table 1.6). The most abundant of these, gp350/220, is responsible for attachment of the virus with high affinity to the complement receptor 2 (CR2 also known as CD21) on B-cells (Fingeroth *et al.*, 1984;Frade *et al.*, 1985;Nemerow *et al.*, 1985;Nemerow *et al.*, 1987;Tanner *et al.*, 1987;Tanner *et al.*, 1988). However recombinant virus lacking gp350/220 can still immortalise B-cells albeit with reduced efficiency perhaps indicating the presence of alternative binding sites (Janz *et al.*, 2000).

Glycoprotein gp350/220 however is probably the dominant binding site as antibodies to gp350/220 and soluble forms of CR2 and gp350/220 can neutralise B cell infection (Moore *et al.*, 1991;Tanner *et al.*, 1988).

Binding of gp350/220 triggers capping of CR2 and endocytosis of the virus (Tanner *et al.*, 1987;Nemerow and Cooper, 1984) and may contribute to positioning the virus

Table 1.6: EBV envelope glycoproteins involved in virus entry

Protein	Gene	Role in virus entry
gp350/220	BLLF1	Attachment to B cell receptor CR2/CD21
gH (gp85)	BXLF2	Fusion: attachment to epithelial cell receptor/co-receptor; complexes with gL and gp42
gL (gp25)	BKRF2	Complexes with gH and gp42; chaperone for gH
gp42	BZLF2	Fusion: interaction with B-cell co-receptor HLA class II
gB (gp110)	BALF4	Fusion
gN	BLRF1	Co-dependent for expression; involved in post fusion events
gM	BBRF3	
BMRF2	BMRF2	Binds integrins

EBV glycoproteins are named for their mass, their homologues or open reading frame

closer to the cell membrane (Hutt-Fletcher, 2007) both of which are essential for fusion of the virus with the B-cell. Four other glycoproteins are necessary for efficient fusion: gH, gL, gB and gp42 (Haddad and Hutt-Fletcher, 1989;Molesworth *et al.*, 2000) which form a non-covalently linked complex within the virus. Glycoprotein gH is the largest of the glycoproteins and depends on gL for folding and transport through the cell (Molesworth *et al.*, 2000). The gHgL and gB proteins form the core fusion machinery. Glycoprotein gp42 also associates with gHgL and interacts with the beta chain of HLA class II molecules (Mullen *et al.*, 2002;Li *et al.*, 1997), essential co-receptors for B-cell infection (Haan *et al.*, 2000;Li *et al.*, 1997). MHC class II-gp42 binding may trigger further signalling events but as yet these are still unknown. The binding of gp42 to specific non-functional HLA-DQ alleles has been reported (Haan *et al.*, 2000) however since HLA-DP, -DQ and –DR alleles can all be used it is unlikely that specific HLA-

DQ alleles would have a major impact in a large outbred population (Hutt-Fletcher, 2007). The overall model proposed for B-cell entry is that following attachment of gp350/220 to the CR2/CD21 receptor, gp42 interacts with HLA class II molecules activating the core fusion machinery consisting of gHgL and gB (Figure 1.7).

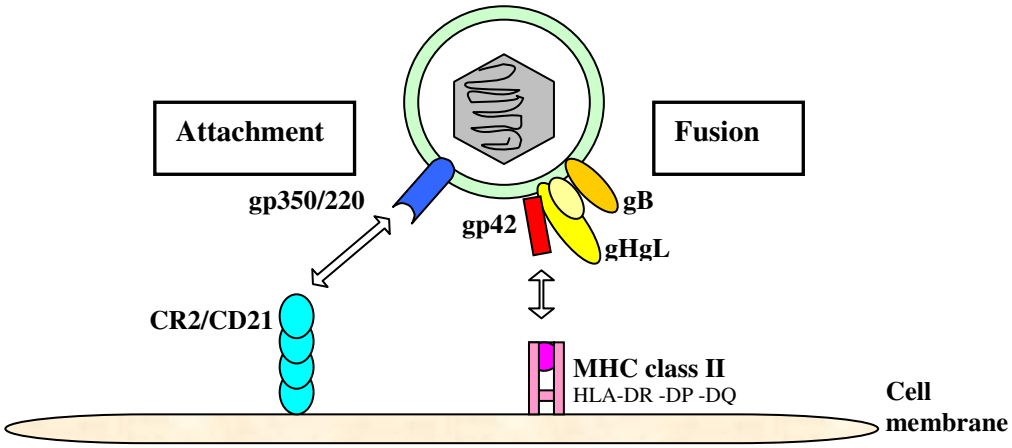


Figure 1.7: Putative model of EBV attachment and fusion with B cell

The virus binds to CR2 via gp350/220, initiating signalling events and triggering endocytosis. Interaction of gp42 with HLA class II triggers the interaction of the gHgLgB complex with the endosomal membrane activating further signalling cascades. The virus and endosomal membranes then fuse allowing entry of the tegumented capsid into the cell cytoplasm. (Adapted from Epstein-Barr virus entry into cells by Speck,P, Hann, KM, Longnecker,R (2000). Virology:277(1): 1-5. By permission of Academic Press)

1.2.3.2 EBV infection of epithelial cell

The attachment and fusion of EBV to epithelial cells is more controversial than that of B-cells, with several mechanisms postulated. CR2/CD21 is expressed at low levels on a small subset of epithelial cells and engineered CR2 expressing epithelial cells are easily infected (Borza *et al.*, 2004;Li *et al.*, 1992). Unlike B-cells epithelial cells lack the machinery associated with the CR2 signalling complex and therefore must signal through an alternative route, possibly through CR2 clustering with homologues of formin; molecular scaffolds that nucleate actin (Gill *et al.*, 2004). However the majority of epithelial cells do not express CR2 so this route of attachment is probably unlikely.

Several alternative attachment strategies have been proposed that do not involve CR2:

- Binding through the polymeric immunoglobulin-A (IgA) receptor. Virus coated with IgA specific for gp350/220 binds to the IgA receptor (Sixbey and Yao, 1992).
- The glycoprotein complex gHgL as a ligand, binding to an as yet unidentified gHgL receptor (gHgLR). Virus derived from a B-cell can bind well to a CR2 deficient epithelial cell whereas recombinant virus lacking the complex cannot (Molesworth *et al.*, 2000;Oda *et al.*, 2000). Also addition of soluble antibody specific for gHgL can reduce virus binding (Borza *et al.*, 2004;Molesworth *et al.*, 2000).
- Binding through the membrane protein BMRF2. In polarised epithelial cells an interaction between BMRF2 and integrins has been demonstrated (Tugizov, Berline, and Palefsky, 2003). Specifically the RGD motif of BMRF2 binds to β1, α5, α3 and αv integrins (Xiao *et al.*, 2008). Antibodies to both integrins and the BMRF2 protein partially block binding and have a significant effect on infection via the basolateral surface of the polarised cell.

Fusion of the virus once attached is also different to that of B-cells. Fusion does not appear to require endocytosis (Borza and Hutt-Fletcher, 2002) or the co-receptor MHC class II binding to gp42. In fact gp42 may act as an inhibitor to fusion in epithelial cells (Wang *et al.*, 1998). The main fusion event is thought to occur through the binding of gHgL to its receptor, however, infection is generally less efficient than that mediated through gp42 in B-cells (Borza and Hutt-Fletcher, 2002). Successful fusion also requires higher levels of the glycoprotein gB to that observed in B-cell fusion, most likely to facilitate fusion via the gHgL-gHgLR interaction (Neuhierl *et al.*, 2002;McShane and Longnecker, 2004).

Once fusion occurs (in both B cell and epithelial cells) the virus genome travels to the cell nucleus, however the post-fusion events leading to this movement are poorly understood. Two glycoproteins gN and gM have been implicated in some immediate post-fusion events. Glycoprotein gM is a transmembrane protein with a long, highly charged cytoplasmic tail, rich in potential phosphorylation sites required for signalling mechanisms. Both gN and gM play important roles in virus assembly and enveloping. It has been postulated that these proteins may also be required for disassembly of the tegument allowing release of the genome to the nucleus (Hutt-Fletcher, 2007).

It is interesting that the main difference between B-cell and epithelial cell entry is the involvement of gp42 (gHgLgp42 complex in B-cell, gHgL in epithelial cell) suggesting that the levels of each complex may affect the tropism of the virion. Indeed virions produced from epithelial cells are rich in the tri-complex and infect B-cells readily whereas virions produced by B-cells are rich in the gHgL complex and infect epithelial cells more efficiently (Borza and Hutt-Fletcher, 2002;Laichalk and Thorley-Lawson, 2005). This alternate tropism strategy may be important for *in vivo* infection and the lifelong persistence of the virus within its host.

1.2.4 EBV primary infection and persistence in vivo

The mechanism by which primary EBV infection resolves into lifelong persistence *in vivo* is controversial. Two differing hypothesis revolve around the first cell type to be infected – B-cell or epithelial cell. The first hypothesis advocates that B-cells underlying the squamous epithelium of the oropharynx are directly infected. The second hypothesis involves the indirect infection of B-cells via the squamous epithelial cell of the oropharynx. Evidence is available to support both mechanisms.

1.2.4.1 B-cell hypothesis

The oropharynx acts as a natural point of entry for most HHVs and exchange of salivary products has long been established as a route of transmission for EBV (Gerber *et al.*, 1972). Waldeyers ring designates a functional unit of lymphoid tissue within the pharynx and includes the adenoids and palatine tonsils. Within the lymphoid tissue loosely bound crypt structures interweave through the tonsil (Macsween and Crawford, 2003;Perry and Whyte, 1998) and B-cells have been found infiltrating these crypts (Faulkner, Krajewski, and Crawford, 2000). These lymphoepithelial crypts are a potential point of entry for EBV to infect B-cells, and conversely, for EBV virion release into saliva and subsequent transmission. Indeed in a recent localisation study EBV was detected in both the adenoid and palatine tonsil tissue with higher viral titres observed in the adenoid tissue (Berger *et al.*, 2007). The current model for EBV infection and persistence within B-cells is closely entwined with the normal biology of B-cells (Figure 1.8).

EBV infects resting naïve B-cells in the lymphoepithelial crypts driving their proliferation into B-cell blasts in an EBNA-2 dependent fashion (latency type III or Growth programme) (Babcock, Hochberg, and Thorley-Lawson, 2000;Babcock and Thorley-Lawson, 2000;Thorley-Lawson, 2005). This reflects B-cell activation upon exposure to antigen. In normal B-cell differentiation the antigen activated B-blast migrates to the follicle to undergo a germinal centre reaction and selection for antibody production. Exposure to T-cells and specific cytokines act to rescue the B-blast into the circulating memory pool. Memory B-cells then enter the circulation and occasionally divide to maintain cell numbers. Upon antigen stimulation memory B-cells can re-enter the lymphoepithelium and differentiate into an antibody producing plasma cell. Virus infected B-blasts follow a similar route. Once in the germinal centre they switch to a

latency type II pattern (default programme) (Babcock and Thorley-Lawson, 2000;Thorley-Lawson, 2005) where LMP-1 provides a CD40-like activation signal and LMP-2A a rescue signal from apoptosis (Caldwell *et al.*, 1998;Gires *et al.*, 1997). Upon entering the peripheral circulation EBV infected memory B-cells down-regulate all latent genes (latency I) occasionally expressing EBNA-1 during homeostatic cell division to ensure viral division with the cell process. Subsequent differentiation into an antibody producing plasma cell triggers EBV to replicate and shed virions along with antibody (Laichalk and Thorley-Lawson, 2005;Thorley-Lawson, 2005;Crawford and Ando, 1986).

1.2.4.2 The epithelial cell hypothesis

The epithelial hypothesis suggests that EBV infects epithelial cells within the oropharynx with subsequent transfer of the virus to underlying B-cells (Allday and Crawford, 1988). There are several reasons to support such a role for epithelial cells. Firstly, EBV has been detected in several carcinomas particularly NPC where nearly all tumours are EBV-positive and usually express a latency type II pattern (Brooks *et al.*, 1992a), suggesting the presence of a latent EBV infection. Secondly, EBV replication has also been detected in epithelial lesions resulting from the disease Oral Hairy Leukoplakia (OHL) (Greenspan *et al.*, 1985). Lastly, as stated above (section 1.2.3.1) the presumed site of entry and exit is the lymphoepithelium of the Waldeyer's ring where the majority of cells are epithelial in nature. The hypothesis suggests that EBV infects epithelial cells within the lymphoepithelium allowing the virus to replicate to high titres thereby increasing the chances of subsequent infection of B-cells within the underlying lymphoid tissue, and increasing the amount of virus shed into saliva.

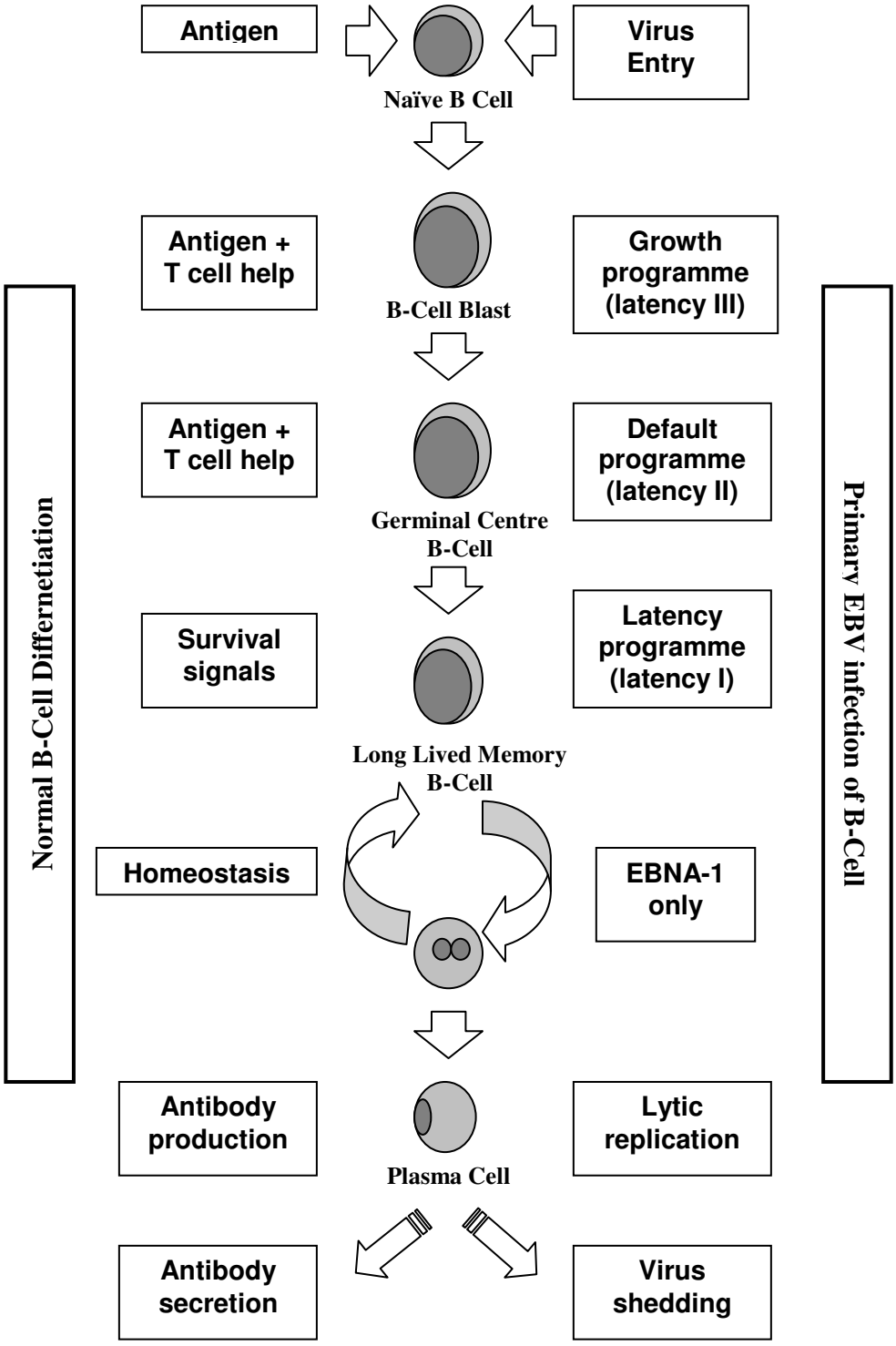


Figure 1.8: Normal B-cell differentiation pathway and EBV persistence
Adapted from 'EBV persistence and latent infection *in vivo' by* Thorley-Lawson,DA. (2005). In *Epstein-Barr Virus*. Robertson,E.S. (ed). By permission of Caister Academic Press.

However the role of the epithelium in this model is controversial with several studies highlighting the importance of the B-cell to persistence. For example, bone marrow transplant patients undergoing complete bone marrow ablation either lose their EBV or acquire the donor strain, depending on donor status (Gratama *et al.*, 1988). Also, patients with X-linked agammaglobulinaemia, a genetic disorder resulting in a lack of B-cells, are unable to be infected with EBV (Faulkner *et al.*, 1999). Both studies indicate that an intact lymphoid system, especially B-cells are required for persistence. However they do not eliminate the involvement of epithelial cells. Indeed the recent advances in our understanding of the mechanisms involved in EBV entry to both B-cells and epithelial cells and the production of dual tropic virions (section 1.2.2) would indicate an important role for epithelial cells in virus egress to the saliva. Further support for this association between B-cells and epithelial cells is obtained from the presence of a unique EBV receptor, α5β1integrin, on epithelial cells which allows infection only through the basolateral surface of the cell (Tugizov, Berline, and Palefsky, 2003). Similarly, EBV can be detected in *ex-vivo* tonsil epithelial cell culture presumably via infection from B-cells prior to or shortly after explantation (Pegtel, Middeldorp, and Thorley-Lawson, 2004). More recently, EBV particles attached to the surface of a B cell have been shown to infect epithelial cells in a cell-to-cell mediated fashion and that this infection was more effective than cell-free virus infection of epithelial cells (Shannon-Lowe *et al.*, 2006). Taking these studies together there is strong evidence that normal naso-oropharangeal epithelium is infected with EBV. This may provide an explanation for the presence of EBV in diseases such as NPC and OHL.

1.3 The Immune Response

EBV establishes a life-long infection in B-cells, generally without causing disease, in over 90% of the world's adult population. This fact is extraordinary as EBV has strong growth transforming capacities for B-cells both *in vivo* and *in vitro*. Our survival as a species is therefore reliant on the efficient immune control of EBV *in vivo*. Co-evolution of humans with the virus has probably shaped the human immune response we see today (McGeoch *et al.*, 1995).

Primary infection generally occurs in early childhood and is usually sub-clinical making investigation of the immune response to acute infection in these subjects difficult. However at an older age primary infection can manifest as infectious mononucleosis (IM), a symptomatic benign lymphoproliferation, observed in approximately 25% of adolescent conversions (Crawford *et al.*, 2006;Crawford *et al.*, 2002). IM patients therefore provide a source for investigation of the acute infection and healthy EBV carriers for persistent infection. Unfortunately *in vivo* investigation of very early events in the immune response is still problematic as IM patients only seek medical help following the development of symptoms therefore, *in vitro* studies are generally used to investigate these early events. From such *in vivo* and *in vitro* studies it has been determined that the immune response to EBV is mediated by both innate and adaptive immune responses.

1.3.1 Innate immunity to EBV

The earliest immune response observed is the production of type I interferons-alpha and -beta (IFN-α, -β). *In vitro* infection of peripheral blood mononuclear cells (PBMCs) demonstrated peak production of IFN-α approximately 24 hours post-infection (Kikuta *et al.*, 1984) with B-cells and natural killer cells (NK) the main producers (Lotz *et al.*,

1986). The secretion of type I IFNs is induced by two types of receptor: toll-like and cytosolic receptors, both of which detect viral nucleic acids. Cytosolic receptors are ubiquitous receptors whereas toll-like receptors are found in endosomes of specialised cells (Stetson and Medzhitov, 2006). Activation of both receptors results in the secretion of type I IFNs which act to induce an antiviral state. For example, IFN-α reduces B-cell outgrowth following EBV immortalisation (Lotz *et al.*, 1985) although this action is only apparent during the first 24 hours, after which time the immortalised B-cell becomes resistant to IFN (Lotz *et al.*, 1985). Several mechanisms for this IFN effect have been postulated: inhibition of CR2 capping during EBV entry (Delcayre, Lotz, and Lernhardt, 1993), repression of EBNA-1 transcription (Nonkwelo, Ruf, and Sample, 1997) and inhibition of translation via modulation of the double-stranded RNA-activated PKR (Gao, Xue, and Griffin, 1999). These mechanisms halt viral replication and lead to apoptosis of the cell thus preventing spread of virus to neighbouring cells. Concurrently, type I IFNs up-regulate the expression of MHC class I molecules on uninfected cells making them more resistant to NK-cell and cytotoxic T-cell activity. Resistance to the effects of type I IFNs coincides with the transcription of EBV latent genes EBNA-2 and EBNA-LP, both of which confer resistance to IFN-α in transfection experiments (Aman and von Gabain, 1990). In addition LMP-1 has been shown to mediate protection from IFN-α induced apoptosis (Henderson *et al.*, 1991) and resistance may also be mediated via the EBERS (Nanbo and Takada, 2002;Nanbo *et al.*, 2002). Therefore the type I IFN response is limited to approximately the first 24 hours of infection and prior to expression of latent proteins. As the adaptive response does not take over until around day 5 other innate systems have to protect until this time.

1.3.1.1 Natural killer cell component

NK-cells account for approximately 10% of the peripheral lymphocyte population and are a major component of the innate immune system. NK-cells can lyse virus infected cells via perforin release and secrete immunoregulatory cytokines that augment the adaptive immune response (Cooper, Fehniger, and Caligiuri, 2001;Biron *et al.*, 1999). The importance of NK-cells in EBV infection was highlighted by the isolation of the mutated gene involved in a fatal primary EBV infection, X-linked lymphoproliferative disease (XLP). In healthy individuals the gene product SAP (signalling lymphocytic activation molecule (SLAM) associated protein) (Coffey *et al.*, 1998;Sayos *et al.*, 1998) acts as a cell activation modulator via interaction with the cell surface co-receptor CD244 (Nakajima 2000). CD244 binds CD48, a molecule highly expressed on B-cells (Brown *et al.*, 1998) activating the NK-cell. However NK-cells derived from XLP patients fail to kill EBV immortalised B-cells (Parolini *et al.*, 2000) emphasising the pivotal role of NK-cells in EBV infection. A number of other receptors are utilised by NK-cells in a cell dependent manner: Natural cytotoxicity receptors (NCRs) and the NKG2D receptor (Pende *et al.*, 2002;Moretta *et al.*, 2001).

During asymptomatic EBV infection the peripheral T-cell repertoire is largely not perturbed (Silins *et al.*, 2001) suggesting that immune control is established at the site of infection, the oropharynx. The oropharynx contains a unique non-cytolytic NK-cell population that secretes cytokines, mainly IFN-γ, upon activation (Ferlazzo and Munz, 2004;Ferlazzo *et al.*, 2004b;Ferlazzo *et al.*, 2004a). In *in vitro* studies IFN-γ is 10-fold more potent at inhibiting EBV immortalisation than IFN-α and can suppress immortalisation for up to 4 days (Lotz *et al.*, 1985) suggesting an antiviral role for IFN-γ producing NK-cells within the oropharynx. NK-cells therefore have the ability to limit infection for several days perhaps until the adaptive specific immune system kicks in.

1.3.2 Adaptive immunity to EBV

Reports on the specific immune response to EBV characterise the already primed adaptive immune response during acute or persistent infection but little is known about the actual events involved in initiation of the specific immune response. EBV immortalised B-cells are unable to elicit EBV-specific T-cell responses in peripheral blood lymphocyte cultures from EBV-negative donors (Calender *et al.*, 1987;Nikiforow, Bottomly, and Miller, 2001;Hurley and Thorley-Lawson, 1988) but can from EBV-positive donors (Wilkie *et al.*, 2004;Rooney *et al.*, 1995). However when CD25-positive T-cells are selected for stimulation or IL-12 added to the EBV-negative culture EBV-specific responses could be observed (Metes *et al.*, 2000;Savoldo *et al.*, 2002) suggesting that immortalised B-cells can act as targets but do not initiate specific immunity (Bickham *et al.*, 2003;Bickham and Munz, 2003). On the other hand, dendritic cells (DCs) when added to EBV-infected cultures derived from negative donors, prevent the outgrowth of immortalised B-cells and elicit an EBV-specific T-cell response: both CD4 and CD8 mediated (Bickham *et al.*, 2003). Cross-presentation of EBV antigens from infected B-cells is thought to be the mechanism used as cross-presentation has been observed in the priming of naïve T-cells and the expansion of memory T-cells (Bickham *et al.*, 2003;Subklewe *et al.*, 2001). Myeloid DCs have also been shown to stimulate NK-cell proliferation, NK-cell IFN-γ production and increase NK-cell cytotoxic activity against MHC class I negative cells (Ferlazzo *et al.*, 2002;Gerosa *et al.*, 2002;Piccioli *et al.*, 2002). DC activation of NK-cells may help limit the EBV infection in the early stages but also assist in the development of EBV-specific T-cell responses via cytokine release (Figure 1.9).

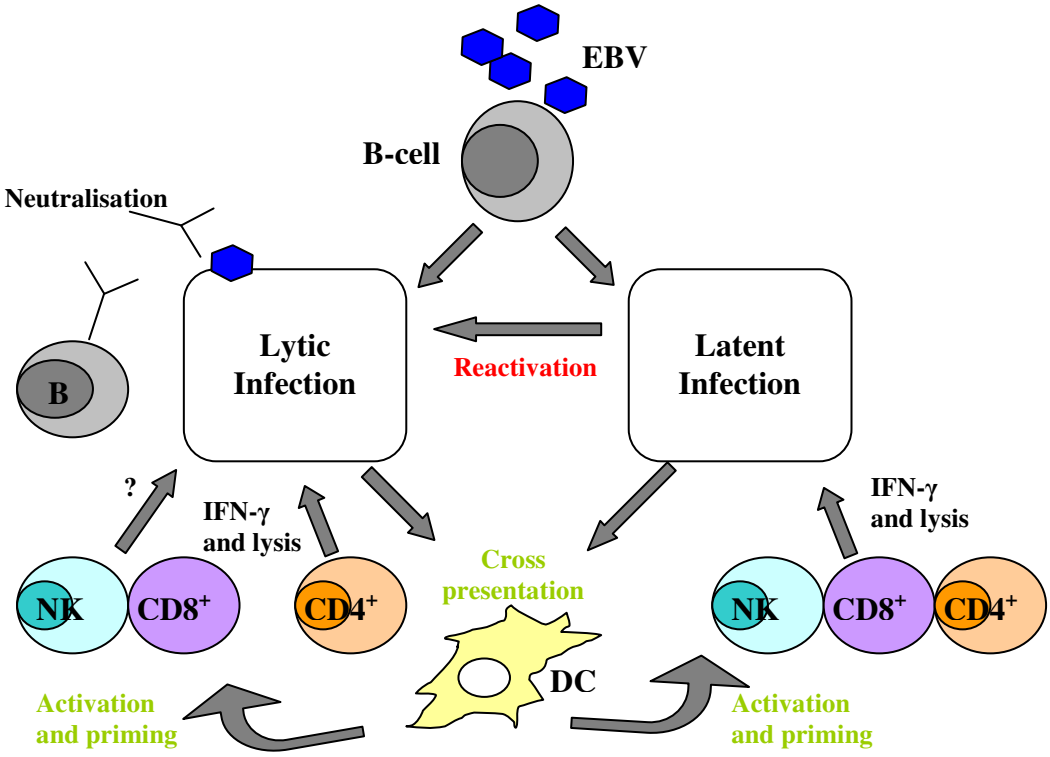


Figure 1.9 Innate and adaptive control of EBV infection.
Initiation of a specific immune response is mediated by DC via cross presentation of EBV antigens. In latent infection NK, $CD8^+$ and $CD4^+$ T-cells target infected B-cells. In lytic infection $CD4^+$ T-cells can target infected B-cells however the role of NK and $CD8^+$ T-cells is still unclear. Antibodies produced by B-cells can neutralise the virus. Figure adapted from 'Immune response and evasion in the host-EBV interaction' by C.Munz; Epstein-Barr Virus edited by ES. Robertson, (2005). By permission of Caister Academic Press.

1.3.2.1 Antibody response to EBV

The specific antibody response to EBV can be divided into acute and persistent responses. Antibody responses against both latent and lytic antigens peak during the acute phase of the infection and include IgM, IgA and IgG antibodies to viral nucleocapsid antigens (VCA), immediate early (IE) and early lytic antigens (EA) (Rickinson and Kieff, 2006). However no peak in response is seen against the latent antigen EBNA-1 or the lytic antigen gp350, one of the more abundant envelope proteins, during this phase. During the persistent phase the antibody pattern changes with IgM and IgA antibodies becoming undetectable and IgG antibodies maintaining a steady state. Both anti-EBNA-1 and gp-350 responses peak during this phase of infection (Henle *et al.*, 1987). The reasons for this late development in response are

unknown. EBNA antigens are generally intracellular therefore a role in protective humoral immunity is highly unlikely. However IgG responses against gp350 do have neutralising capabilities and may contribute to the immune regulation of the infection (Thorley-Lawson and Geilinger, 1980;Hoffmann, Lazarowitz, and Hayward, 1980). These changes in antibody response are used diagnostically to assess if an individual is infected with EBV. An acute infection is defined by the presence of IgM antibodies against VCA and a lack of IgG antibodies against EBNA-1, whilst a persistent infection is defined by the presence of IgG antibodies against EBNA-1 and VCA in the absence of an IgM response. Sero-negative individuals lack responses to all of these antigens. IgA antibodies against gp350 can be detected in the serum and saliva of a small percentage of infected individuals and may have a role in prevention of virus spread (Yao *et al.*, 1991) however they are not routinely used for diagnosis of infection. IgA antibody responses are however associated with EBV-positive NPC with increased levels of EA and VCA IgA readily detected (Henle and Henle, 1976). Glycoprotein gp350 specific IgA may facilitate EBV infection of epithelial cells in this situation (Sixbey and Yao, 1992) (see page 24).

1.3.2.2 CD8+ T-cell response to EBV

Following IM patients from the acute phase through convalescence to a persistent state of infection has allowed the study of the T-cell response in great detail. The important effector role of the CD8+ T-cell response has been elucidated from such studies. During the acute phase of IM an atypical lymphocytosis is observed. Several studies have speculated that this expansion is due to a non-specific bystander effect (Welsh *et al.*, 2000;McNally and Welsh, 2002) or as a response to a virus induced superantigen (Sutkowski *et al.*, 2001), however the expanded CD8+ population is in fact oligoclonal

in T-cell receptor (TCR) usage (Annels *et al.*, 2000;Callan, Steven, and Krausa, 1996) and is EBV epitope-specific (Steven *et al.*, 1996;Steven *et al.*, 1997). In the acute infection up to 50% of the CD8+ population recognise EBV-specific lytic antigens (Callan *et al.*, 1998a), in particular the IE antigens BRLF-1 and BZLF-1, and the EAs BMRF-1, BMLF-1 and BALF-2 (Steven *et al.*, 1997). These cells are cycled rapidly during the acute phase (Macallan *et al.*, 2003) and decrease dramatically as the acute infection progresses (Hislop *et al.*, 2002;Callan *et al.*, 2000).

In contrast the CD8+ T-cell population recognising latent antigens is lower during the acute phase of the infection and reach their highest frequencies during the convalescent phase (Hislop *et al.*, 2002;Callan *et al.*, 1998b) although they never reach the high frequencies observed against lytic antigens during the acute phase. The EBNA -3 proteins tend to be the dominant antigens recognised by CD8+ T-cells (Steven *et al.*, 1996;Khanna *et al.*, 1992;Murray *et al.*, 1992) although strong responses have been reported for several eptiopes identified from the LMP-2 protein. The levels of lytic and latent CD8+ T-cell specificity tend to follow the natural course of the EBV infection from lytic to latent states.

During persistent infection both lytic and latent antigen specific CD8+ T-cells can be detected. The immunodominant eptiopes recognised by CD8+ T-cells have been mapped to specific HLA types (Munz, 2005;Moss *et al.*, 2001). HLA-B*08 restricted CD8+ T-cells appear to be one of the more dominant with as much as 5% of the total CD8+ population, from persistently infected individuals, directed against the lytic antigen BZLF-1 and as much as 1% against an EBNA-3A epitope (Tan *et al.*, 1999;Burrows *et al.*, 1990). In initial screens CD8+ T-cell responses to EBNA-1 could not be detected (Khanna *et al.*, 1992;Murray *et al.*, 1992) presumably due to the presence of its gly-ala repeat domain which prevents degradation by proteosome and

mRNA translation (Levitskaya *et al.*, 1997;Yin, Manoury, and Fahraeus, 2003), thus escaping T-cell presentation. However, in subsequent investigations EBNA-1 CD8+ T-cells have been detected in individuals with HLA-B*35 and -B*07 haplotypes (Blake *et al.*, 1997;Blake *et al.*, 2000;Lee *et al.*, 2004). MHC class I presentation of EBNA-1 via proteosomal degradation of defective ribosomal products (DRiPs) is one suggested mechanism for EBNA-1 specific T-cell responses in these haplotypes (Munz, 2004). Latent antigen-specific CD8+ T-cells tend to have the differentiation phenotype CD28+/CD27+ whilst lytic antigen-specific cells sometimes harbour a CD28-/CD27- phenotype (Hislop *et al.*, 2001;Appay *et al.*, 2002). Latent antigen-specific T-cells also frequently express CCR7, a homing marker for secondary lymphoid organs (Appay *et al.*, 2002) which could facilitate the regulation of EBV reactivation within the tonsils. Changes in phenotype tend to be associated with the T-cell potential to proliferate and their cytotoxic capabilities. For example CD28-/CD27- CD8+ T-cells have greater cytotoxic potential and a reduced ability to proliferate.

Two main mechanisms, cytolytic and anti-viral cytokine secretion, are employed by CD8+ T-cells (Figure 1.9). Early investigation concentrated on the use of the cytolytic molecule perforin to lyse EBV immortalised B-cells (Khanna *et al.*, 1992;Murray *et al.*, 1992). However, in some cases this cytolytic lysis was weak especially with EBNA-1 specific T-cells (Hill *et al.*, 1995;Blake *et al.*, 2000). More recently, anti-viral cytokine production has been investigated with reports of EBNA-3C specific T-cells controlling B-cell outgrowth via IFN-γ secretion (Shi and Lutz, 2002). Similarly IFN-γ production has been detected from EBNA-1 specific T-cells in response to LCL stimulation (Lee *et al.*, 2004). Moreover, IFN-γ production is increased upon removal of the gly-ala domain (Lee *et al.*, 2004).

1.3.2.3 CD4+ T-cell response to EBV

CD4+ specific T-cells prime both the humoral and cellular mediated immune responses discussed above but despite their crucial function they have not been studied in detail until recently. This has probably been due to the low frequencies of CD4+ T-cells in peripheral blood. During IM latent and lytic antigens, EBNA-1, EBNA-3A, BZLF-1 and BMLF-1, stimulate only 0.3% of the CD4+ T cell population (Precopio *et al.*, 2003) whilst in persistent infection this percentage drops to 0.1% (Amyes *et al.*, 2003). On average the frequency of CD4+ T-cells reaches one tenth that of CD8+ T-cells during both acute and persistent infection (Munz, 2005).

Investigation of epitope specificity revealed that CD4+ T-cells recognise different latent antigens to that of CD8+ T-cells. Most notable is the recognition of EBNA-1 (Munz *et al.*, 2000;Leen *et al.*, 2001;Mautner *et al.*, 2004) which is consistently recognised in persistent carriers and is more frequently recognised than other latent antigens. EBNA-3B and -3C antigens are recognised by approximately 50% of persistent carriers however in contrast to CD8+ T-cells EBNA-3A specific CD4+ T-cells are rarely detected (Paludan and Munz, 2003). Similarly LMP-1 specific responses are more readily detected in CD4+ compared to CD8+ T-cell populations (Marshall, Vickers, and Barker, 2003). Unfortunately little is known regarding the specificity toward lytic antigens although CD4+ T cell responses have been reported for gp350, BZLF-1, BMLF-1 and BHRF-1 antigens (Precopio *et al.*, 2003;Landais *et al.*, 2004).

The majority of EBV-specific CD4+ T-cells secrete Th1 ctyokines such as IFN-γ (Amyes *et al.*, 2003). Both EBNA-1 and EBNA-3C specific CD4+ T-cells have been shown to secrete IFN-γ *in vitro* (Bickham *et al.*, 2001) and also *in vivo* in the case of EBNA-1 (Bickham *et al.*, 2001). Th1 polarisation of CD4+ T-cells may help to mediate efficient protection against viral infections (Rentenaar *et al.*, 2000). Interestingly LMP-1

specific CD4+ T-cells from persistent carriers are not Th1 polarised but secrete IL-10 (Marshall, Vickers, and Barker, 2003) suggesting a role in regulation of T-regulatory cells (Treg). Moreover, CD4+ T-cells from persistently infected individuals have been shown to have an effector role in immune regulation. The addition of CD4+ T-cells prevented the proliferation of EBV infected B-cells in culture and conversely the removal of CD4+ T-cells allowed the B-cells to grow (Nikiforow, Bottomly, and Miller, 2001). This was attributed to IFN-γ secretion and the Fas/FasL interaction between B cell and CD4+ T-cells (Nikiforow, Bottomly, and Miller, 2001;Paludan *et al.*, 2002). Interestingly, Burkitt lymphoma cells expressing only EBNA-1, largely undetected by CD8+ T-cells, can be targeted by EBNA-1 specific CD4+ T-cells (Paludan *et al.*, 2002;Fu, Voo, and Wang, 2004). These studies indicate the important effector role of CD4+ T-cells.

1.3.3 Cytokine interactions

Cytokines are protein factors produced by cells to act on cells. They are generally soluble, have a similar structure to hormones and growth factors, and bind to specific receptors to activate intracellular signalling pathways. Normally they are tightly controlled and are induced in response to host challenge from microbial infections. As we can see from the previous sections they have a pivotal role to play in both innate and adaptive immunity. Cytokines can be divided into 4 main groups based upon induction and function: initial and innate, adaptive, chemokines, and haematopoietic growth factors. The first 3 mediate defence against infection through a complex network while the fourth mediates leucocyte growth and differentiation. Some cytokines such as IFN-γ belong to more than one group.

Unique microbial structures, such as double-stranded RNA in virus infected cells, recognised by cell receptors, are thought to elicit the initial and innate response cytokine cascades (Medzhitov and Janeway, 1998;Yang *et al.*, 1998). Upon recognition several positive and negative regulatory gene elements are activated that bind to specific transcription factors activating downstream elements such as NFκB (Maran *et al.*, 1994) which is required for transcription of IFN-β and some IFN-α genes. Interferon regulatory factors (IRFs) are also activated and bind to specific DNA elements in IFN genes activating their transcription which in turn activates transcription of other IRFs (Marie, Durbin, and Levy, 1998). Therefore initial IFN production in response to infection can induce further innate responses. Production of IFN-α/β can also regulate expression of a number of other innate cytokines such as IL-15 (Waldmann and Tagaya, 1999) and IL-12 (McRae *et al.*, 1998) as well as modify immune cell distribution and function. Changes associated with IFN-α/β include activation of NK-cell cytotoxicity, inhibition of NK-cell responsiveness to IL-12, enhancement of MHC-class I presentation, and facilitation of T-cell IFN-γ responses (Cousens *et al.*, 1999;Nguyen *et al.*, 2000;Biron *et al.*, 1999). Thus the IFN-α/β cascade has the potential to regulate a variety of innate and adaptive immune responses.

A second innate cytokine cascade, which has been extensively studied in bacterial infection, includes production of TNF, IL-1 and IL-6. This pathway may also be utilised in viral infection, particularly with herpes virus infection (Pulliam, Moore, and West, 1995;Kanangat *et al.*, 1996;Orange and Biron, 1996). TNF-α mediates several immunoregulatory and anti-viral functions including apoptosis, monocyte activation, dendritic cell migration, enhancement of IFN-γ responses and up-regulation of MHC-class I expression (Balkwill, 2006;Biron and Sen, 2001). IL-1 also enhances IFN-γ and IL-6 production whilst IL-6 promotes B-cell growth (Biron and Sen, 2001). IL-12

induces production of IFN-γ by NK-cells and also promotes some CD4+ T-cell responses associated with the production of IL-2 and IFN-γ (Biron and Sen, 2001). During the adaptive immune response a major function for IL-2 is to promote T-cell proliferation however it can also induce IFN-γ production by T-cells and promote antibody synthesis by B-cells (Stark *et al.*, 1998). Likewise IFN-γ has several immunoregulatory functions including upregulation of MHC class I and II expression, it promotes expression of the IL-12 receptor, and can influence immunoglobulin class switching on B-cells (Biron and Sen, 2001). During the adaptive response T-cells also secrete IL-4, -5, and -13 that promote B-cell responses and activate eosinophils. T-cells also produce TGF-β, IL-10 and lymphotoxin (LT). LT has similar effects to that of TNF-α and is often produced along with IFN-γ and functions to promote lymphomorphogenesis (Chaplin and Fu, 1998).

The interaction of the various cytokines produced during both the innate and adaptive immune response is therefore an important one with components of the cascade dependent upon each other for activation and regulation. Alterations to these cascades could affect several aspects of the immune response. During lytic EBV infection a viral homologue of IL-10 (vIL-10) is expressed which can suppress immune responses through inhibition of IL-12 and IFN-γ in vitro (Moore *et al.*, 2001). Also expressed during lytic infection is BARF1, a soluble receptor for colony stimulating factor-1(CSF-1), which blocks CSF-1 stimulation of monocyte proliferation and the release of cytokines such as IFN-α (Cohen and Lekstrom, 1999).

1.4 EBV-associated disease

In order to counterbalance the immune surveillance mechanisms discussed in section 1.3 EBV has developed several strategies to avoid clearance from the human host. As already discussed, EBNA-1 contains a cis-acting gly-ala repeat domain that limits the amount of antigen displayed by MHC class I molecules and EBNA-1 specific T-cells possibly depend upon derived DRiPs for recognition of infected cells (Munz, 2004). However, perhaps the most efficient escape mechanism is the number of antigens expressed at low copy numbers per cell. Indeed frequency estimates of epitopes, for example the RRIYDLIEL epitope from EBNA-3C, are as little as 1 copy per cell (Crotzer *et al.*, 2000). Low antigen expression may be problematic for efficient antigen presentation to T-cells. In addition antigen expression is further restricted depending on the stage of B-cell differentiation with only EBNA-1, LMP-1 and LMP-2 expressed in germinal centre B-cells or EBNA-1 alone in long-lived memory B-cells (discussed in section 1.2.3.1). EBV depends upon such evasive measures for survival and in the vast majority of immunocompetent individuals causes no disease. However, in some cases T-cell immunity can be comprised either through diversion/alteration of these EBV-specific evasive manoeuvres or via immunosupression of the host. Some of the resultant diseases are listed in Table 1.7 and discussed in subsequent sections of this chapter.

1.4.1 Infectious mononucleosis (IM)

Infectious mononucleosis (IM) is an acute self-limiting lymphoproliferation that was first described in the medical literature towards the end of the 19th century when Filatov and Pfeiffer simultaneously described an illness characterised by fever, pharyngitis, lymphadenopathy, hepatosplenomegaly and abdominal discomfort, which later became known as Glandular Fever (Schooley, 1995). However due to a lack of diagnostic

Table 1.7 EBV-associated disease of lymphoid and epithelial cell origin

Disease	Population at risk	EBV association
Lymphoid origin		
Infectious Mononucleosis (IM)	Young adults with high socio-economic status	>90%
B-lymphoproliferative disease (BLPD)	Immunosupressed individuals: Post-transplant lymphoproliferative disease (PTLD) HIV-infected individuals: Primary CNS lymphoma Peripheral lymphoma	~90% 100% ~50%
Burkitt's lymphoma (BL)	African children (endemic) HIV-infected individuals	>90% ~25%
Hodgkin's lymphoma (HL)	Children in developing countries Young adults from high socio-econimic groups and history of IM	~65% 25-50%
X-linked lymphoproliferative disease (XLP)	Males with mutation in XLP gene	>90%
Primary effusion lymphoma (PEL)	HIV-infected individuals	~70%
T/NK cell lymphoma	Individuals with chronic active EBV HIV-infected individuals	10-100% 10-100%
Epithelial origin		
Nasopharangeal carcinoma (NPC)	Southern Chinese and Inuit races	~100%
Oral hairy leukoplakia (OHL)	HIV-infected individuals Immunosupressed individuals	~100%
Gastric carcinoma	Unknown	~10%

techniques the disease was not fully recognised as a clinical entity until 1921 when a mononuclear lymphocytosis was first described and the characteristic atypical lymphocyte identified (discussed in section 1.3.2). A major advance in the diagnosis of IM occurred in the 1930's when Paul and Bunnell observed the presence of non-specific IgM antibodies, called heterophile antibodies, in the blood of IM patients (Schooley, 1995). Diagnostic techniques were developed based upon detection of these antibodies: the monospot test, which is still used today in conjunction with the EBV-specific antibody profile (discussed in section 1.3). Perhaps the most significant development in the history of IM was the observation that EBV was the causative agent. Sequential

serum samples obtained from a technician with IM in the laboratory of Werner and Gertrude Henle were analysed for EBV-specific antibodies (Henle, Henle, and Diehl, 1968). The results suggested that acute EBV infection was associated with the illness. Subsequent epidemiological studies demonstrated that IM occurred in EBV sero-negative individuals who upon EBV infection developed an antibody response to EBV (Evans, Niederman, and McCollum, 1968;Sawyer *et al.*, 1971;Niederman *et al.*, 1968). We now know that approximately 90% of all IM cases are aetiologially linked to primary infection with EBV; the remaining 10% are linked to other agents, most frequently cytomegalovirus.

In most countries IM is not a notifiable disease and as a result determining the incidence can be difficult. However in 1955 a pilot study asking hospital laboratories in England and Wales to report serologically confirmed IM cases to the Public Health Laboratory Service estimated an incidence rate of 50 cases per 100,000 persons per year (Newell, 1956). A similar rate of 45 cases per 100,000 persons per year was obtained from a study carried out in the USA in the 1970's (Heath, Jr., Brodsky, and Potolosky, 1972). Ireland is one of the few countries to notify IM on a regular basis and results between 1988 and 2003 estimate a rate of 40 cases per 100,000 persons per year suggesting that the incidence rate has remained fairly stable over the past 50 years. More recently, an epidemiological study investigating IM in university students demonstrated an annual incidence rate of 3.7% among those who were sero-negative (Crawford *et al.*, 2006). However, there are reports that the number of hospital admissions due to IM in France is increasing from 1.4/year to 7/year and that more cases are being admitted to intensive care units (Tattevin *et al.*, 2006) suggesting that severe IM may be increasing. Epidemiological data from several studies suggest that the highest incidence rates for IM are seen in the 15-24 year old age group, in those with a higher socio-economic

status, and in those who are sexually active (Crawford *et al.*, 2002;Niederman *et al.*, 1970;Hallee *et al.*, 1974;Hesse *et al.*, 1983).

The classical symptoms of IM (pharyngitis, fever and lymphadenopathy) usually appear following an incubation period of 4-7 weeks (Hoagland, 1964) and resolve within 2-6 weeks of development (Williams and Crawford, 2006). However in some cases the symptoms can persist as in chronic active EBV (CAEBV) resulting in a high viral load within the peripheral blood and abnormal antibody responses (Macsween and Crawford, 2003;Cohen, 2005). CAEBV has a high morbidity and a high mortality rate (>40%) resulting from complications such as lymphoma, sepsis and haemophagocytic syndrome (Macsween and Crawford, 2003). Complications from classic IM can also occur such as airway obstruction, splenic rupture, liver failure and secondary bacterial infections (Macsween and Crawford, 2003;Cohen, 2006) and epidemiological data has also linked IM with the development of HL (Jarrett *et al.*, 2003;Hjalgrim *et al.*, 2007;Hjalgrim *et al.*, 2000).

The immune response during IM is characterised by a massive expansion of activated lymphocytes (discussed in section 1.3.2). The majority of activated lymphocytes observed in IM are oligoclonal, EBV-specific CD8+ cytotoxic T-cells (Annels *et al.*, 2000;Callan, Steven, and Krausa, 1996;Steven *et al.*, 1996;Steven *et al.*, 1997). This CD8+ T-cell expansion is believed to control the infection through lysis of infected cells and to cause the symptoms of IM via excessive cytokine secretion particularly the TH1 cytokines IL-2 and IFN-γ (Foss *et al.*, 1994;Biglino *et al.*, 1996). Indeed recent studies show a correlation between the level of activated T-cells and the severity of symptoms during IM with higher levels associated with more severe symptoms (Williams *et al.*, 2004). The resolution of symptoms has also been associated with the number of EBV antigens recognised by T-cells: a broader T cell response resulted in quicker resolution

of symptoms (Bharadwaj *et al.*, 2001). During convalescence the level of virus within the peripheral blood drops to baseline levels however the levels of virus within saliva remains elevated for up to 6 months (Fafi-Kremer *et al.*, 2005b;Fafi-Kremer *et al.*, 2005a) suggesting that patients can remain infectious for some time following resolution of symptoms.

Although much is known about the incidence of IM and the cellular immune response during IM it is still unclear why some individuals develop symptoms upon primary EBV infection and others do not. IM is rare in children and it has been postulated that a less developed immune system (children) may prevent the development of IM and conversely that a more mature immune system (adolescents) would allow development of IM upon primary infection. However, this does not fully explain why sero-conversion in adolescence is largely asymptomatic. It has also been suggested that the level of virus transferred to a sero-negative individual may be important however results suggest that there is no difference in viral load between asymptomatic and symptomatic individuals (Silins *et al.*, 2001). Genetic differences in components of the immune response may also be important and these shall be discussed in chapter 5.

1.4.2 Burkitt's lymphoma (BL)

As stated previously BL was first identified in African children by Denis Burkitt in 1958 and it remains one of the commonest childhood malignancies of Equatorial Africa (Magrath, 1990). There are 3 types of BL as defined by the World Health Organisation (WHO): endemic BL which occurs mainly in Africa, sporadic BL and immunodeficiency-related BL. BL was originally designated a sarcoma of the jaw, but was later determined to be a lymphoid tumour giving rise to tumours in other sites such as liver, kidney, the ovary, and mammary glands. Histologically, BL is characterised by

a rapid proliferation of B-cells with small round or oval nuclei and prominent basophilic nucleoli. Macrophages are often present within the tumour mass giving rise to the classic 'starry sky' pattern observed. The cellular origin is thought to be a germinal centre B-cell as B-cell surface markers are expressed and there is evidence of somatic hypermutation in IgG genes (Gregory *et al.*, 1987;Kuppers, 1999;Kuppers and Hansmann, 2005). The development of BL is a multi-step process involving at least 3 factors: malarial infection, dysregulation of the *c-myc* oncogene and EBV infection. Based on the geographical distribution of endemic BL and the age of onset it was postulated that an infectious agent may be linked with the development of BL. It was evident that the highest incidences of endemic BL occurred in regions with the highest rate of malaria transmission by *Plasmodium falciparum* (Morrow, 1985). Furthermore endemic BL levels were reduced in areas where mosquito eradication measures were introduced and in those with malarial resistance due to the sickle cell trait (Crawford, 2001). However the mechanism for the interaction between the two is unclear. *P.falciparum* malaria can lead to defects in cell-mediated responses (Ho *et al.*, 1998;Hviid *et al.*, 1992) and to alterations in B-cell homeostasis (Nagaoka *et al.*, 2000) therefore 2 mechanisms have been proposed: suppression of T-cell immunity and activation/expansion of B-cells. In support of the T-cell suppression theory it has been demonstrated that PBMCs isolated from patients with acute malaria are unable to control outgrowth of EBV immortalised cells and that healthy adults living in endemic regions have impaired EBV-specific T-cell responses (Whittle *et al.*, 1984;Whittle *et al.*, 1990;Moormann *et al.*, 2005). However, it is argued that the immunosuppressive effect of malaria does not explain the relationship fully. Lam et al (1991) demonstrated that the number of EBV-infected B-cells increases during acute malarial episodes and

young children living in endemic areas had EBV loads typical to that seen in acute IM (Moormann *et al.*, 2005;Lam *et al.*, 1991).

A molecular hallmark of all 3 types of BL is the activation of the *c-myc* gene on chromosome 8. Chromosomal breaks that result in the translocation of the *c-myc* gene to sites involved in Ig gene expression on chromosomes 2, 14 and 22 can result in aberrant expression of the *c-myc* gene (Magrath, 1990). Constitutively active *c-myc* is a prominent feature in BL cases. *c-myc* is a transcription factor that regulates cell growth, differentiation and apoptosis and may contribute to the development of BL by promoting cell cycle progression (Hecht and Aster, 2000;Lindstrom and Wiman, 2002). EBV is associated with >90% of endemic BL (Labrecque *et al.*, 1994). The association of EBV with sporadic BL and immunodeficiency-related BL is around 25% and 30% respectively (Griffin and Rochford, 2005). This variation has led to speculation that EBV is merely a passenger in BL pathogenesis however there is strong evidence that EBV is linked to BL development. A large scale prospective study carried out in Uganda in the 1970's demonstrated very high antibody titres directed against EBV VCA prior to development of BL suggesting the EBV infection was poorly controlled and contributed to the development of BL (de-The, 1977). The EBV genome is present in BL cells as multiple nuclear episomes and has been shown to be clonal, suggesting that infection is an early event (Raab-Traub and Flynn, 1986;Neri *et al.*, 1991). Viral antigen expression is limited to EBNA-1, EBERs and BARTs (Rowe *et al.*, 1986) which may explain the BL cell's ability to avoid immune detection. As with malaria the mechanisms involved with the association are still unclear. BL cell lines lacking EBERs are more sensitive to apoptotic signals then EBERs positive cell lines suggesting that EBV may block apoptosis (Takada and Nanbo, 2001). The expression of Tcl-1, a protein originally detected in T-cells, can be stimulated by EBV in BLs thereby

promoting cell survival (Kiss *et al.*, 2003). Upon EBV infection and during B-cell expansion translocation of the c-myc gene may occur rendering expression of EBV latent antigens redundant (Griffin and Rochford, 2005).

The pathogenesis of BL is probably due to all 3 factors. Firstly EBV-infection leading to viral persistence within B-cells, followed by recurring bouts of malaria resulting in increased viral production and/or T cell suppression. Expression of viral antigens protects from apoptosis and promotes cell cycle progression. Rapid proliferation and prolonged expression of viral antigens may lead to increased incidence of c-myc translocations leading to outgrowth of malignant cells with a restricted EBV antigen expression. Therefore EBV-infection alone may not be sufficient, but rather a necessary co-factor, in the development of endemic BL.

1.4.3 Hodgkin's lymphoma (HL)

HL is one of the commonest tumours in young adults in the West where its incidence is increasing (Swerdlow, 2003). Approximately 1500 new cases occur each year in the UK, and HL now accounts for one in eight of all lymphomas diagnosed. The lymphoma is characterised by the disruption of normal lymphnode architecture and the presence of the malignant Reed-Sternberg (RS) cell comprising less than 2% of the tumour mass (Harris *et al.*, 1994). Identification of non-functional re-arranged Ig genes and somatic mutation suggests that RS cells are of B-cell origin, more specifically of post-germinal centre origin (Kuppers and Rajewsky, 1998;Tamaru *et al.*, 1994). The RS cells are surrounded by a complex mixture of immune cells, fibroblasts and stromal cells that cross-talk via a cytokine network to favour tumour survival (Pinto *et al.*, 1998). HL comprises 4 subtypes: nodular sclerosing (NS), mixed cellularity (MC), lymphocyte depleted (LD) and lymphocyte predominant (LP).

The incidence of HL is bimodal with a peak in childhood and another in late adulthood however there are socio-economical and geographical variations in age of onset. The childhood peak occurs later (15-35yrs) in more prosperous countries compared to an earlier peak (5-10yrs) in less prosperous countries (Macsween and Crawford, 2003). This disparity in age is similar to that seen for EBV sero-conversion and the development of IM suggesting an association between the two. Indeed in less affluent countries HL is predominantly EBV-associated. However in more affluent countries HL is predominantly of the non-EBV-associated NS variety. A recent UK based study confirmed the non-EBV-associated peak but also demonstrated a smaller bimodal distribution for EBV-associated HL in the younger age groups (Jarrett, 2002;Jarrett *et al.*, 2005).

The detection of raised antibody titres to EBV antigens in HL patients prior to treatment provided the first evidence that EBV was associated with HL pathogenesis (Mueller *et al.*, 1989;Levine *et al.*, 1971). In addition IM was identified as a risk factor for the development of HL (Gutensohn and Cole, 1980) and recent reports suggest that EBV-associated HL is increased four-fold following IM, usually developing on average 3yrs after resolution of IM symptoms (Hjalgrim *et al.*, 2003;Hjalgrim *et al.*, 2007;Hjalgrim *et al.*, 2000). Advances in molecular technology led to the identification of the EBV genome in the RS cells (Weiss *et al.*, 1989;Wu *et al.*, 1990) of approximately 25-50% of HL cases from affluent areas (Andersson, 2006) and in around 65% of HL cases from less prosperous areas (Dinand *et al.*, 2007;Glaser *et al.*, 1997). Other immuno-suppressive factors may account for this increased HL development in underdeveloped areas. Higher EBV-positive HL rates observed in HIV-infected subjects would support this theory (Uccini *et al.*, 1990). EBV is detected most readily in MC and LD type HL (60-80% of cases) and less so in LP type HL (<10%) (Rickinson and Kieff, 2006). The

viral genome is normally clonal suggesting an early infection event (Anagnostopoulos *et al.*, 1989) and the virus persists at multiple HL sites (Coates, Slavin, and D'Ardenne, 1991). EBV-positive RS cells display a type II form of latency (Grasser *et al.*, 1994;Deacon *et al.*, 1993;Pallesen *et al.*, 1991), in particular LMP-1and LMP-2A are expressed at high levels suggesting an important role in disease progression. LMP-1 upregulates B-cell activation markers, IL-10, cell adhesion molecules and anti-apoptotic genes; and acts as a constitutively activated member of the TNF receptor family activating a number of signalling pathways. LMP-2A acts as a constitutively activated BCR driving proliferation and protecting from apoptosis (discussed in section 1.2.2.2). Between them, LMP-1 and -2A provide the necessary signals for EBV-infected B-cells, and in the case of HL, RS cells, to undergo proliferation in the germinal centre and protect from apoptosis. LMP-1 and -2A are also potential targets for CTL (Khanna *et al.*, 1998;Lee *et al.*, 1997). The survival of EBV-infected RS cells expressing these antigens would suggest that mechanisms are employed to evade CTL detection. This is supported by the observation that IL-10 is increased in HL serum as is the expression of thymus and activated regulated cytokine (TARC) (Herbst *et al.*, 1996;Poppema *et al.*, 1998) both of which act to skew the environment from a Th1 type to a Th2 type of response.

Interestingly there are few differences between EBV-positive HL and EBV-negative HL therefore in the absence of EBV other cellular genetic events may be required to elicit a similar pathogenesis.

1.4.4 Post transplant lymphoproliferative disease (PTLD)

PTLD encompasses a heterogeneous group of disorders that can follow solid organ transplantation (SOT) or bone marrow transplantation (BMT) as a consequence of

immunosupression and range from reactive hyperplasia to malignant monoclonal entities (Gottschalk, Heslop, and Rooney, 2005;Burns and Crawford, 2004). Classification by the WHO divides PTLD into early lesions (reactive plasmacytic hyperplasia and mononucleosis-like syndrome), polymorphic lesions, monomorphic lesions and Hodgkin's like lesions. Progression from early lesions to the more aggressive monomorphic lesions can occur (Larratt *et al.*, 2001).

The incidence of PTLD varies with type of transplant, level of immunosupression and EBV sero-status. PTLD occurs in approximately 1-3% of all renal, heart or liver, 7-33% of lung, intestine and multi-organ, and less than 1% of bone marrow transplants (Cockfield, 2001). The highest incidence occurs in the first few years following transplant and may involve the grafted organ as well as other sites such as the gastrointestinal tract, lungs and central nervous system (Gottschalk, Heslop, and Rooney, 2005). High levels of immunosuppressive agents such as cyclosporine and tacrolimus have been linked with an increased incidence of PTLD (Brumbaugh *et al.*, 1985;Sokal *et al.*, 1997) as has the use of anti-CD3 antibodies and anti-thymocyte globulin (Sokal *et al.*, 1997;Swinnen *et al.*, 1990). Furthermore, the incidence of PTLD rises from 1% to 24% in BMT recipients receiving T-cell depleted bone marrow, suggesting that removal of T-cells further promotes PTLD development (Shapiro *et al.*, 1988). EBV sero-status is also important in PTLD development. EBV sero-negative transplant recipients constitute a major risk group with estimations of a 10-76 fold increase in incidence compared to sero-positive recipients (Cockfield, 2001). Indeed around 50% of PTLD cases are associated with primary EBV infection and present with IM-like symptoms soon after transplantation (Ho *et al.*, 1985). A sero-negative status may account for the particularly high PTLD incidence in children who are less likely to have encountered the virus. However, not all PTLD cases are associated with EBV.

EBV-negative lesions are well documented and tend to occur several years after transplantation (Swerdlow *et al.*, 2000;Leblond *et al.*, 1998).

Immunosuppressive drug therapy to prevent graft rejection following transplantation results in severely reduced T-cell function, leading to increased virus replication and an accumulation of latently infected B lymphocytes which in some individuals may result in uncontrolled EBV driven B-cell lymphoproliferation and the onset of PTLD. EBV may be the key factor in the development of disease, particularly the hyperplastic and polyclonal forms in which the infected B-cells express all of the latent genes required to drive B-cell immortalisation. However, viral gene expression can vary within and between tumours with restricted expression patterns such as those seen in HL and BL observed in some cases (Cen *et al.*, 1993). Moreover, immunosupression increases the number of circulating infected B-cells but only a small proportion of individuals develop PTLD suggesting that only a few infected cells have the ability to immortalise. Therefore, other factors in addition to EBV may be required.

PTLD tumours may originate from donor cells, as predominantly occurs in the BMT situation, or from recipient cells as generally occurs in the SOT scenario (Shapiro *et al.*, 1988). More recently, investigation of somatic mutations in Ig heavy chain genes has shown that tumours can arise from naïve or post GC memory B-cells as opposed to the situation in healthy persistently infected individuals where infection is contained with the memory compartment only (Timms *et al.*, 2003). Moreover when tumours arose from memory B-cells frequent Ig gene mutations were observed suggesting that EBV infection provided rescue signals to abnormal B-cells, preventing apoptosis, and encouraging proliferation. A similar mechanism has been proposed in the development of HL (section 1.4.3).

T-cells and the production of cytokines may be an important co-factor in the development of disease as cytokines can support the growth of EBV infected B-cells. Elevated serum levels of IL-4, -6 and -10 have been reported as well as the detection of IL-4 and IL-10 messenger RNAs (Mathur *et al.*, 1994;Nalesnik *et al.*, 1999;Tosato *et al.*, 1993). Interestingly, PTLD biopsies show an infiltrate of CD4+ T-cells which are a potential source of these cytokines (Perera *et al.*, 1998). The population of CD4+ T-cells are also important for the development of tumours from PBMCs in severe combined immunodeficiency (SCID) mouse models of PTLD, as reduced of numbers CD4+ T-cells in PBMC result in reduced tumour incidence (Veronese *et al.*, 1992;Johannessen, Asghar, and Crawford, 2000). In addition, polymorphisms within cytokine genes have been associated with the development of PTLD. Polymorphisms leading to low level expression of IFN-γ have been shown to be more frequent in PTLD subjects following renal and liver transplantation and may predispose to its development (VanBuskirk *et al.*, 2001). However, this observation has been refuted in other studies (Thomas *et al.*, 2005). Other genetic abnormalities may play a role, particularly in the case of the monomeric forms of the disease. Mutations of the oncogenes *ras* and *c-myc* and the tumour suppressor p53 have been detected in some monomeric PTLD cases perhaps contributing to the more aggressive nature of this tumour (Knowles *et al.*, 1995;Chadburn, Cesarman, and Knowles, 1997).

A number of strategies are employed in the treatment of PTLD. Reduction of immunosupression with the aim of restoring EBV-specific immunity is usually the first line of treatment in solid organ recipients. However tumour regression is only observed in approximately 20-50% of cases (Paya *et al.*, 1999) and may be limited by the onset of graft rejection due to reduced immunosuppression (Swinnen *et al.*, 1995). Other forms of treatment such as chemotherapy, radiotherapy, rituximab (anti-CD20 monoclonal

antibody) are often utilised but often have debilitating side effects (Swinnen *et al.*, 1995;Choquet *et al.*, 2006). However, despite these treatments the overall mortality from PTLD in solid organ transplantation is still around 50% (Armitage *et al.*, 1991;Opelz and Dohler, 2004). In recent years the use of adoptive cellular immunotherapy has been developed for the treatment of PTLD and has shown promising results without significant side effects or the risk of graft rejection (section 1.5).

1.5 Adoptive immunotherapy

The concept of immunotherapy, which involves harnessing or restoring immune mechanisms to treat disease, is an attractive one, since controlled manipulation of natural defences promises to be less invasive and/or less toxic than other available therapeutic options. Most immunotherapeutic approaches use antibodies or cellular components of the immune system to target specific antigens on diseased tissues, particularly those expressed on virus infected or tumour cells. Our increased understanding of the gene expression in tumour cells and the mechanisms by which the immune system, in particular T-cells, recognises antigens, has facilitated the development of adoptive CTL immunotherapy for the treatment of EBV-associated malignancies.

T-cell immunotherapy using antigen specific cytotoxic T-cell lines (CTL) was first pioneered in the USA for the treatment of cytomegalovirus (CMV) infection in BMT patients. Early studies using a murine (M) CMV model showed that large numbers of virus specific CD8+ memory T-cells could be grown *ex vivo* from latently infected immunocompetent mice, that they retained their cytotoxic activity, and were able to prevent virus replication in vivo when transferred to immunodepleted mice challenged with MCMV (Reddehase *et al.*, 1988;Reddehase *et al.*, 1987). Riddell et al (1992)

reported the first adoptive transfer of CMV antigen specific CTLs in humans, demonstrating that CTL from BMT donors grown ex vivo and then administered to the BMT recipients remained detectable for up to 1 month post infusion and retained their cytolytic potential (Riddell *et al.*, 1992). This study set the scene for the use of *ex vivo* expanded CTL to treat virus infections with high morbidity and mortality in immunodeficient patients such as EBV-associated PTLD.

1.5.1 Adoptive T-cell immunotherapy for EBV-associated PTLD

Early studies using unselected populations of peripheral blood leucocytes from EBV seropositive BMT donors to treat PTLD in the BMT recipients suggested that these transferred cells could control the EBV driven lymphoproliferation. In one study 3/5 patients showed some tumour regression (Papadopoulos *et al.*, 1994), presumably due to the presence of EBV-specific cytotoxic T-cell precursors in the infusions. However, this treatment was associated with potentially fatal complications, including pneumonitis and graft-versus-host disease, probably mediated by non-specific cell populations, such as NK-cells, activated macrophages and alloreactive T-cells, within the infusions. The emphasis therefore shifted to refining the infused cell populations by *ex vivo* expansion of EBV-specific T-cells from peripheral blood prior to infusion.

Generation of EBV-specific T-cell lines for infusion

EBV-specific CTL can be routinely grown *ex vivo* from the peripheral blood of healthy EBV sero-positive donors. EBV immortalised LCL generated by EBV infection of peripheral blood B lymphocytes serve as antigen presenting cells for EBV latency III antigens while the addition of IL-2 to the culture system stimulates T-cell growth. CTL are normally expanded for 8-10 weeks with weekly LCL stimulation to ensure specificity, after which flow cytometry and cytotoxicity analyses generally show

expanded CTL to contain predominately activated CD8+ cells, often with a minority population of CD4+ cells, and good HLA class 1 restricted killing specific for EBV-positive target-cells expressing a latency III pattern.

Autologous EBV-Specific CTL

Rooney *et al* (1998) were the first to demonstrate the effective use of donor derived EBV-specific CTL for the prevention of PTLD following T-cell depleted BMT (Rooney *et al.*, 1995;Rooney *et al.*, 1998). Of the 39 patients treated with CTL none developed PTLD compared to a 11.5% PTLD rate in a historical control group with no CTL prophylaxis. The same group also showed that donor derived EBV-specific CTL could be used to successfully treat established PTLD in BMT patients with complete resolution of tumour observed in two cases (Rooney *et al.*, 1998). Studies have shown that infused CTL can be detected within the tumour mass post treatment and survive long term in their adoptive hosts (Heslop *et al.*, 1996).

Similar studies on PTLD in solid organ transplant (SOT) patients have used autologous EBV-specific CTL expanded from the patients themselves prior to transplant. This has induced tumour regression in several cases, including lung and renal transplant recipients, with no infusion related toxicity (Comoli *et al.*, 2005a;Khanna *et al.*, 1999). However there are a number of drawbacks to the use of autologous CTL as a first line therapy, particularly in the SOT situation where generally the donor can not be used as a source of CTL. Due to the length of time required to grow CTL *ex vivo* there may be a delay of up to 3 months before treatment can be administered, and this is not acceptable for an aggressive tumour. It is also difficult to expand CTL from EBV sero-negative individuals, who are at high risk of developing PTLD following transplant, and

generating LCL for CTL stimulation from patients treated with B lymphocyte depleting regimens such as rituximab is not always possible.

Allogeneic EBV-Specific CTL

The problems inherent in the use of autologous CTL, where the treatment must be tailored to each individual patient, mean that this expensive, labour intensive form of therapy is not suitable for general use. An alternative option which overcomes some of these problems is to use allogeneic EBV-specific CTL expanded from healthy EBV sero-positive blood donors. Haque *et al* (2007) employed this approach in a phase II clinical trial treating PTLD in patients with partially HLA-matched CTL from a frozen bank of 100 EBV-specific CTL of known HLA type (Wilkie *et al.*, 2004;Haque *et al.*, 2002;Haque *et al.*, 2007). No CTL related toxicity was detected and complete or partial response was observed in 52% of patients, all of whom had failed on conventional treatment regimens. Successful outcome correlated with a high percentage of CD4+ cells in the infused CTL suggesting that these 'helper' T-cells promote the survival and cytotoxic function of the majority CD8+ population *in vivo*. During the trial CTL from the bank were sent to locations as distant as Australia where they were used successfully to treat PTLD patients (Gandhi *et al.*, 2007). Thus a single bank of CTL can provide for a large area making this a feasible option for routine clinical practice.

1.5.2 Adoptive T-cell therapy for EBV-associated diseases other than PTLD

Following the success of CTL therapy for PTLD, both autologous and allogeneic EBV-specific CTL have been used to augment patient immune responses in NPC and HL. However, as expected, EBV-specific CTL grown in the conventional way are mainly directed against the EBNA-3 complex, with only a small proportion of T-cells within the polyclonal population recognising the subdominant epitopes on LMP-1, -2 and

EBNA-1 which are the major 'tumour antigens' expressed by HL and NPC tumour cells. Not surprisingly success is limited with only a few cases showing complete regression of tumour (Straathof *et al.*, 2005;Comoli *et al.*, 2005b;Bollard *et al.*, 2004;Lucas KG *et al.*, 2004;Comoli *et al.*, 2004).

It is clear that CTL targeting sub dominant epitopes in LMP-1, -2 molecules are required for successful immunotherapy of malignancies with restricted EBV gene expression. New ways of expanding EBV-specific CTL *ex vivo* are now providing CTL specific for these rare viral epitopes by altering antigen presentation to EBV-specific CTL precursors. Various methods of presenting these antigens have been employed including the use of LCL loaded with EBV peptides, transduction of LCL or dendritic cells with vectors expressing specific EBV antigens, and EBV peptide coated beads (Gottschalk *et al.*, 2003;LU *et al.*, 2006;Ayako Demachi-Okamura *et al.*, 2006). In all cases EBV-specific CTL reactive against sub-dominant EBV epitopes have been generated which display similar growth properties and phenotypes to those expanded by established methods and show good EBV-specific cytolytic activity.

Although improvements in cell targeting and expansion are extending the potential use of CTL immunotherapy from PTLD to other EBV-associated malignancies with more restricted gene expression, some caution is still required when directing CTL against individual epitopes. An EBV (EBNA-3B) deletion mutant has been described following CTL treatment which allowed the tumour cells to evade CTL killing and continue to grow (Gottschalk *et al.*, 2001). Such mutants may potentially contribute to some of the treatment failures observed in various studies. However, even with such concerns CTL immunotherapy for EBV-associated malignancies is becoming a practical option in the clinical setting.

1.6 Aim

As discussed in previous sections the host immune response to EBV infection has a major role to play in the development of disease, especially with regard to IM and PTLD. Understanding both the viral and host immuno-regulatory mechanisms involved in the development of disease is crucial for devising new and improved treatments. The aim of this thesis was to investigate several aspects of the immune response to EBV-associated disease. This resulted in 3 independent studies.

Study 1: investigated the epitope specificity and T-cell receptor (TCR) clonality of CTL used to successfully treat a panel of PTLD patients. Characterisation at this level may improve matching procedures between donor and recipient when using immunotherapy based treatments as well as provide insights into the recognition process of virus-infected cells by activated T-cells.

Study 2: investigated single nucleotide polymorphisms (SNPs) in several cytokine genes (TNF, IL-1, -6, -10) in both IM and PTLD cases and compared these with relevant control groups. As discussed the cytokine network has a pivotal role in the immune response to infection and variation in cytokine genes, and ultimately cytokine levels, may alter this response.

Study 3: analyzed two microsatellite markers and two SNPs located near the HLA class I locus in IM, PTLD and control subjects to determine whether HLA genes may affect development of EBV-associated diseases. The HLA locus, particularly the class I region, is primarily involved with antigen presentation to T-cells. Variation in the genes of this locus could potentially alter how viral peptides are presented to the immune system thereby dictating the overall level of the immune response.

Chapter 2:
Materials and Methods

2.1 Suppliers and Manufacturers

Autoimmun Diagnostika GmbH	Ebinger Strasse 4, 72479 Strassberg, Germany
GE Healthcare UK Ltd	Amersham Place, Little Chalfont, HP7 9NA, UK
Applied Biosystems	7 Kingsland Grange, Woolston, Warrington, WA1 4SR UK
BD Biosciences/PharMingen	21 Between Towns Road, Cowley, Oxford, OX4 3LY, UK
Beckman Coulter	Oakley Court, Kingsmead Business Park, London Road, High Wycombe, HP11 1JU, UK
Bioline Ltd	16 The Edge Business Park Humber Road, London, UK
Biometra GmbH i. L.	Rudolf-Wissell-Straβe 30, 37079 Goettingen, Germany
Chiron Corporation	4560 Horton Street, Emeryville, 94608-2926 California,USA
DAKO Ltd	Denmark House, Angel Drive Ely, CB7 4ET
Dynex Technologies	Billinghurst, West Sussex, UK
European collection of Animal cell culture (ECACC)	Porton Down, UK
Exalpha Biologicals Inc	20 Hampden St, Boston, MA 02119, USA
Greiner BioOne Inc	1205 Sarah St, Longwood, 32750 Florida, USA
Invitrogen	3 Fountain Drive, Inchinnan, Business Park, Paisley, PA4 9RF, UK
Miltenyi Biotec Ltd	Almac House, Church Lane, Bisley, Surrey, GU24 9DR, UK
Oxoid Limited	Wade Road, Basingstoke, RG24 8PW, UK
Perkin Elmer Life Sciences	Chalfont Road, Seer Green, Beaconsfield, HP9 2FX, UK

Promega UK	Delta House, Chilworth Science Park, Southampton, SO16 7NS, UK
Qiagen Ltd	Qiagen House, Fleming Way, Crawley, RH10 9NQ, UK
R&D Systems	19 Barnton Lane, Abingdon Science Park, Abingdon, Oxon, OX14 3NB, UK
Roche	Roche Diagnostics Ltd, Bell Lane, Lewes, BN7 1LG, UK
Sandoz Ltd	Unit 37, Woolmer Way, Bordon, GU35 9QE, UK
Sanyo Gallenkamp (MSE)	Monarch Way, Belton Park, Loughborough, LE11 5XG, UK
Sigma	Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, SP8 4XT, UK
Sigma-Genosys Ltd	Sigma-Aldrich House, Homefield Business Park, Homefield Road, Haverhill, CB9 8QP
Scientific Laboratory Supplies (SLS)	Coatbridge Business Centre, 204 Main Street Coatbridge, ML5 3RB, UK
Stratagene	11011 N. Torrey Pines Road, La Jolla, California CA92307, USA
Sterilin	Barloworld Scientific Ltd. Beacon Road, Stone, ST15 0SA, UK
Stuart Scientific	Barloworld Scientific Ltd. Beacon Road, Stone, Staffordshire, ST15 0SA, UK
ThermoFisher Scientific	Bishop Meadow Rd, Loughborough, Leicestershire, LE11 5RG, UK
ThistleResearch	27 Westbourne Crescent Glasgow, G61 4HB
Ultra-Violet Products Ltd	Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge, CB4 1TG, UK
Vector Laboratories	3 Accent Park, Bakewell Road, Orton,Southgate, Peterborough PE2 6X3, UK
VWR Intl Ltd	Merck House, Poole, Dorset, BH15 1TD, UK

2.2 Consumables and Equipment

2.2.1 Consumables

All tissue culture flasks, 96-well, 48-well, 6-well tissue culture plates and cryovials were obtained from Nunc.

0.2ml PCR tubes were supplied by Greiner, 0.5ml and 48-well PCR tubes by Anachem.

LP-2 gamma counter tubes were supplied by Thermo.

Unless otherwise stated all other plastic-ware was obtained from either SLS or Sterilin.

2.2.2 Equipment

Several types of centrifuge were used in the course of this work and are listed below:

Sanyo MSE Mistral 3000E (Rotor 43124129) for cell spins.

Sanyo MES Mistral 3000i (Rotor43124129) for cell spins with temperature control.

Beckman Coulter TJ-6 for spins with chromium 51 loaded cells.

Sigma SciQuip 1-15K (Rotor 12124) for general temperature controlled spins and DNA/RNA extractions.

Two thermocyclers were used for all PCR and reverse transcriptase reactions: T3 Thermocyler from Biometra and the Stratagene Robocycler 96. PCR products were visualised on a UVP transilluminator whilst fluorescent PCR amplicons were analysed in the ABI 3730 sequencer with ABI Genemapper software.

FACS analysis was performed on the BD FACScan machine with CellQuest Software. Chromium release from cells in cytotoxicity assays was analysed on the 1480 Wizard 3" Automatic Gamma Counter from Perkin Elmer.

The AID Elispot Reader (version 3.2.3) [Autoimmun Diagnostika] was used to count interferon gamma producing cells in Elispot assays.

All ELISA plate readings were performed on the MRX II ELISA plate reader from Dynex Technologies.

2.3 Reagents and solutions

The following is a list of media, solutions and reagents used in the course of this work.

2.3.1 Tissue culture media and solutions

All media used are detailed in Table 2.1.

Table 2.1: Tissue culture media preparations.

Cell type	Medium	L-Glut	P/S	NEAA	FBS	HEPES	BrdU
CTL	RPMI 1640	2mM	100IU/ml	-	20%(v/v)	-	-
LCL/ B958/ K562	RPMI 1640	2mM	100IU/ml	-	10%{v/v)	-	-
TK143B	DMEM	2mM	100IU/ml	1%(v/v)	10%(v/v)	-	15µg/ml
DC	RPMI 1640	4mM	100IU/ml	-	10%(v/v)	25mM	-
RVacc	DMEM	2mM	100IU/ml	1%(v/v)	10%(v/v)	-	-
Supplier	*Invitrogen*	*Invitrogen*	*Invitrogen*	*Invitrogen*	*Invitrogen*	*Invitrogen*	*Sigma*

Abbreviations used: CTL: cytotoxic T lymphocyte, LCL: lymphoblastoid cell line, TK^-143B: thymidine kinase deficient 143B cell, DC: dendritic cell, RVacc: recombinant vaccinia virus, RPMI: Rosewell Park memorial institute, DMEM: Dulbecco's modified essential medium, L-Glut: L-glutamine, P/S: penicillin/streptomycin, NEAA: non-essential amino acids, FBS: foetal bovine serum, HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, BrdU: bromodeoxyuridine.

***Toluidine blue stock (1%)*:** 1g toluidine blue [Sigma] diluted with 10ml ethanol [VWR] and 90ml distilled water with filtration through a 0.4µm filter.

***Phosphate buffered Saline (PBS)*:**1 PBS tablet [Oxoid] diluted in 100ml distilled water and autoclaved to sterilise.

***4% Neutral buffered formalin (NBF)*:** 50ml of 10% NBF stock solution [VWR] diluted in 75ml PBS.

***Magnetic activated cell sorting (MACS) buffer*:** PBS (pH 7.2) containing 0.5% bovine serum albumin (BSA) [Sigma] and 2mM EDTA [Sigma].

***Fluorescent activated cell scanning (FACS) buffer*:** PBS (pH 7.2) containing 1% BSA, 0.1% sodium azide [VWR] and 0.1mM EDTA.

***FACS fix*:** 1ml BD CellFix™ (10%) [BD] in 9ml sterile distilled water.

2.3.2 Molecular solutions

***TE (x0.5) buffer*:** Water containing 45mM Tris-borate [Sigma] and 1mM EDTA.

***Agarose gels*:** TE (x0.5) buffer containing 2% agarose [Bioline] and 100µg/ml Ethidium Bromide [ThermoFisher Scientific].

Quantitative polymerase chain reaction (QPCR) wash buffer: Distilled water containing 600mM sodium chloride [Sigma], 60mM sodium citrate [Sigma] and 0.5% Tween 20.

Cresol Red buffer: Nuclease free water [VWR] containing 0.6% cresol red [Sigma].

2.3.3 Antibodies

Fluorescent activated cell scanning antibodies and working dilutions are detailed in Table 2.2.

Table 2.2: Details of fluorescent activated cell scanning antibodies.

Antibody	Clone	Conjugate	Volume/10^6 cells (µl)	Supplier
CD14	rmC5-3	PE	20	*BD Pharmingen*
DC-SIGN	120507	FITC	20	*R&D Systems*
IgG_1,k	MOPC-21	PE	20	*BD Pharmingen*
IgG_{2b},k	27-35	FITC	20	*BD Pharmingen*

Abbreviations used: PE: phycoerythrin; FITC: fluorescent isothiocyanin; IgG_1: immunoglobulin isotype 1; IgG_{2b}: immunoglobulin isotype 2b; CD: clusters of differentiation; DC-SIGN: dendritic cell-specific ICAM-3 grabbing non-integrin

2.3.4 Recombinant Vaccinia Virus Constructs

Recombinant vaccinia virus constructs were obtained from Professor Alan Rickinson and Dr Stephen Lee, Cancer Research UK, Institute for Cancer Studies, University of Birmingham, Birmingham, UK. The recombinant vaccinia virus constructs expressed the following Epstein Barr virus-antigens: Epstein Barr nuclear antigen (EBNA) -1, EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C, EBNA-Leader protein (LP), Latent membrane protein (LMP) -1, LMP-2 and matrix antigen (MA:gp350). A recombinant vaccinia virus construct with no EBV antigen insertion was included as a control in all experiments where these constructs were used**.**

2.4 Tissue Culture Methods

2.4.1 Density Gradient Separation of Peripheral Blood Mononuclear cells (PBMC)

In a 50ml tube Histopaque®-1077 Hybri-Max® [Sigma] was overlaid with an equal volume of blood or cell suspension and centrifuged in a MSE refrigerated centrifuge for 15 minutes at 800g. The resulting cell layer was removed into a fresh tube and 40ml of Hank's balanced salt solution (HBSS) medium [Invitrogen] added with further centrifugation for 7 minutes at 190g. The cell pellet was then re-suspended in an appropriate volume of culture medium and counted using trypan blue (see section

2.4.2). Cells were then either used fresh or viably frozen for use in functional assays, or frozen in aliquots ready for DNA/RNA extraction.

2.4.2 Cell Counts with Trypan Blue Exclusion

Cells were counted using the trypan blue exclusion method, where a 1:1 mixture of cell suspension and 0.4% trypan blue [Sigma] was applied to a haemocytometer and both the live (unstained) and dead cells (stained) were counted in the 25 central squares. This number was multiplied by the dilution factor of cells and trypan blue and multiplied by 10^4 to give the number of cells per ml of cell suspension.

2.4.3 Cryopreservation of Cells

The same cryopreservation protocol was observed for all cell types. Cells were centrifuged for 7 minutes at 190g and pellet re-suspended in 1ml (10% v/v dimethyl sulphoxide (DMSO) [Sigma], 90% v/v FBS) and transferred to 1.8ml cryovials. Cryovials were stored overnight at -80°C and then transferred to liquid nitrogen for long-term storage.

2.4.4 Monocyte Isolation

PBMC were isolated by density gradient centrifugation, re-suspended in RPMI 1640, passed through a 30µm nylon mesh [BD] to obtain a single cell suspension and counted using trypan blue. Monocytes were isolated via a negative selection method using the Monocyte Isolation Kit II [Miltenyi Biotec] as per manufacturer's instructions. Briefly, counted cells were centrifuged and re-suspended in 30µl of MACS buffer per10^7 total cells and 10µl Fc receptor (FcR) Blocking reagent (human IgG, supplied with kit) together with 10µl of Biotin Antibody Cocktail (containing biotinylated anti- CD3,

CD7, CD16, CD19, CD56, CD123 and glycophorin A antibodies, supplied with kit) per 10^7 total cells added. After a 10 minute incubation at 4˚C a further 30µl of MACS buffer and 20µl of Anti-Biotin Microbeads (supplied with kit) per 10^7 total cells were added. Cells were incubated at 4˚C for 15 minutes and then washed with MACS buffer (10 x labelling volume) and centrifuged for 10 minutes at 190g. Cells were re-suspended in 500µl MACS buffer for every 10^8 cells and applied to a pre-washed MS MACS column [Miltenyi Biotec] attached to MACS magnet [Miltenyi Biotec]. Monocytes (unlabeled cells) were collected from the run-through and the column was washed x3 with MACS buffer (washes collected and added to run-through). The column was then removed from the magnet, 500µl MACS buffer added and cells eluted with pressure to produce the non-monocyte fraction. Monocyte and non-monocyte fractions were re-suspended in appropriate medium and the purity estimated by FACS analysis (see section 2.4.12). Purified monocyte fractions were then cultured (see section 2.4.5).

2.4.5 Dendritic Cell Culture

Isolated monocytes were resuspended in 10-15ml of DC culture medium containing granulocyte-macrophage colony stimulating factor (GM-CSF) (20ng/µl) [R&D Systems] and IL-4 (20ng/µl) [R&D Systems] and incubated for 7 days at 37˚C with 5% carbon dioxide (CO_2). Three millilitres of fresh culture medium was added on days 3 and 6. On day 7 the culture medium was collected into a tube and cells centrifuged for 7 minutes at 190g to pellet. Immature DC were then re-suspended in 10ml of fresh medium, counted, and the purity assessed by FACS analysis (see section 2.4.12) before use in the standard cytotoxicity assay (see section 2.5.1).

2.4.6 TK$^-$143B Cell Culture

TK$^-$143B cells (thymidine kinase deficient human bone osteosarcoma cell line ECACC-91112502) were seeded at 2-3x10^6 cells per 175cm^2 culture flask with 50mls of TK$^-$ 143B culture medium. Cells were incubated for 2-3 days at 37˚C with 5% CO_2 until the monolayer was 100% confluent before harvesting with 20mls of trypsin-versene (1:1volume) mix. Cells were then washed with culture medium and counted. For culture in a 6 well plate 1x10^5 cells in 3ml of medium per well were used.

2.4.7 Lymphoblastoid cell line (LCL) Culture

EBV-immortalised LCLs were initially setup and grown by Dr Tanzina Haque and Mrs Gwen Wilkie (Wilkie *et al.*, 2004). Briefly, LCLs were established by mixing a loosened cell pellet of 2x10^6 PBMC with 100µl of EBV B95.8 virus preparation and 10µl of 100µg/ml cyclosporine A [Sandoz]. The total volume was made up to 1ml with LCL culture medium and transferred into one well of a 48-well plate. PBMCs were incubated at 37°C with 5% CO_2. Cultures were fed weekly, split when required and cells transferred to tissue culture flasks upon establishment of the culture before cryopreservation in liquid nitrogen.

2.4.8 Cytotoxic T-cell line (CTL) Culture

CTL cultures were established and maintained by Dr Tanzina Haque and Mrs Gwen Wilkie following the method described by Wilkie *et al*, (2004). Briefly, γ-irradiated autologous LCLs were used to stimulate PBMC at a 40:1 PBMC:LCL ratio. The stimulated PBMC were re-suspended at a concentration of 1x10^6 cells/ml in CTL culture medium. Ten days after initial stimulation CTLs were re-stimulated using γ-irradiated autologous LCLs at a 4:1 CTL:LCL ratio and subsequently re-stimulated on a

weekly basis. 20IU/ml of recombinant IL-2 [Chiron] was added to the culture 14 days after the initial stimulation and every 3 days thereafter. Once shown to be EBV-specific cells were frozen in liquid nitrogen.

2.4.9 B95.8/K562 Cell Culture

B95.8 and K562 cells (Lozzio and Lozzio, 1975;Miller *et al.*, 1974) were maintained in culture medium at a concentration of $1x10^6$ cells per ml. Cells were centrifuged at 190g for 7 minutes to pellet, washed with PBS and counted. Viable cells were cryopreserved in liquid nitrogen. Aliquots of B95.8 cells were prepared containing 10 million cells/ml PBS and centrifuged for 5 minutes at 15500g. The supernatant was removed and the cell pellet frozen at -80˚C for DNA/RNA extraction.

2.4.10 Recombinant Vaccinia Virus Culture

TK^-143B cells were grown in $175cm^2$ culture flasks until they reached confluence. Culture medium was removed and cells overlaid with recombinant vaccinia virus ($2x10^7$ plaque forming units (pfu)/3.5ml sterile PBS) and incubated at 37°C with 5%CO_2 for 2 hours. After incubation, 45ml of growth medium was added and flasks incubated for a further 3 days until the cell monolayer detached. The detached cells were then collected into a 50ml Falcon tube and centrifuged at 500g for 15 minutes and the pellet re-suspended in 1ml PBS. Cells were then freeze-thawed multiple times to release virus, and then centrifuged at 225g for 5 minutes before collecting supernatant into a fresh tube and storing at -80°C.

2.4.11 Recombinant Vaccinia Virus Titration

TK$^-$143B cells were grown to confluence in a 6 well plate and culture medium removed prior to addition of recombinant vaccinia virus. Dilutions (10^{-1}-10^{-7}) of recombinant vaccinia virus stock were prepared in PBS and 400µl of each dilution was added to the wells and the plate incubated at 37°C with 5%CO_2 for 2 hours. Three millilitres of growth medium were then added to each well and the plate incubated for a further 3 days. On day 3 the medium was removed and 1ml of 4% NBF added and incubated at room temperature for 30mins to fix the cells. The NBF was removed and 1ml of 0.1% toluidine blue added for a further 30 minutes before washing the plate with 2ml PBS and allowing it to air dry. Unstained viral plaques were then counted using a light microscope and the number of PFU per ml estimated.

2.4.12 Fluorescent activated cell scanning (FACS) Antibody Staining

Cells were distributed into 5ml FACS tubes to give $1x10^5$ cells per 100µl per tube and 1ml FACS buffer added prior to centrifugation at 190g for 7 minutes. The supernatant was aspirated and appropriate volumes of FACS antibody or isotype control antibody added and tubes incubated for 20 minutes at 4˚C. One millilitre of FACS buffer was then added and cells centrifuged as before. The resultant cell pellet was re-suspended in 0.5ml of FACS fix and kept covered in the dark until analysis was performed using the BD FACScan [Bectin Dickinson] in conjunction with Cellquest software.

2.5 Cell Cytotoxicity Methods

2.5.1 Chromium Release Assay

Target cells (LCL and K562 cell line) were distributed into 15ml Falcon tubes to give $0.5x10^6$ cells per tube and centrifuged at 130g for 5 minutes to pellet cells. Fifty

microcuries (μCi) of ^{51}Chromium (Chromium-51 supplied as sodium chromate in sterile sodium chloride solution at 1mCi/ml, GE Healthcare) were added to each pellet and incubated for 1 hour at 37˚C. Cells were washed with RPMI (x2) and re-suspended in 5ml of CTL culture medium to give a concentration of 1x 10^5 cells per ml. Effector cells (CTL) were re-suspended to 2x10^6 cells per ml with CTL culture medium and a 1 in 2 dilution series prepared. One hundred microlitres of each dilution was added in triplicate to a 96 well round bottom plate to give 2x10^5, 1x10^5 and 5x10^4 cells per well. Control wells containing 100μl culture medium or 100μl 1% triton-X were also prepared in triplicate. One hundred microlitres of chromium labelled target cells were then added to each well and the plate incubated for 4 hours at 37°C with 5%CO_2. Centrifugation at 200g for 7 minutes was performed to pellet the cells and 100μl of supernatant removed to LP-2 gamma counter tubes [ThermoFisher] for analysis in a gamma counter. Triplicate values were averaged and the percent specific lysis for each effector:target ratio was calculated as follows:.

$$\frac{\text{Test release – Spontaneous release (Medium only)}}{\text{Maximum release (Triton X) – Spontaneous release}} \times 100$$

2.5.2 Chromium Release Assay Using Recombinant Vaccinia Virus Infected Dendritic Cells

Dendritic cells were re-suspended in DC medium to give 1x10^6/ ml and 100μl (1x10^5 cells) added to 11 wells of a 96 well plate. Recombinant vaccinia virus constructs were added to each well at a multiplicity of infection (MOI) of 10:1 (1x10^6pfu) and incubated overnight at 37˚C with 5%CO_2. Cells were transferred to a 15ml Falcon tube containing

5ml of medium and centrifuged for 7 minutes at 190g to pellet the cells. Ten microcuries of ^{51}Chromium were added to each pellet and incubated for 1 hour at 37˚C. Cells were washed with RPMI and re-suspended in 900µl of CTL culture medium. Effector cells were re-suspended at $1x10^6$ cells per ml and 100µl added in triplicate to a 96 well round bottom plate to give $1x10^5$ cells per well and the method continued as per standard chromium release assay (see section 2.5.1).

2.5.3 Human Interferon-gamma Elispot Assay

Elispot assays were carried out using the Human Interferon-gamma kit (IFN-γ) from R&D systems. Plates (coated with monoclonal antibody specific for human IFN-γ) were equilibrated with 200µl of sterile CTL culture medium (SCM) and incubated at room temperature for 30mins. Cell combinations were prepared as follows: LCL ($1x10^4$/50µl SCM) with CTL ($5x10^4$/50µl SCM); PBMC ($1x10^5$/50µl SCM with or without peptide) with CTL ($5x10^4$/50µl SCM). The peptides used are detailed in Table 2.3. One hundred microlitres of prepared cell combination was added to triplicate wells and incubated at 37˚C with 5%CO_2 for 24 hours. Wells were washed with 250µl of 1x kit wash buffer 4 times and blotted dry before addition of 100µl of detection antibody (biotinylated polyclonal antibody specific for human IFN-γ) and a further incubation overnight at 4˚C. The plate was washed as before and 100µl of streptavidin-AP added to each well and incubated for 2 hours at room temperature. After a further series of washes, 100µl of BCIP/NBT (5-Bromo-4-Chloro-3'Indolyphosphate p-Toluidine salt/ Nitro blue Tetrazolium Chloride) chromagen solution was added to each well, and the plate was protected from light and incubated for 1 hour at room temperature. The plate was then washed with de-ionised water and allowed to air dry. Interferon-γ producing cells were then counted using an AID Elispot reader [Autoimmun Diagnostika].

Table 2.3 Peptide sequences and HLA restriction

Peptide sequence	Abbreviated sequence	HLA restriction	EBV protein
VLKDAIKDL	VLK	A2	EBNA 1
SVRDRLARL	SVR	A2	EBNA 3A
QAKWRLQTL	QAK	B8	EBNA 3A
FLRGRAYGL	FLR	B8	EBNA 3A
IVTDFSVIK	IVT	A11	EBNA 3B
AVFDRKSDAK	AVF	A11	EBNA 3B
VEITPYKPTW	VEI	B44	EBNA 3B
GQGGSPTAM	GQG	B62	EBNA 3B
LLDFVRFMGV	LLD	A2	EBNA 3C
QPRAPIRPI	QPR	B7	EBNA 3C
KEHVIQNA	KEH	B44	EBNA 3C
QNGALAINTF	QNG	B62	EBNA 3C
YLLEMLWRL	YLL	A2	LMP 1
LLWTLVVLL	LLW	A2	LMP 2

Abbreviations used: HLA: human leukocyte antigen; EBNA: Epstein Barr nuclear antigen; LMP: latent membrane protein

2.6 Molecular Methods

2.6.1 Deoxyribonucleic Acid (DNA) Extraction

Extractions were carried out using the Invitrogen Easy DNA kit as per manufacturer's instructions. Frozen cell pellets (approximately $5x10^6$ cells) were thawed and re-suspended in 200µl PBS immediately before extraction. Briefly, 350µl of solution A (lysis solution) was added to cells, vortexed to mix and then incubated at 65˚C for 10 minutes. One hundred and fifty microlitres of solution B (precipitation solution) was then added to form a precipitate of proteins and lipids, followed by the addition of 500µl chloroform [Sigma] and micro-centrifugation at maximum speed (15500g) for 15mins at 4˚C to separate layers. After centrifugation the top layer of the tri-phasic separation containing the DNA was removed to a clean tuce and 1ml of 100% ethanol added before incubating overnight at -20˚C. The DNA solution was then centrifuged as above to pellet and washed with 80% ethanol with a further centrifugation for 5 minutes. The

DNA pellet was allowed to air dry prior to the addition of 50µl nuclease free water. The DNA concentration was then measured (see section 2.6.3) and aliquots prepared for storage at -20˚C.

2.6.2 Ribonucleic Acid (RNA) Extraction

Extractions were carried out using the Qiagen RNeasy Mini Kit. Three hundred and fifty microlitres of RLT buffer (RNeasy lysis buffer containing guanidine thiocyanate) was added to a PBMC cell pellet ($5x10^6$ cells) and vortexed to mix. The lysate was then added to a Qiashredder spin column [Qiagen] and centrifuged for 2mins at 15500g to homogenize. Three hundred and fifty microlitres of 70% ethanol were added to the lysate and mixed before applying to an RNeasy spin column. The column was centrifuged for 15 seconds at 9000g before addition of 700µl RW1 buffer (RNeasy wash buffer containing ethanol) and further centrifugation. Two more washes with 500µl RPE buffer (RNeasy wash buffer containing ethanol) were performed with a final centrifugation for 1 minute. To elute RNA 30µl of RNase free water was added and the column centrifuged at 15500g for 1 minute. Eluted RNA concentration was then measured (see section 2.6.3) and 1µg RNA treated with the RQ1 RNase-Free DNase kit [Promega] to remove any contaminating DNA. The resultant DNase free RNA was frozen in aliquots at -70˚C prior to complementary DNA synthesis (see section 2.6.4).

2.6.3 DNA/RNA Spectrophotometer Measurement

DNA and RNA concentration was determined on a GeneQuant ultraviolet (UV) spectrophotometer [GE Healthcare] using the following formulas:

DNA concentration = Absorption at 260nm (A^{260}) x 50 x dilution factor

where an A^{260} of 1.0 is equivalent to 50mg/ml of DNA.

RNA concentration = Absorption at 260nm (A^{260}) x 40 x dilution factor

where an A^{260} of 1.0 is equivalent to 40mg/ml of DNA.

The spectrophotometer was blanked at A^{260} using 4µl of nuclease-free water. Four microlitres of eluted DNA/RNA were added to a quartz capillary [GE Healthcare] to determine the concentration and quality of extracted product.

2.6.4 Complementary DNA (cDNA) Synthesis

The ThermoScript™ Reverse Transcriptase kit (Invitrogen) was used for the synthesis of first-strand cDNA from the RNA and this was performed following the manufacturer's instructions. Briefly, components of the kit (random hexamers, dNTP and DEPC treated water) were added to 1µg RNA and denatured for 5 minutes at 65˚C. The denatured solution was then added to the kit reaction mix and incubated for 10 minutes at 25˚C, followed by 50 minutes at 50˚C with a final 5 minute incubation at 85˚C. RNase H was then added and incubated for 20 minutes at 37˚C before storing cDNA at -20˚C.

2.6.5 Polymerase Chain Reaction (PCR)

Unless otherwise stated, DNA was diluted with nuclease free water to 100ng/ml for use in PCR and all PCR was carried out on a T3 Thermocycler [Biometra] or a Robocycler 96 [Stratagene]. Promega and GE Healthcare reagents were used throughout unless otherwise stated and all primers were obtained from Sigma Genosys. PCR of house keeping genes was performed to verify the quality of extracted DNA and cDNA: the β-

globin gene was used for DNA and β-actin for cDNA. Primer sequences are shown in Table 2.4 with reaction conditions and cycling times detailed in Table 2.5.

Table 2.4: Primer sequences and product size.

Primer	Primer Sequence (5'-3')	Base pair size
β-globin forward[b]	ACA CAA CTG TGT TCA CTA GC	110
β-globin reverse[b]	CAA CTT CAT CCA CGT TCA CC	
β-actin forward[d]	CTC CTT AAT GTC ACG CAC GAT TTC	540
β-actin reverse[d]	GTG GGG CGC CCC AGG CAC CA	
EBNA 1 forward[a]	GAT GGA GAT GAG GGT GAG GA	218
EBNA 1 reverse[a]	GGA GCT GAG TGA CGT GAC AA	
EBNA 2 forward[a]	CTC TGC CAC CTG CAA CAC TA	171
EBNA 2 reverse[a]	GAG GGT GCA TTG ATT GGT CT	
EBNA 3a forward[a]	GCC CTG AGC CAG AGT GTT AG	193
EBNA 3a reverse[a]	GAT GTT GGA CCA CGT CAG TG	
EBNA 3b forward[c]	TTC CAT GTT GCA ATC GGA CC	398
EBNA 3b reverse[c]	AAA GTG ACC TAG CAC GAC GT	
EBNA 3c forward 1° [d]	GGC TGT CAA GAA TCG CAC CT	198
EBNA 3c reverse 1° [d]	GTG TTT AGA GTT CGT GCC GC	
EBNA 3c forward 2° [d]	CAT CTT GTG CTT CGT GAT GG	
EBNA 3c reverse 2° [d]	TAA CAT GAT GCT GTC AGC CC	
EBNA LP forward[a]	CAC AAA TGG GAG ACC GAA GT	
EBNA LP reverse[a]	ACC GCT TAC CAC CTC CTC TT	
LMP-1 forward 1° [e]	ACA CAC TGC CCT GAG GAT GG	104
LMP-1 reverse 1° [e]	ATA CCT AAG ACA AGT AAG CA	
LMP-1 forward 2° [e]	CTT CAG AAG AGA CCT TCT CT	
LMP-1 reverse 2° [e]	ACA ATG CCT GTC CGT GCA AA	
LMP-2 forward 1° [d]	ATG ACT CAT CTC AAC ACA TA	197
LMP-2 reverse 1° [d]	TCA CCA GAA CGT AAA TGC CT	
LMP-2 forward 2° [d]	CTC GTG TTT CAC GGC CTC AG	
LMP-2 reverse 2° [d]	AAG GTG GGT CCT CAA TCC TC	
GP 350 forward [f]	CAC AGG CCC CAC TGT ATC	142
GP 350 reverse [f]	GAG GTG GAG CTG GTC ATT G	
QP 1 [g]	GCC GGT GTG TTC GTA TAT GG	N/A
QP 2 biotin [g]	CAA AAC CTC AGC AAA TAT ATG AG	
Wild type probe [g]	TCT CCC CTT TGG AAT GGC CCC TG	
Internal standard probe [g]	CTA TAT GCC TGC TTC CTC CGG CG	

Abbreviations used: 1°: primary PCR round; 2°: secondary PCR round. [a] Primers designed in house using Primer 3 software; [b] (Saiki *et al.*, 1985); [c] (Joseph, Babcock, and Thorley-Lawson, 2000); [d] (Hopwood *et al.*, 2002); [e] (Brooks *et al.*, 1992b); [f] (Rochford and Mosier, 1995); [g] (Stevens *et al.*, 1999); N/A: not applicable

For β-globin amplification, 5µl of DNA were used and for β-actin 1µl of cDNA. Appropriate positive and negative controls were included in all reactions. PCR products were run on a 2% agarose gel containing ethidium bromide with TBE (0.5) buffer and visualised under a UV light source [UVP transilluminator, Ultra Violet Products].

Table 2.5: PCR reaction mix and cycling conditions for β-globin and β-actin.

β-globin	β-actin	Supplier
1 x reaction buffer	1 x reaction buffer	*Promega*
1.5mM $MgCl_2$	1.5mM $MgCl_2$	*Promega*
200µM dNTP's	200µM dNTPs	*GE Healthcare*
50pmol forward primer	20pmol forward primer	*Sigma genosys*
50pmol reverse primer	20pmol reverse primer	*Sigma genosys*
0.3U *Taq* polymerase	0.625U *Taq* polymerase	*Promega*
NF water: final volume 50µl	NF water: final volume 25µl	
95˚C for 5' 28 cycles of: 94˚C for 1' 49˚C for 2' 72˚C for 2' final extension: 72˚C for 10'	95˚C for 5' 35 cycles of: 95˚C for 1' 65˚C for 2' final extension: 72˚C for 10'	

Abbreviations used: $MgCl_2$: magnesium chloride; dNTPs: deoxy-nucleoside triphosphates; NF: nuclease free, U: units; reaction buffer contains 10mM tris (pH 9), 50mM KCl and 0.1% Triton X-100

2.6.5.1 Latent and Lytic EBV Gene PCR

Latent (EBNA 1, 2, 3a, 3b, 3c, LP, LMP-1, 2) and lytic (gp350) genes were amplified using the primers detailed in Table 2.4. Five microlitres of cDNA was amplified in 50µl of each reaction shown in Table 2.6. Where nested PCR was performed, 2µl of primary product was added to second round of PCR.

Cycling conditions for each PCR are detailed in Table 2.7. Ten microlitres of amplified product were visualised on a 2% agarose-ethidium bromide gel under UV illumination.

Table 2.6: PCR reaction conditions for EBV latent and lytic genes

Reagent	EBNA 1, 2, 3A, LP	EBNA 3B	EBNA 3C, LMP-1,-2	gp350
Reaction buffer (Promega)	1x	1x	1x	1x
$MgCl_2$ (Promega)	1.5mM	2.5mM	1.5mM	2mM
dNTP's (GE Healthcare)	200µM	200µM	200µM	200µM
Forward Primer	30pmol	25pmol	50pmol	50pmol
Reverse Primer	30pmol	25pmol	50pmol	50pmol
Taq polymerase (Promega)	1U	1U	1U	1U

Abbreviations used: $MgCl_2$: magnesium chloride; dNTP's: deoxy-nucleoside triphosphates; U: units; reaction buffer contains 10mM tris (pH 9), 50mM KCl and 0.1% Triton X-100

Table 2.7: Cycling conditions for EBV latent and lytic genes

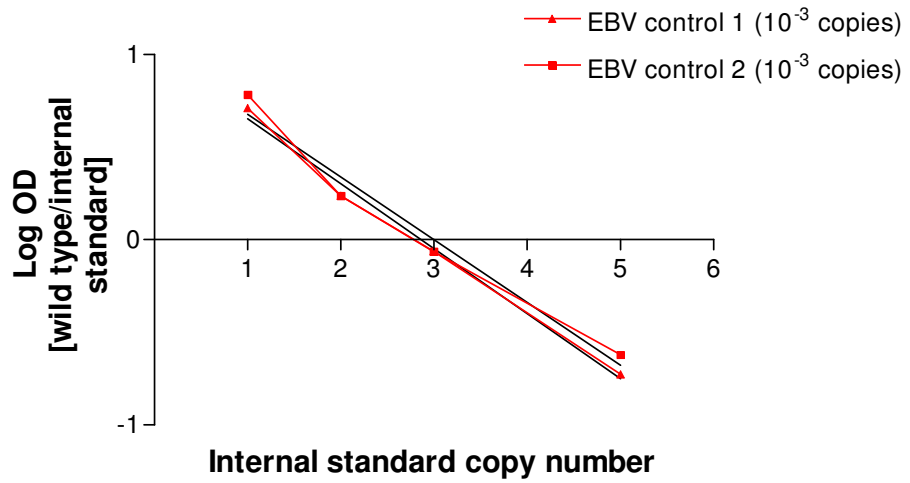
	EBNA 1, 2, 3A, LP	EBNA 3B	LMP 2 (1˚)	LMP 1 (1˚)	EBNA 3C (1˚)	LMP 1 (2˚), LMP 2A (2˚), EBNA 3C (2˚)
Denaturation	94˚C:10mins	94˚C:10mins	94˚C:10mins	94˚C:10mins	94˚C:10mins	94˚C:10mins
Cycle	94˚C:30sec 61˚C:45sec 72˚C:45sec	94˚C:30sec 62˚C:30sec 72˚C:45sec	94˚C:1min 55˚C:2min 72˚C:2min	94˚C:1min 50˚C:1min 72˚C:1min	94˚C:1min 54˚C:1min 72˚C:1min	94˚C:1min 57˚C:2min 72˚C:2mins
Cycle Number	x 40	x 40	x 35	x 35	x 35	x 35
Final Extension	72˚C:10mins	72˚C:5mins	72˚C:10mins	72˚C:10mins	72˚C:10mins	72˚C:10mins

Abbreviations used: 1˚: first round; 2˚: second round

2.6.5.2 Quantitative EBV PCR

Quantitative EBV PCR was performed on 200-500ng of extracted DNA using the method described by Stephens *et al* (1999). DNA was amplified in a 50µl reaction containing 10mM tris (pH 9), 50mM KCl, 0.1% triton x-100, 1.5mM $MgCl_2$, 200µM dNTPs, 25pmol of QP1 forward and QP2 reverse primers (Table 2.4) and 2U *Taq* polymerase. Each sample reaction contained an internal standard with a set number of EBV copies ($1\text{-}10^6$). Initial denaturation was performed for 5mins at 95˚C followed by 40 cycles of 1 min each of 95˚C, 55˚C and 72˚C, with a final extension of 5mins at 72˚C. PCR product was diluted 10 fold and 50µl added to a streptavidin coated plate [Streptowell, Roche] with incubation at 37˚C for 1 hour. The plate was then washed with QPCR wash buffer and distilled water before denaturation of DNA with 0.2M sodium hydroxide for 15mins at room temperature. Further washing was performed followed by the addition of 50pmol/ml of digoxigenin (DIG) labelled probe (made using the Roche DIG labelling kit) specific for either wild type EBV or the internal standard (Table 2.4). After incubation at 37˚C for 1 hour the plate was again washed and anti-DIG AP [Roche] added for another hour. Final wash steps were carried out and nitrophenyl phosphate (pNPP) substrate [Sigma] added for 1 hour to develop colour on the plate. The plate was read on a plate reader [MRX II reader, Dynex] at 405nm. EBV copy number was estimated by plotting the log optical density (OD) [wild type/internal standard] against the log number of internal standard copies. The EBV copy number in the sample is equivalent to where graph cuts the x-axis (Figure 2.1).

Figure 2.1 Estimation of copy number in EBV control sample



	EBV control 1	EBV control 2
Variables		
Slope	-0.3391 ± 0.04054	-0.3513 ± 0.02185
Y-intercept	1.016 ± 0.1266	1.005 ± 0.06824
X-intercept	2.996	2.862
1/slope	-2.949	-2.847

Log OD [wild type/internal standard] versus internal standard copy number for duplicate EBV control samples containing 10^{-3} copies of the EBV genome. Linear regression was performed to calculate x-intercept and the copy number calculated as the inverse log of the x-intercept.

2.6.6 Cytokine Polymorphism PCR

DNA was amplified in 47 separate PCR reactions using primers (detailed in Table 2.8) amplifying TNF-α and -β, IL-1, IL-6, IL-10 cytokine genes and the equivalent cytokine receptor genes.

Primers were reconstituted to 2µg/ml and diluted to working concentrations in 1ml of NF water (Table 2.9). Each primer mix also contained a set of control primers (housekeeping genes) depending on the base pair size of the specific primer set, and cresol red buffer for visual purposes.

Table 2.8 Cytokine primer sequences and genes

Primer label	Primer Sequence (5'– 3')	Gene
1971	CAAAGGAGAAGCTGAGAAGAT	TNF alpha
1972	CAAAGGAGAAGCTGAGAAGAC	TNF alpha
1973	CGAGTATGGGGACCCCCC	TNF alpha
1974	GAGTATGGGGACCCCCA	TNF alpha
1986	CCGGGAATTCACAGACCCC	TNF alpha
1988	AAGGATAAGGGCTCAGAGAG	TNF alpha
1975.2	CTACATGGCCCTGTCTTCG	TNF alpha
1976.2	TCTACATGGCCCTGTCTTCA	TNF alpha
1359	GCAGTGGTCGAGTCTGCAG	IL-1 receptor
1360	GCAGTGGTCGAGTCTGCAA	IL-1 receptor
1361	CCAGCCTGGATTTGTCCGG	IL-1 receptor
2405	GAAGACCCCCCTCGGAATCG	TNF alpha
2406	GAAGACCCCCCTCGGAATCA	TNF alpha
2403	ATAGGTTTTGAGGGGCATGG	TNF alpha
2404	ATAGGTTTTGAGGGGCATGA	TNF alpha
710	GCATCCCCGTCTTTCTCCAC	TNF alpha
711	GCATCCCCGTCTTTCTCCAT	TNF alpha
706	GAGCAGCAGGTTTGAGGT	TNF beta
707	GAGCAGCAGGTTTGAGGG	TNF beta
638	GGGGTCGGGGGGTGCTG	TNF beta
641	GGGGTCGGGGGGTGCTC	TNF beta
1414	GCTTTCCAAGCCTCCTGAGC	TNF receptor I
1453	AGAGCAGAGGCAGCGA	TNF receptor I
1454	AGAGCAGAGGCAGCGG	TNF receptor I
1478	AGAGGCAGCGAGTTGT	TNF receptor I
1479	AGAGGCAGCGGGTTGT	TNF receptor I
1480	AGAGGCAGCGAGTTGG	TNF receptor I
1481	AGAGGCAGCGGGTTGG	TNF receptor I
1476	GCCTCTGCTGCCATGGC	TNF receptor I
1477	GCCTCTGCTGCCATGGT	TNF receptor I
T362T	GACGTGCAGACTGCATCCA	TNF receptor II
T362g	GACGTGCAGACTGCATCCC	TNF receptor II
T341	GAGAACCGCATCTGCACCT	TNF receptor II
PAN7	TGGAAAACAGATCCAGACAGG	TNF receptor I promoter
PAN8	ATTGGAAAACAGATCCAGACAGT	TNF receptor I promoter
PAN-9	GTTATGTGTCTGAGAAGTTCATTTG	TNF receptor I promoter
PAN-10	AGTTATGTGTCTGAGAAGTTCATTTA	TNF receptor I promoter
PAN-12	TCCCTGGTCTCACCAGC	TNF receptor I promoter
PAN-13	GTCCCTGGTCTCACCAGT	TNF receptor I promoter
PAN-21	TCTTCTTGCACAGTGGACCG	TNF receptor II promoter
PAN-22	TCTTCTTGCACAGTGGACCA	TNF receptor II promoter
PAN-27	CGGCACAGCTAAAGGAGG	TNF receptor II promoter
PAN-28	GCGGCACAGCTAAAGGAGA	TNF receptor II promoter
1030	CTACTAAGGCTTCTTTGGGAA	IL-10
1031	CTACTAAGGCTTCTTTGGGAG	IL-10
1034	CAAACTGAGGCACAGAGATG	IL-10

Primer Label	Primer Sequence (5'– 3')	Gene
1035	GCAAACTGAGGCACAGAGATA	IL-10
778	CTGTTGAAAGACCACTGATCT	IL-6
779	CTGTTGAAAGACCACTGATCC	IL-6
784	GAGGAACAAGCCAGAGCTGT	IL-6
IL6P-G	AATGTGACGTCCTTTAGCATG	IL-6
IL6P-C	AATGTGACGTCCTTTAGCATC	IL-6
IL6OX	TCGTGCATGACTTCAGCTTTA	IL-6
IL10RAb-Conc:	TGGTCATAACTCAGCCCTTTG	IL-10 receptor
IL10RAb-A:	AGCAGAACCTCACATCCCTAT	IL-10 receptor
IL10RAb-G:	GCAGAACCTCACATCCCTAC	IL-10 receptor
1165	CTTTAATAATAGTAACCAGGCAACAC	IL-1 alpha
1164	CTTTAATAATAGTAACCAGGCAACAT	IL-1 alpha
1166	AAGTAGCCCTCTACCAAGGA	IL-1 alpha
211	TTCATCAGTTGCTGCCCCTC	Control
210	ATGATGTTGACCTTTCCAGGG	Control
63	TGCCAAGTGGAGCACCCAA	Control
64	GCATCTTGCTCTGTGCAGAT	Control

Primer sequences were obtained from Professor Kenneth Welsh, the National Heart and Lung Institute, Imperial College, London and have been published previously (Grutters *et al.*, 2002;Grutters *et al.*, 2003;Fanning *et al.*, 1997;Koss *et al.*, 2000)

Between 60 and 80ng DNA were amplified in a reaction mix containing Bio*Taq* DNA polymerase and reagents [Bioline]: 1x NH_4 buffer; 1.5mM $MgCl_2$; 200µM dNTPs and 0.35U Taq polymerase. Five microlitres of each primer mix were added to give a final volume of 13µl. Cycling parameters were staged as follows: 96˚C for 1min; 4 cycles of 96˚C for 20sec, 75˚C for 45sec, 72˚C for 25sec; 20 cycles of 96˚C for 25sec, 65˚C for 50sec, 72˚C for 30sec; 3 cycles of 96˚C for 30sec, 55˚C for 60sec, 72˚C for 90sec; 5˚C for 10mins. The resultant PCR product was visualised on a 2% agarose- ethidium bromide gel under UV illumination.

Table 2.9 Cytokine polymorphism primer mix preparations

PCR No.	Gene position and base	Product Size (bp)	Primer 1	Vol (ul)	Primer 2	Vol (ul)	Control Primers
1	TNF-1031 T	433	1971	10	1986	10	63/64
2	TNF-1031 C	433	1972	8	1986	8	63/64
3	TNF-863 C	263	1973	8	1986	8	63/64
4	TNF-863 A	262	1974	8	1986	8	63/64
5	TNF-857 G	270	1988	5	1975.2	5	63/64
6	TNF-857 A	270	1988	5	1976.2	5	63/64
7	IL1-R1 1359 C	288	1359	20.7	1361	22.7	63/64
8	IL1-R1 1359 T	288	1360	19	1361	22.7	63/64
9	TNF g-g-g haplotype	835	710	1.5	2403	1.5	210/211
10	TNF g-g-a haplotype	835	710	5	2404	5	210/211
11	TNF a-g-g haplotype	763	711	1.5	2405	1.5	210/211
12	TNF g-a-g haplotype	763	710	2.5	2406	2.5	210/211
13	TNFβ GGT haplotype	390	707	8.7	641	11.4	63/64
14	TNFβ TCC haplotype	390	706	8.9	638	9.1	63/64
15	TNFβ GCT haplotype	390	707	8.7	638	9.1	63/64
16	TNFβ TGT haplotype	390	706	8.9	641	11.4	63/64
17	TNF receptor 1663 A	430	1453	43.9	1414	2.25	63/64
18	TNF receptor 1663 G	430	1454	11.6	1414	2.25	63/64
19	TNF receptor 1668 T	424	1478 1479	21.8 18.9	1414	2.25	63/64
20	TNF receptor 1668 G	424	1480 1481	22.8 16.7	1414	2.25	63/64
21	TNF receptor 1690 C	404	1476	14.7	1414	2.25	63/64
22	TNF receptor 1690 T	404	1477	12	1414	2.25	63/64
23	TNF-RII ex-6 T	1127	T362T	5	T341	5	210/211
24	TNF-RII ex-6 G	1127	T362G	5	T341	5	210/211
25	TNF-R promoter 201G-x-845G	681	PAN-7	7	PAN-12	7	210/211
26	TNF-R promoter 201G-x-845A	681	PAN-7	7	PAN-13	7	210/211
27	TNF-R promoter 201T-x-845G	681	PAN-8	7	PAN-12	7	210/211
28	TNF-R promoter 201T-x-845A	681	PAN-8	7	PAN-13	7	210/211
29	TNF-R promoter x-230G-845G	657	PAN-9	7	PAN-12	7	210/211
30	TNF-R promoter x-230G-845A	657	PAN-9	7	PAN-13	7	210/211
31	TNF-R promoter x-230A-845G	657	PAN-10	7	PAN-12	7	210/211

PCR No.	Gene position and base	Product Size (bp)	Primer 1	Vol (ul)	Primer 2	Vol (ul)	Control Primers
32	TNF-R promoter x-230A-845A	657	PAN-10	7	PAN-13	7	210/211
33	TNF-RII promoter x-x-839G-x-1135C	300	PAN-21	5	PAN-27	5	63/64
34	TNF-RII promoter x-x-839A-x-1135C	300	PAN-22	4	PAN-27	4	63/64
35	TNF-RII promoter x-x-839G-x-1135T	300	PAN-21	5	PAN-28	5	63/64
36	TNF-RII promoter x-x-839A-x-1135T	300	PAN-22	4	PAN-28	4	63/64
37	IL10-1082A--819T	303	1030	10	1035	10	63/64
38	IL10-1082A--819C	303	1030	10	1034	10	63/64
39	IL10-1082G--819C	303	1031	10	1034	10	63/64
40	IL6 intron 4 A	~800	778	23.1	784	18.8	210/211
41	IL6 intron 4 G	~800	779	21.5	784	18.8	210/211
42	IL6-174 C	156	IL6P-G	10	IL6OX	10	63/64
43	IL6-174 G	156	IL6P-C	10	IL6OX	10	63/64
44	IL10RAb-G	>800	IL10R-G	5	IL10RX	5	210/211
45	IL10RAb-A	>800	IL10R-A	5	IL10RX	5	210/211
46	IL1 alpha C	150	1165	40	1166	10	63/64
47	IL1 alpha T	150	1164	40	1166	20	63/64

47 different PCR reactions were prepared containing one set of control primers (63/64 or 210/211: 1.5µl of each primer per ml of nuclease free water) and 2 or more specific primers (primer 1 and 2) for each gene analysed. Volume used is based on a 2µg/ml primer stock and is diluted in 1ml of nuclease free water. bp: base pair.

2.6.7 Human Leukocyte Antigen (HLA) PCR

Three microsatellite markers, 2 from the HLA class I (D6S265 and D6S510) and 1 from the HLA class III regions (D6S273) were amplified using PCR performed in a total volume of 10µl containing 25ng of DNA. The reaction mix contained 1x GoTaq Flexi buffer (pH8·5), 2·5mM $MgCl_2$, 200µM dNTP [GE Healthcare], 25µM of forward and reverse primer and 0·5 units of GoTaq Flexi polymerase [Promega]. The reverse primer was 5' labelled with fluorochrome 6-(fluorescein-5 carboxamido)hexanoate (6-

FAM) (Table 2.10). The cycling conditions consisted of an initial denaturation at 94˚C for 5mins followed by 35 cycles of 94˚C for 30secs, 55/62˚C for 30secs, 72˚C for 45secs and a final extension at 72˚C for 5mins. Five microlitres of PCR product were diluted in 45µl of nuclease free water and then further diluted 1 in 10 with Hi-Di formamide (containing Genescan 500LIZ size standard [ABI]) before electrophoresis in an ABI 3730 (Dye set 5) automated sequencer. ABI Genemapper software (version 3·7) was used to analyse the data.

Table 2.10 Primer and PCR information for microsatellite markers

Locus	HLA Class	Primer Sequence (5' – 3')	Number of alleles	Annealing Temp.
D6S510	I	Forward AATGTTCCTGCTTTCATTTCTTT Reverse 6FAM-GTCAAAACTGCAATGGGCTACTA	10	62˚C
D6S265	I	Forward ACGTTCGTACCCATTAACCT Reverse 6FAM-ATCGAGGTAAACAGCAGAAA	13	55˚C
D6S273	III	Forward GCAACTTTTCTGTCAATCCA Reverse 6FAM-GACCAAACTTCAAATTTTCGG	9	55˚C

Abbreviations used: FAM**:** 6-(fluorescein-5 carboxamido)hexanoate. Primer sequences published in Diepstra *et al* (2005).

2.6.8 T Cell Receptor (TCR) PCR

Functionally re-arranged TCR β-chain variable (BV) gene sub-families were amplified across the complementarity determining region 3 (CDR3) encoding regions using 23 subfamily-specific primers and a FAM conjugated β-chain constant region specific primer (Table 2.11). PCR amplifications were performed on 1µl of cDNA in a total volume of 20µl containing 10mM-Tris-HCL (pH 8.3), 50mM-KCl , 2mM $MgCl_2$, 200µM of each dATP, dTTP, dGTP, dCTP, 1µM of variable and constant primers and

0.5U of Amplitaq Gold polymerase [ABI]. One microlitre of PCR product was diluted in 10µl of nuclease free water and then further diluted 1 in 10 with ABI Hi-Di formamide (containing ABI Genescan 500LIZ size standard) before electrophoresis in an ABI 3730 (Dye set 5) automated sequencer. ABI Genemapper software (version 3.7) was used to analyse the data.

2.6.9 Single nucleotide polymorphism (SNP) PCR

SNP PCR and analysis were performed by Ms Annette Lake and Professor Ruth F Jarrett at the LRF Virus Centre, Institute of Comparative Medicine, University of Glasgow, Glasgow. SNP analysis was carried out using TaqMan SNP Genotyping Assays from Applied Biosystems. Reactions were performed in a final volume of 25µl containing 1x TaqMan Universal Master Mix with no AmpEraseUNG, 900nM of each primer (ABI), 200nM of probes labelled with either FAM or VIC and 20ng of extracted DNA. Thermal cycling (2min at 50˚C, 10min at 95˚C followed by 40 cycles of 15sec at 95˚C and 1min at 60˚C) and allelic discrimination were performed on an ABI 7500 Real Time PCR System.

Table 2.11 TCR β-chain variable gene primer sequences

TCR (BV)	Primer Sequence (5'-3')	Base pair size
1	GCACAACAGTTCCCTGACTTGCAC	195-207
2	TCATCAACCATGCAAGCCTGACCT	195-207
3	GTCTCTAGAGAGAAGAAGGAGCGC	190-208
4	ACGATCCAGTGTCAAGTCGAT	334-346
5	CTGATCAAAACGAGAGGACAGCA	354-375
6	TCAGGTGTGATCCAATTTC	329-347
7	CCTGAATGCCCCAACAGCTCTC	190-214
8	GGTGACAGAGATGGGACAAGA	355-373
9	CACCTAAATCTCCAGACAAAGCT	194-212
11	TGTTCTCAAACCATGGGCCATGAC	321-333
12	GrCATGGGCTGAGGCTGAT	267-290
13	CTCTCCTGTGGGCArGTC	408-425
14	ACCCAAGTACCTCATCACAG	328-383
15	AGTGTCTCTCGACAGGCACAG	193-208
16	AAAGAGTCTAAACAGGATGAGCC	241-256
17	TTTCAGAAAGGAGATATAGCT	226-241
18	AGCCCAATGAAAGGACACAGTCAT	325-337
20	CTCTGAGGTGCCCCAGAA	218-227
21	GGCTCAAAGGAGTAGACTCC	185-200
22	ATGAAATCTCAGAGAAGTCT	234-252
23	GATCAAAGAAAAGAGGGAAAC	358-370
24	TACCCAGTTTGGAAAGC	353-368
25	CAGGTATGCCCAAGGAAAGA	226-241
Cb(FAM)	6 FAM-TTCTGATGGCTCAAACAC	

Abbreviations used: FAM**:** 6-(fluorescein-5 carboxamido)hexanoate. Primer sequences consist of 23 Vβ forward primer sequences and 1 reverse constant chain (Cβ) primer conjugated to FAM (Foster *et al.*, 2004)

2.7 Study Cohorts

2.7.1 Cytotoxic T-Cell Lines

A bank of cytotoxic T-cell lines was grown as part of a phase II multicentre clinical trial carried out at Edinburgh University. The CTL and the study have been detailed elsewhere (Wilkie *et al.*, 2004;Haque *et al.*, 2002). Briefly, PBMC were obtained from HLA typed, EBV sero-positive blood donors (Scottish National Blood Transfusion Service) and LCL and CTL grown as detailed (sections 2.4.7 and 2.4.8). CTL were tested in standard cytotoxicity assays against phytohaemagglutinin stimulated blasts, autologous and HLA mismatched LCL, and K562 cells. CTL specific for autologous LCL were analysed by FACS for B-cell, NK-cell and a wide range of T-cell activation and differentiation markers and then frozen in vials containing $20x10^6$ cells in liquid nitrogen. A small panel of CTL (those used to treat patients with EBV-associated tumours in the phase II trial) was chosen for further characterisation in this thesis.

2.7.2 PTLD Patient and Control Cohorts

A cohort of EBV-associated post transplant lymphoproliferative disease (PTLD) patients was accrued as part of the pilot study and phase II multi-centre clinical trial detailed in Haque *et al* (2007). Patients from 19 transplant centres were recruited to the trials with informed written consent from patients or guardians. The study was approved by the Lothian Research Ethics Committee. The diagnosis of PTLD and its classification was determined by histological examination. EBER staining was performed by *in situ* hybridisation using a commercial kit from DAKO and immunohistochemistry carried out using commercial antibodies for EBNA 1, 2, and LMP 1 expression [DAKO]. Assessment of tumour cell clonality was performed using a

DAKO in situ hybridisation kit for kappa and lambda mRNA. PTLD patients were monitored at regular intervals for tumour regression and PBMC samples were taken pre and post CTL treatment, and frozen viably in liquid nitrogen or as a cell pellet at -70˚C. DNA, RNA and cDNA were prepared from frozen PBMC pellets as described (sections 2.6.1, 2.6.2 and 2.6.4) then frozen until required.

An anonymised control cohort of EBV sero-positive heart transplant patients with no development of PTLD was obtained from Dr Paul Hopwood (Hopwood *et al.*, 2002). DNA, RNA and cDNA were prepared and frozen in a similar manner to the PTLD cohort.

PTLD and control samples were analysed in the HLA microsatellite and the cytokine polymorphism studies. PTLD patient outcome/tumour regression was also assessed in relation to CTL characterisation.

2.7.3 IM and Control Study Cohorts

EBV seropositive and seronegative individuals were recruited as part of an epidemiological study carried out at Edinburgh University (Crawford *et al.*, 2002;Crawford *et al.*, 2006). The study was approved by the Lothian Research Ethics Committee and all participants provided written signed consent. In brief, all students enrolling at the University during 1999 and 2000 were approached to take part in the study and upon recruitment provide a blood sample for EBV serology. EBV serostatus was determined by routine indirect immunofluorescence of IgG antibody against EB viral capsid antigen. EBV seropositive individuals formed the EBV seropositive group used in this thesis but were not followed further. EBV seronegative individuals were monitored for development of IM during their university career (approximately 4 years).

A diagnosis of IM was made upon detection of IgM antibodies to EB viral capsid antigen and/or a positive monospot test in a known EBV seronegative. Upon diagnosis IM patients were examined and asked to provide a blood sample for full blood count (FBC) which was carried out on a Coulter counter (Coulter) and these patients formed the IM subject group used in this thesis. Those students seronegative at enrolment and who did not report symptoms of IM were asked to return for further testing upon exit from university. Students who remained EBV negative formed the seronegative group used in this thesis whilst students who tested EBV-positive were regarded as asymptomatic sero-convertors and joined the EBV seropositive group. The EBV seropositive, seronegative and IM groups formed the subject groupings for investigation in the HLA microsatellite study and the cytokine polymorphism study.

2.8 Statistical Methods

Each HLA microsatellite marker, cytokine and cytokine receptor gene for each group of subjects was tested for Hardy Weinberg disequilibrium by comparing the observed allele frequency with the expected frequency if equilibrium applied. Classical association analysis was conducted to compare allele frequencies between the groups of subjects (EBV- positive with symptoms of IM, EBV-positive without symptoms of IM, EBV-negative, PTLD positive and PTLD negative post transplant cohorts) and tested using the Fisher's Exact or Chi-square test (this was not adjusted for multiple testing). Clinical characteristics such as total lymphocyte count, neutrophil count and viral load were compared across groups using the Mann-Whitney U test. Within the HLA microsatellite study the Fisher's Exact test was used to compare the severity of

symptoms among IM cases who were positive compared to those who were negative for particular alleles.

Advice on use of statistical parameters was obtained from Dr Craig Higgins, Infectious Disease Epidemiology Unit, Department of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, London.

Chapter 3:
Results I

Epitope specificity and T-cell receptor clonality of EBV-specific cytotoxic T-lymphocytes used for the treatment of post transplant lymphoproliferative disease (PTLD)

3.1 Introduction

We recently investigated the use of partially HLA-matched allogeneic CTL for the treatment of EBV-positive PTLD. A frozen bank of 107 HLA-typed polyclonal CTL lines specific for EBV was established from healthy blood donors (Wilkie *et al.*, 2004). Donor CTL lines partly matched to the recipient on HLA-A, -B and –DR alleles (a maximum of 6 allele matches) were tested *ex vivo* and the lines showing maximum specific and minimum non-specific killing of recipient cells were chosen for infusion. Our initial pilot study demonstrated complete remission of tumour in 3/5 PTLD patients treated and also highlighted the safe, cheap and rapid use of our bank of allogeneic CTL (Haque *et al.*, 2002). A subsequent phase II trial using the same bank of CTL further demonstrated the effective use of HLA-matched allogeneic CTL, with complete or partial response observed in 52% of patients 6 months post treatment. Significantly better tumour responses were seen in patients infused with CTL with a high degree of donor CTL-recipient HLA matching and containing high proportions of CD4+ T-cells (Haque *et al.*, 2007). During the phase II trial no attempt was made to characterise the epitope specificity of the infused CTL or to correlate this with patient outcome. However, the observed importance of donor CTL-recipient HLA matching suggests that further refinement of the donor CTL-recipient matching procedure may be beneficial. The present study was undertaken to investigate the HLA-restricted epitope specificity at the protein and peptide level, and to determine the T-cell receptor clonality, of donor CTL used to treat 28 EBV-positive PTLD patients (Appendix I) who completed the phase II trial, and to correlate the results with tumour response.

3.2 Optimisation of modified chromium release assay

The standard chromium release assay, using LCL as targets for estimation of CTL killing, was modified to use DCs infected with recombinant vaccinia virus constructs as an alternative target. Each recombinant vaccinia construct expressed one of eight latent EBV proteins: EBNA -1, -2A, -3A, -3B, -3C, -LP, LMP-1, -2 (Murray *et al.*, 1990).

3.2.1 Establishment and characterisation of dendritic cell cultures

We used a magnetic bead method to isolate monocytes from PBMCs (detailed in section 2.4.4) for culture of DCs. Magnetic isolation of monocytes routinely enriched monocyte populations giving >90% purity when stained with an anti-CD14 antibody (Figure 3.1 and Table 3.1). After 1 week of culture with IL-4 and GM-CSF the CD14 positive population decreased and the proportion of cells positive for the DC marker CD209 (DC-SIGN) increased (Figure 3.1 and Table 3.1). This method proved consistent and produced adequate numbers of viable DCs to act as targets in our chromium release assay.

Table 3.1: Percentage of CD14 and CD209 cells in monocyte rich pre- and post-culture

	Median CD14 % (Range)	**Median CD209 (DC-SIGN) % (Range)**
Monocyte Rich Fraction	95.5 (90-99)	9 (1-74)
Monocyte Rich Culture (DC)	8 (1-40)	97 (82-99)

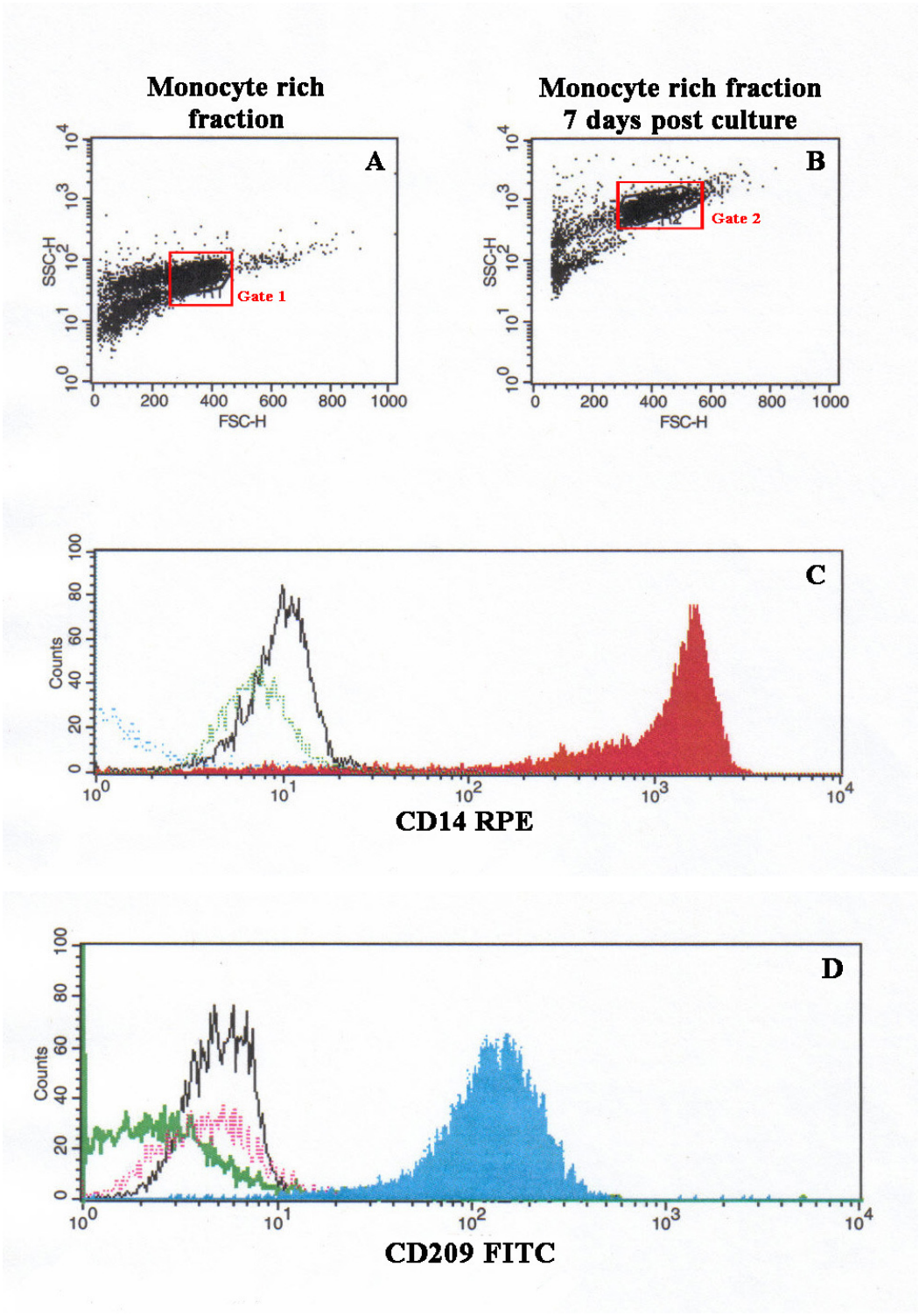


Figure 3.1: FACS analysis of monocyte rich fraction pre- and post-culture
Panel A: monocyte rich fraction – gate 1; panel B: cultured DC fraction – gate 2; panel C: CD14 expression, monocyte rich fraction isotype control - black, DC culture isotype control – green, monocyte rich fraction CD14 – red, DC culture CD14 – blue; panel D: CD209 expression, monocyte rich fraction isotype control - black, DC culture isotype control – pink, monocyte rich fraction CD209 – green, DC culture CD209 – blue

3.2.2 Confirmation of recombinant vaccinia infection of dendritic cells

All 8 recombinant vaccinia virus constructs containing EBV latent genes (EBNA-1, -2, -3A, -3B, -3C, -LP and LMP-1, -2) were grown and the virus titre estimated on thymidine kinase deficient 143B cells (section 2.4.10, 2.4.11). Infection and expression of viral transcripts in 143B cells was confirmed by RT-PCR (Figure 3.2).

Dendritic cells were isolated and grown as detailed in section 2.4.4 and 2.4.5. RT-PCR was performed on infected DCs to determine that infection and expression of EBV proteins was successful. Approximately $1x10^6$ cells were infected with the recombinant vaccinia EBV constructs at an MOI of 10:1 and incubated overnight. Infected cells were then harvested, RNA extracted and reverse transcribed prior to amplification in a specific RT-PCR. Transcripts for all 8 EBV proteins were detected in the infected cells (Figure 3.3). It was therefore concluded that infection with recombinant vaccinia constructs at MOI 10:1 was sufficient for infection and expression of EBV proteins in DCs.

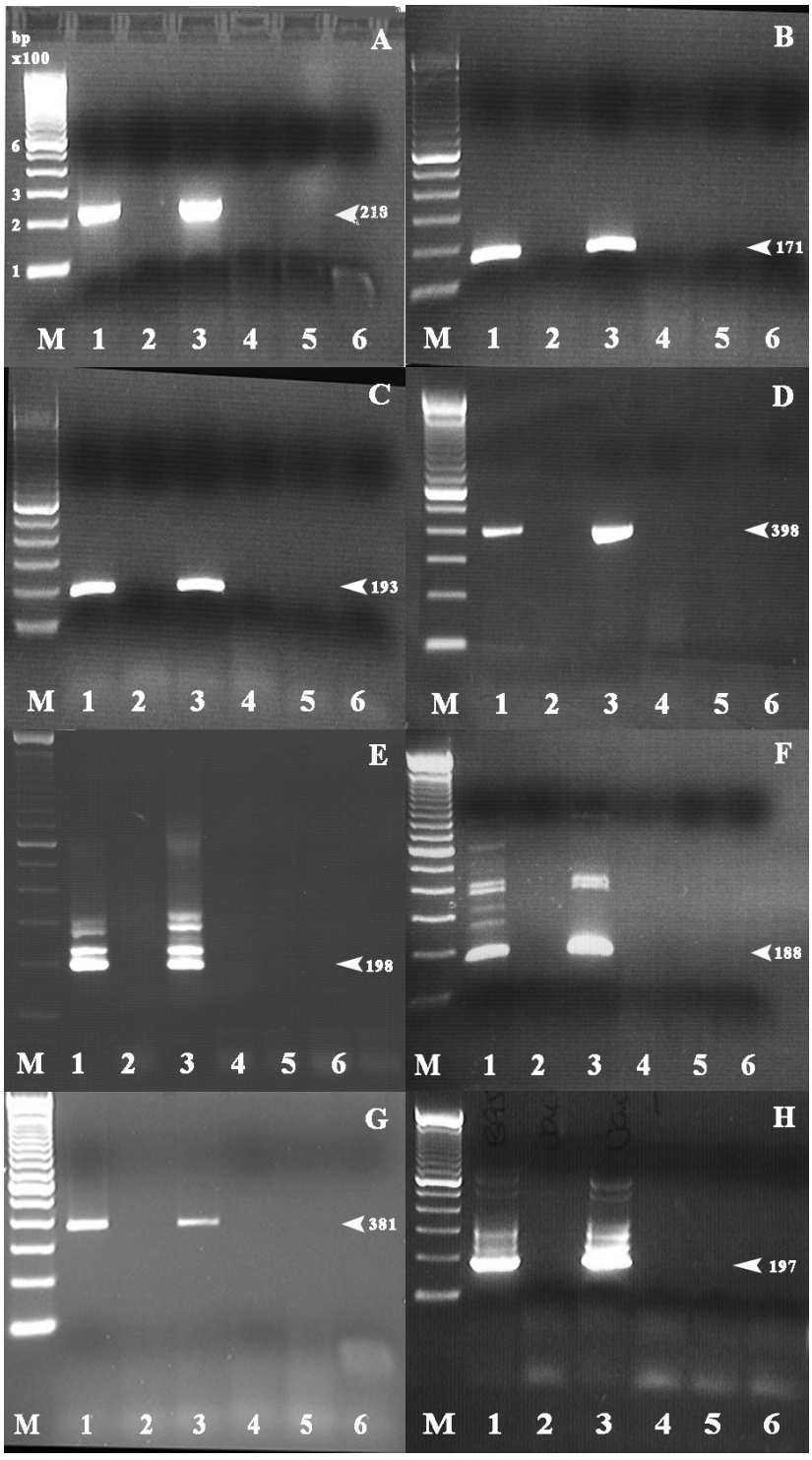


Figure 3.2: RT-PCR analysis of 143B cells infected with recombinant vaccinia EBV constructs.

Panel A: EBNA-1 construct (218bp); panel B: EBNA-2A (171bp); panel C: EBNA-3A (193bp); panel D: EBNA-3B (398bp); panel E; EBNA-3C (198bp); panel F: EBNA-LP (188bp); panel G: LMP-1 (381bp); Panel H: LMP-2 (197bp). B958 positive control: lane 1; 143B cells infected with empty recombinant vaccinia construct: lane 2; 143B cells infected with EBV-specific recombinant vaccinia construct: lane 3; uninfected 143B cells: lane 4; RNA negative control (no RT step): lane 5; negative water control: lane 6; 100bp marker: lane M.

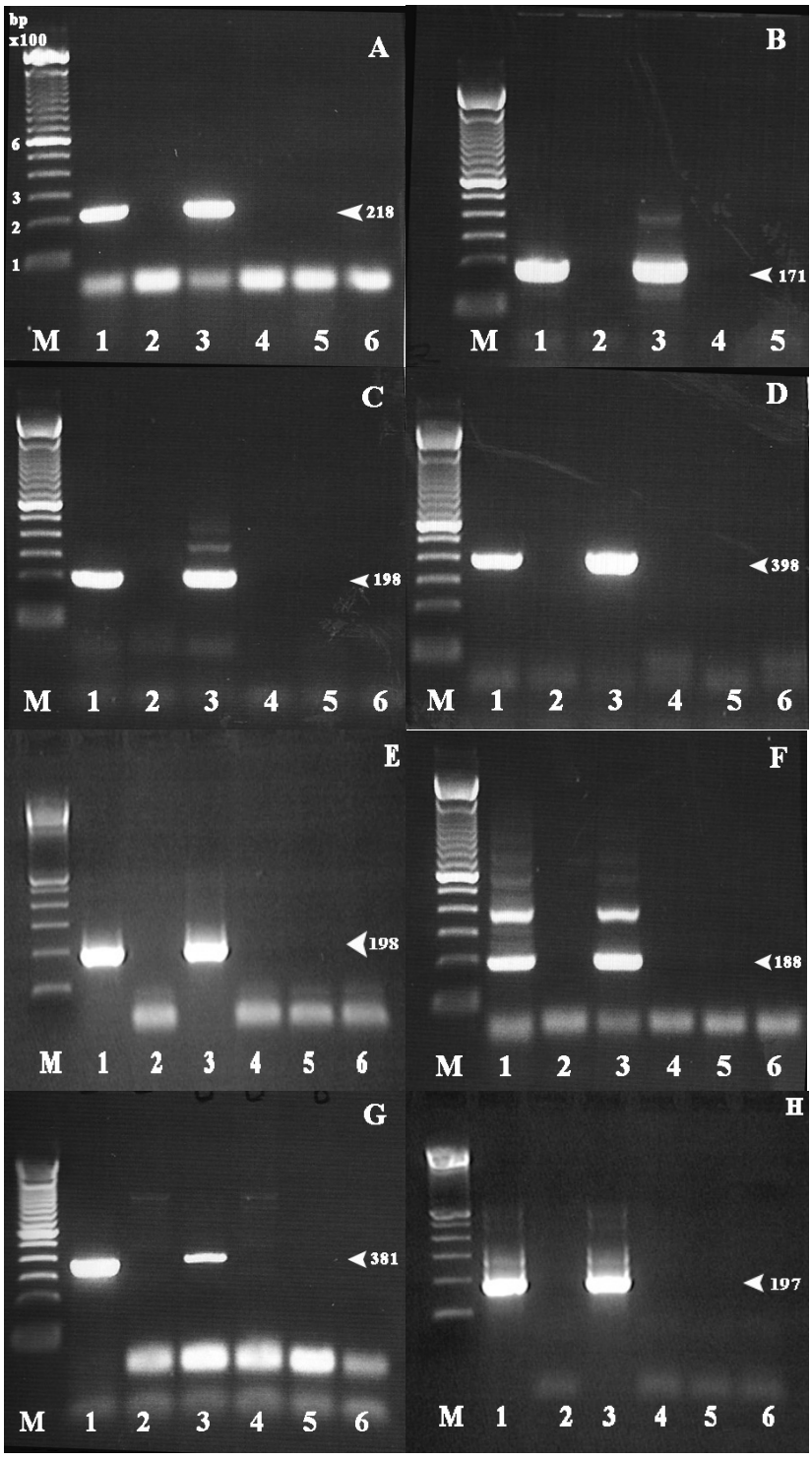


Figure 3.3: RT-PCR analysis of dendritic cells infected with recombinant vaccinia EBV constructs.

Panel A: EBNA-1 construct (218bp); panel B: EBNA-2A (171bp); panel C: EBNA-3A (193bp); panel D: EBNA-3B (398bp); panel E; EBNA-3C (198bp); panel F: EBNA-LP (188bp); panel G: LMP-1 (381bp); Panel H: LMP-2 (197bp). LCL positive control: lane 1; uninfected DCs: lane 2; DCs infected with EBV-specific recombinant vaccinia construct: lane 3; DCs infected with empty recombinant vaccinia construct: lane 4; RNA negative control (no RT step): lane 5; negative water control: lane 6; 100bp marker: lane M.

3.3 EBV latent protein specificity of CTL

The modified chromium release assay was used to determine which of the 8 EBV latent proteins were preferentially recognised by the CTL lines (n=21). Peripheral blood DCs from the CTL donors were infected with recombinant vaccinia virus constructs containing one of the 8 EBV latent proteins and used as targets in the assay. In a few cases donor DCs were unavailable and so DCs partially matched on HLA-A, -B and –DR alleles were used instead. Autologous LCL and an empty vaccinia virus construct were also included as controls. Figure 3.4 depicts the results obtained from one line (CTL 14). A response was deemed positive if the percentage of specific lysis was greater than that obtained with the empty vaccinia virus control plus 3%. The highest percentage of specific lysis obtained was termed a dominant response, whilst specific lysis percentages between the control and maximum levels, were termed sub-dominant. CTL 14 displayed a dominant response to EBNA-3C and a sub-dominant response to LMP-1.

In general EBV latent protein recognition by CTL lines was polyclonal in nature. Of the 21 CTL lines tested 19% (4/21) produced a response against 1 EBV protein, 43% (9/21) produced a response against 2 EBV proteins and 38% (8/21) against 3 or more EBV proteins. The majority (95%) of the CTL lines tested recognised at least one of the EBNA 3 proteins with 28% recognising EBNA-3A, 28% recognising EBNA-3B and 71% recognising EBNA-3C (Figure 3.5). Sub-dominant responses were also seen in a number of CTL lines against the LMP proteins with 24% recognising LMP-1 and 33% recognising LMP-2 (Figure 3.5). Sub-dominant responses were observed in a small number of lines against the EBNA-1 (19%), -2 (9%) and -LP (24%) proteins, with 1 line displaying a dominant response towards EBNA-1 (70% EBNA-1 specific lysis). Appendix II details the protein specificity for each individual line.

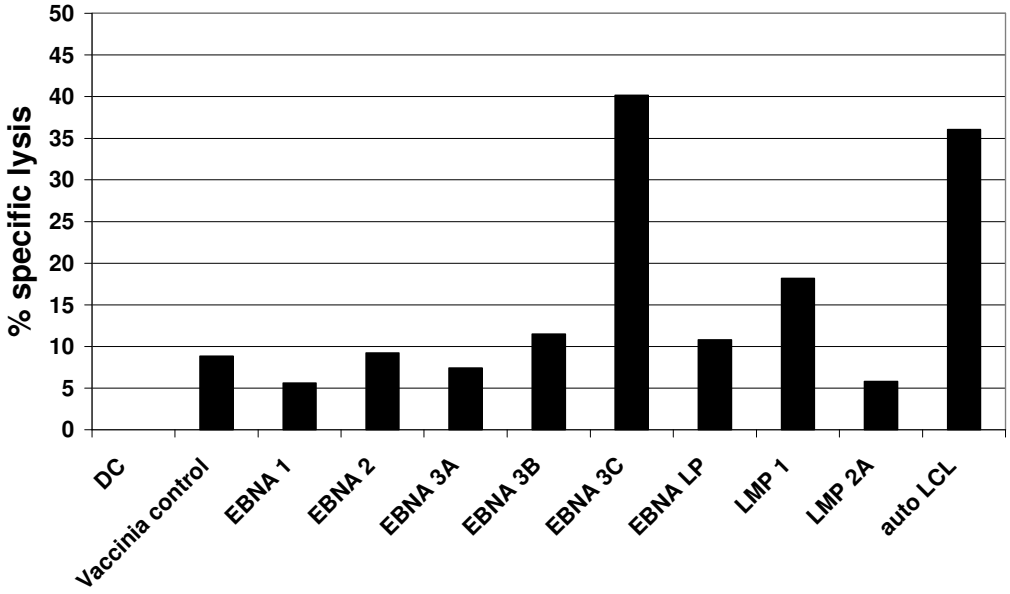


Figure 3.4 Modified chromium release assay of CTL 14
Approximately $1x10^5$ DCs were infected at an MOI of 10:1with each specific construct. Triplicate wells for each construct were averaged and the percent specific lysis estimated. Autologous LCLs were included as a positive control. Percent specific lysis greater than lysis obtained from vaccinia control plus 3% was designated a response

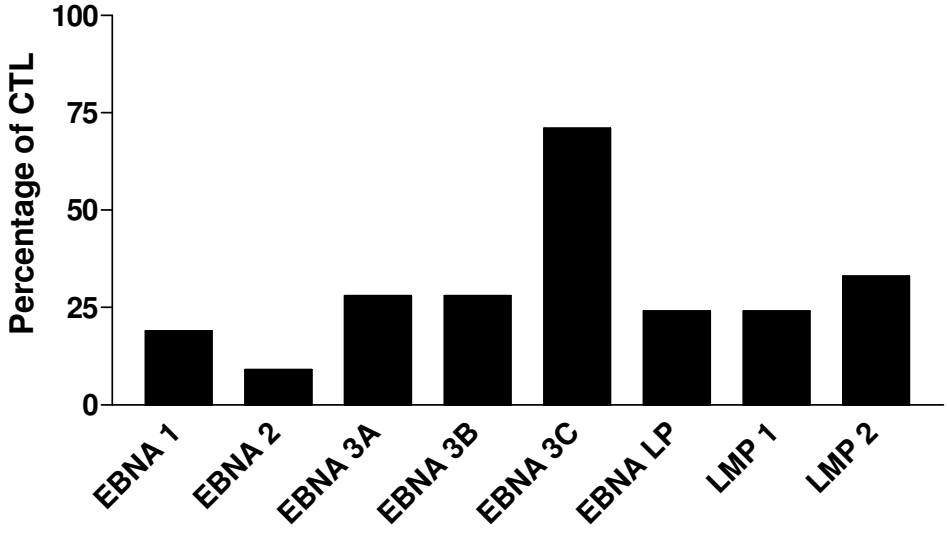


Figure 3.5 Protein specificity of CTL.
CTL were tested in a modified chromium release assay using recombinant vaccinia constructs containing EBV latent genes as targets. The percentage of CTL lines recognising each latent protein was calculated.

3.4 Optimisation of peptide Elispot assay

A human IFN-γ elispot assay was established and optimised for determination of CTL peptide specificity. Two kits were initially tested as per maunfacturer's instructions: the

R&D Systems Human IFN-γ kit (EL285) and the Endogen Human IFN-γ kit (FF72474). Both kits were screened using LCL as targets for the CTL. Three concentrations of LCL were used: $1x10^5$, $1x10^4$ and $1x10^3$ together with CTL at CTL:LCL ratios of 1:1, 2:1, 5:1,10:1 and 20:1. For both kits the maximum number of IFN-γ positive spots was observed at a CTL:LCL ratio of 20:1 (3-129 spots for the R&D kit; 1-50 for the Endogen kit) with decreasing spot formation seen for lower CTL:LCL ratios. Increased numbers of spots were also observed with increasing numbers of LCL although it was difficult to count spots at the $1x10^5$ LCL concentration due to smudging, particularly for the Endogen kit (Figure 3.6).

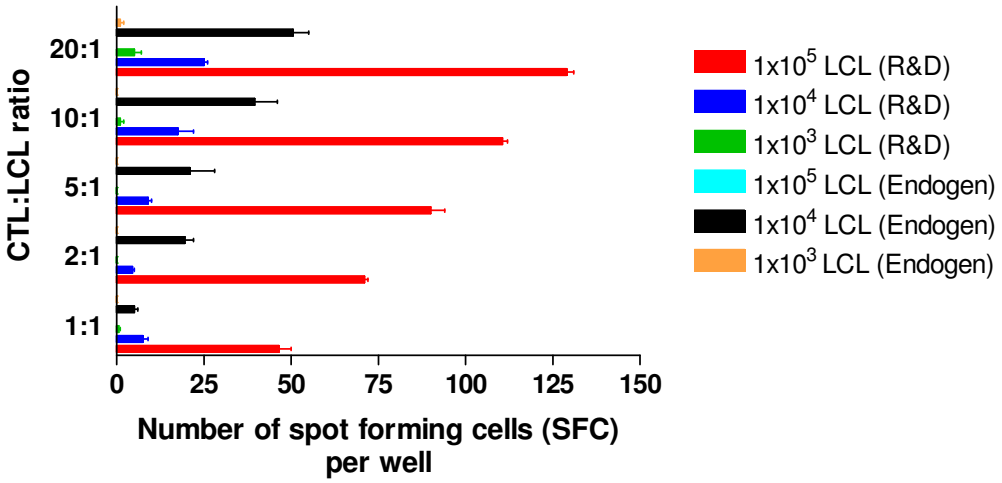


Figure 3.6 Comparison of Human IFNγ kits (R&D Systems and Endogen).
CTL were tested using increasing numbers of LCL and increasing CTL:LCL ratios. Where possible IFNγ producing spots were counted and the average of duplicate wells compared between kits.

No major differences were observed between the kits. The R&D Systems kit was quicker to process and was therefore selected for further optimisation.

3.4.1 Optimal peptide presentation

Autologous PBMC were chosen as the vehicle for antigen presentation of peptide. For initial experiments a concentration of $1x10^5$ cells was selected based upon the numbers

of PBMC available for use from our frozen stock. PBMC were incubated with varying concentrations of peptide: 1µg/ml, 5µg/ml and 10µg/ml and used as targets in the assay. Targets were incubated with 5-fold dilutions of CTL from $5x10^4$ to no CTL and the assay performed in triplicate. The number of spot forming cells was counted and the average compared. The highest number of spot forming cells (average 42 spots) was observed at a CTL concentration of $5x10^4$ and a peptide concentration of 10µg/ml (Figure 3.7).

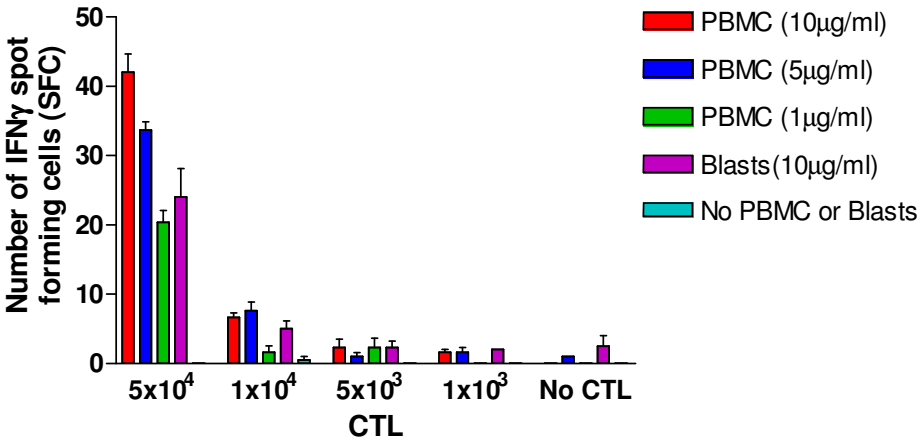


Figure 3.7 Optimisation of peptide concentration
CTL 14 (EBNA-3C specific, HLA-A*02 positive) was tested against the the HLA-A*02 restricted, EBNA-3C specific peptide LLDFVRFMGV. Autologous PBMC ($1x10^5$) and PHA blasts ($1x10^5$) with various peptide concentrations were used a targets

In case of autologous PBMC being unavailable the CTL were also tested against peptide loaded PHA blasts ($1x10^5$) with 10µg/ml of peptide. The highest number of spot forming cells (average 24 spots) was again seen at the highest concentration of CTL although the number was slightly reduced compared to the autologous PBMC (Figure 3.7). In light of this result we opted to use $1x10^5$ PBMC or blasts in conjunction with $5x10^4$ CTL and 10µg/ml of peptide for subsequent experiments.

3.4.2 Confirmation of peptide specificity

To confirm that our selected conditions were appropriate and specific we obtained a CTL with known peptide specificity from Dr Stephen Lee, Department of Cancer Studies, University of Birmingham, UK. The CTL obtained was HLA-A*11 restricted with specificity for the EBNA-3B peptide AVFDRKSDAK. We tested the CTL in triplicate using the optimised conditions (see section 3.4.1)

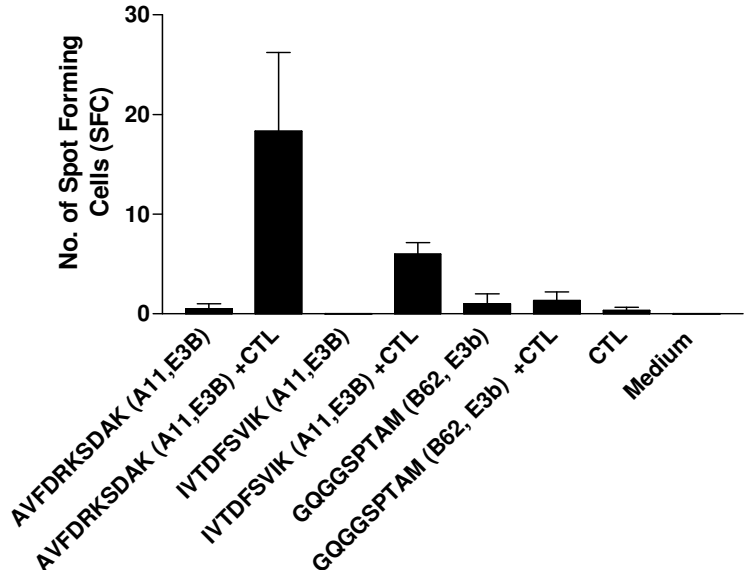


Figure 3.8 Elispot analysis of CTL with known specificity
The CTL was tested using optimised conditions of $1x10^5$ PBMC, 10µg/ml of EBNA-3B, HLA-A11 restricted peptides AVF and IVT or EBNA-3B, HLA-B62 restricted control peptide GQG, with or without the presence of $5x10^4$ CTL. CTL alone and medium were also included as controls.

The results indicated that the CTL was specific for the HLA-A*11 restricted EBNA-3B peptides with maximal spot forming cells observed for the AVF peptide (Figure 3.8). This was in line with the specificity observed by Dr S.Lee and therefore our conditions were deemed acceptable to continue with the analysis of the CTLs.

3.5 EBV peptide specificty of CTL

Human IFN-γ elispot assays were used to confirm the protein specificity at the peptide level in 20 of the 24 CTL lines (PBMC and PHA blast numbers were insufficient in 4

lines). CTLs were tested against a variety of peptides with the same HLA-A or HLA-B restriction as detailed in Table 2.3, and a control peptide with a mismatched HLA allele restriction. LCLs were also included as a positive control for IFN-γ release. A total of 8 CTL lines displayed a positive response towards peptides with the same HLA subtype restriction and to those peptides derived from proteins corresponding to CTL protein specificity (Table 3.2). In the case of three CTL lines (CTL 40, 91 and 67) more than one HLA-restricted peptide response was recognised (Table 3.2). The remaining CTL lines did not respond to our panel of peptides (Appendix II).

3.6 CTL protein and peptide specificity did not correlate with patient response

A total of 28 PTLD patients were treated with CTL and monitored for tumour regression 6 months post treatment. Protein specificity data were available for all CTL lines used to treat the 11 non-responders and for CTL lines from 14 of the 17 responders. Within the responder group 21% (3/14) of CTL lines recognised 1 EBV protein, 57% (8/14) recognised 2 EBV proteins and 21% (3/14) recognised 3 or more EBV proteins. No significant difference was noted for the non-responder group with 9% (1/11) of CTL line recognising 1 EBV protein, 36% (4/11) recognising 2 and 54% (6/11) recognising 3 or more EBV proteins ($p=0.22$).

Table 3.2: Peptide specificity of CTL.

CTL	CTL HLA	CTL protein specificty	CTL peptide specificty	Peptide HLA restriction
50	A3,11; B7,51; DR15(2), 4	EBNA-3B	IVT AVF	A11 A11
55	A2,68; B51,62; DR4, 13(6)	EBNA-3C	LLD	A2
	A2,68; B51,62; DR4, 13(6)			
58	A3,11; B55,64; DR15(2), 4	EBNA-3B	IVT	A11
48	A1,2; B7,8; DR15(2), 17(3)	EBNA-3A	QAK	B8
40	A2; B7,44: DRBr, 4	EBNA-3C LMP-2	LLD LLW	A2 A2
95	A2; B44,49; DR4, 7	EBNA-3C	LLD	A2
91	A2,68; B44,62: DR4	EBNA-3B EBNA-3C	VEI GQG LLD	B44 B62 A2
67	A2,11; B7,62: DR15(2), 4	LMP-2 EBNA-3B	LLW GQG	A2 B62

CTL protein specificity was determined via a modified chromium release assay. Peptides corresponding to CTL specific proteins and matched to CTL HLA were tested in an IFNγ Elispot assay.

Analysis of the specific proteins recognised showed that the majority of CTL lines from both the responder and the non-responder groups recognised the EBNA-3C protein (64% and 73% respectively). The number of CTL specific for EBNA-3A and EBNA-3B proteins was slightly increased in the responder group compared to the non-responder group (EBNA-3A: 36% responders, 18% non-responders; EBNA-3B: 36% responders and 18% non-responders) but not significantly so (p=0.4 for both EBNA-3A and -3B). In comparison the number of CTL displaying a response to EBNA-2 and EBNA-LP was decreased in the responder group compared to the non-responder group (EBNA-2: 7%

responders, 27% non-responders; EBNA-LP: 14% responders and 36% non-responders) but again not significantly so (p=0.28 and p=0.35, respectively). CTL responses to LMP-1, LMP-2 and EBNA-1 were similar in both treatment groups (LMP-1: 21% responders and 27% non-responders; LMP-2: 29% responders and 36% non-responders; EBNA-1: 14% responders and 18% non-responders) (Figure 3.9).

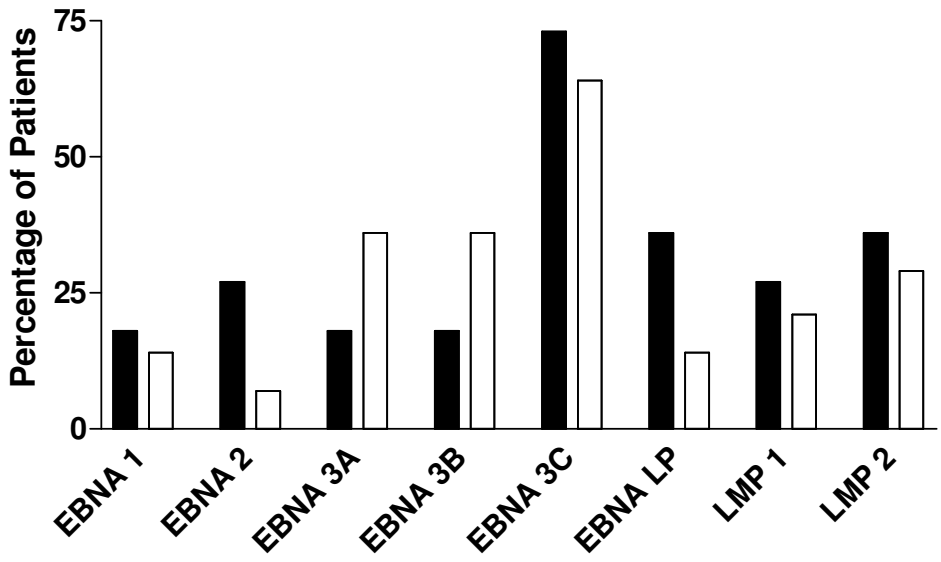


Figure 3.9 CTL protein specificity in responder and non-responder groups.
The percentage of CTL within each group recognising each latent protein was estimated. Non-responders: black bar; responders: white bar.

Where available CTL specificity at the peptide level was compared between treatment responders (n=5) and non-responders (n=4). Within the non-responder group infused CTL peptide specificity was restricted to 1 protein (EBNA-3 complex proteins) and 1 HLA allele. In comparison 3/5 responders displayed multiple peptide specificities (EBNA-3 complex and LMP proteins) (Table 3.3).

Table 3.3: Peptide specificity of CTL: comparison with patient response

CTL	CTL HLA	CTL protein specificty	CTL peptide specificty	Peptide HLA restriction	Tumour response: 6 month
50	A3*,11; B7*,51; DR15(2), 4*	EBNA 3B	IVT AVF	A11 A11	Non-responder
55	A2*,68; B51*,62*; DR4*, 13(6)	EBNA 3C	LLD	A2	Non-responder
	A2*,68; B51,62*; DR4*, 13(6)				Non-responder
58	A3,11*; B55*,64; DR15(2)*, 4	EBNA 3B	IVT	A11	Non-responder
48	A1*,2; B7,8*; DR15(2), 17(3)*	EBNA 3A	QAK	B8	Responder
40	A2*; B7,44*: DRBr, 4*	EBNA 3C LMP 2	LLD LLW	A2 A2	Responder
95	A2*; B44*,49; DR4*, 7*	EBNA 3C	LLD	A2	Responder
91	A2*,68*; B44*,62: DR4*	EBNA 3B EBNA 3C	VEI GQG LLD	B44 B62 A2	Responder
67	A2*,11; B7*,62*: DR15(2)*, 4*	LMP 2 EBNA 3B	LLW GQG	A2 B62	Responder

CTL protein specificity was determined via a modified chromium release assay.
CTL peptide specificity was compared between responder and non-responder groups.
* Recipient-CTL HLA matched alleles

3.7 CTL epitope specificity did not correlate with EBV antigen expression of tumour cells

Although the majority of PTLDs showed the classic histological features of the disease (classified as hyperplastic (n=4), polymorphic (n=8), or monomorphic (n=10) type), 5 patients with Hodgkin's-type PTLD and 1 with a Burkitt-like PTLD were included in the trial (Appenndix 1). These tumours are known to have a restricted pattern of viral

gene expression which is less likely to be recognised by CTLs selected only on the basis of the best HLA-match (Rowe *et al.*, 1986;Grasser *et al.*, 1994;Deacon *et al.*, 1993). We therefore analysed viral gene expression in tumour biopsy, and compared this with protein specificity of infused CTL and tumour response in these 6 cases.

All 6 tumours were EBER-positive, and 5/5 Hodgkin's PTLDs expressed monoclonal κ or λ mRNA, however the Burkitt-like PTLD stained for neither κ or λ mRNA. Four out of five Hodgkin's-type PTLDs tested stained LMP-1 positive and EBNA-2 negative, whereas the Burkitt-like PTLD was negative for both markers (Table 3.4).

At 6 month after the last infusion 4 of the 5 Hodgkin's-type PTLDs, and the one Burkitt-like tumour, showed a complete response to CTL therapy, whereas the remaining Hodgkin's-type tumour showed no response. All 6 recipients were treated with CTL lines with a dominant EBNA-3 specificity: 4 received CTL lines with multiple protein specificities and 2 received lines specific for EBNA 3A alone (Table 3.4). Each CTL was chosen on the best recipient-CTL HLA match (between 2 and 5 matching alleles), however, analysis of HLA-restricted peptide specificity showed that the treatment non-responder (number 3, Table 3.4) was infused with a CTL line restricted through HLA-A*11; a mismatched allele (Table 3.4).

Table 3.4: CTL epitope specificity: comparison with restricted tumour cell expression.

Recipient	PTLD histology	EBERS	LMP-1	EBNA-2	Clonality	CTL HLA	Response: 6 month	Protein specificity	Peptide specificity
1	Hodgkin's	+	+	-	mono	A2, 3*; B35*, 44(12); DRBr, 15(2)	R	EBNA-3C; EBNA-1; LMP-1; LMP-2	Nd
2	Hodgkin's	+	+	-	mono	A1*, 2; B7, 8*; DR15, 17(3)*	R	EBNA-3A; EBNA-LP	QAK: B8
3	Hodgkin's	+	+	-	mono	A3*, 11; B7*, 51(5); DR4*, 15(2)	NR	EBNA-3B; EBNA-1; EBNA-3C	IVT: A11; AVF: A11
4	Hodgkin's	+	nd	nd	mono	A2*; B44(12)*, 49(21); DR4*,7*	R	EBNA-3A; EBNA-3C	LLD: A2
5	Burkitt's	+	-	-	neg	A1*; B8*; DR4, 17(3)*	R	EBNA-3A	Nd
6	Hodgkin's	+	+	-	mono	A1*; B8*; DR4*, 17(3)	R	EBNA-3A	Nd

* Recipient-CTL HLA matched alleles; nd: not determined; neg: negative; R: responder; NR: non-responder

3.8 Optimisation of T-Cell receptor PCR product analysis

The T-cell receptor PCR was adapted from Peggs *et al* (2001) with no modifications to the published PCR conditions. However the subsequent analysis of the PCR products by the ABI 3730 automated sequencer and Genemapper software in our laboratory required optimisation.

cDNA from PBMC was amplified as described in methods section 2.6.4. The PCR products were then diluted 10, 50 and 100 times. Approximately 1µl of neat and diluted product was further diluted 1 in 10 with Hi-Di formamide/size marker mix prior to running on the sequencer. For each individual β-variable sub-family the 1 in 50 and 1 in 100 dilutions produced peak heights below that of the base pair size marker making these dilutions unreliable for detection (Figure 3.10). The opposite was observed for undiluted product with peak heights vastly exceeding the height of the marker. The 1 in 10 dilution was in line with the base pair size marker peak height and was therefore chosen for all subsequent analysis (Figure 3.10).

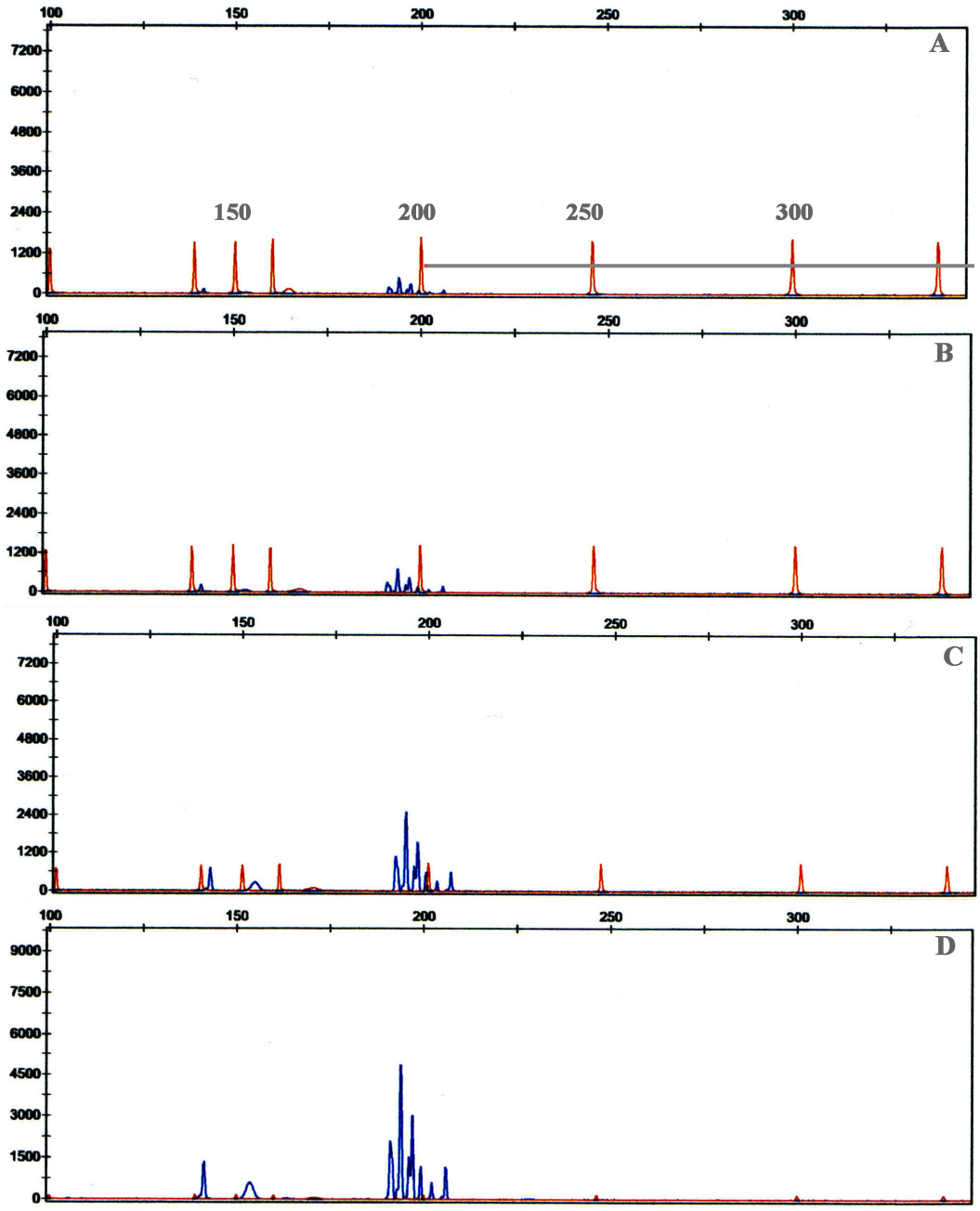


Figure 3.10 Dilution of sub-family BV-21 PCR product (PBMC sample)
Sample was diluted 1 in 100: panel A; 1 in 50: panel B; 1 in 10: panel C and undiluted: panel D. Diluted sample was then further diluted 1 in 10 for screening on ABI 3730 sequencer. Size standard (bp) labelled in panel A.

3.9 PBMC T-cell receptor (TCR) clonality

cDNA extracted from 5 PBMC samples from healthy volunteers was prepared as described in methods section 2.6.4. Integrity of the cDNA samples was checked in a β-actin PCR. All 5 cDNA samples were successfully amplified (Figure 3.11).

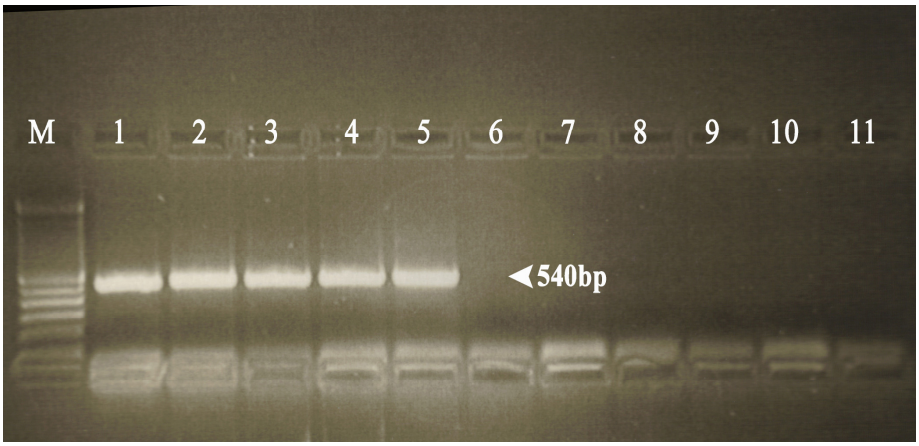


Figure 3.11 B-actin amplification of PBMC cDNA.
Lanes 1-5: cDNA from PBMC donors 1-5; lanes 6-10: RNA from PBMC donors 1-5; lane 11: nuclease free water; M: 100bp marker

Following β-actin PCR the cDNA was analysed in a TCR spectratyping PCR as described in section 2.6.8. Sub-family usage was designated according to the number of specific peaks observed: 3 or more peaks in a Gaussian or skewed-Gaussian distribution was termed polyclonal usage, a single or double peak was termed clonal (Figure 3.12).

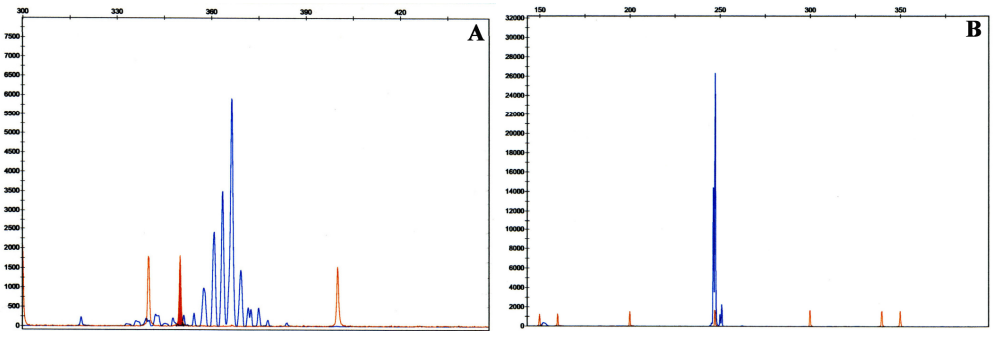


Figure 3.12 Polyclonal (A) and clonal (B) distribution patterns
Distribution pattern shown in blue and size standard shown in orange

Across each individual sub-family all 5 PBMC samples were polyclonal in nature with few if any clonal distributions observed. Figure 3.13 is an example of of the PBMC profiles obtained.

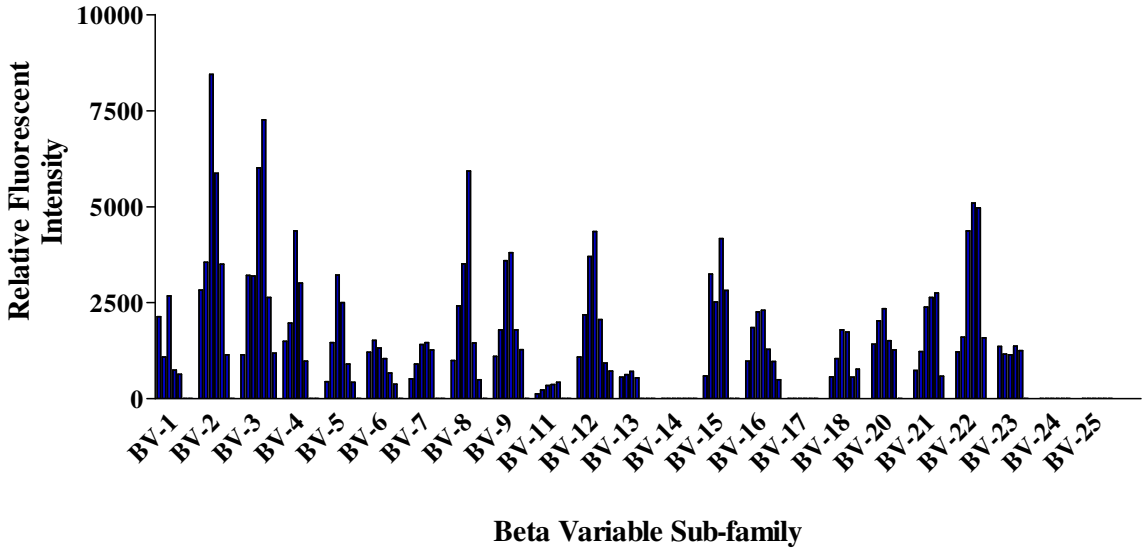


Figure 3.13: TCR spectratyping profile of PBMC sample
Polyclonal distribution patterns were obtained for 20 of 23 sub-families tested. 4 sub-families (-14, -17, -24 and -25) were undetectable.

3.10 CTL T-cell receptor (TCR) clonality

CTL lines (n=22) were analysed for use of the TCR β-chain variable gene sub-families using the spectratyping PCR. Polyclonal and clonal peaks were observed for 22 of the 23 sub-families; no polyclonal peaks were seen for sub-family 25. No one particular sub-family was preferentially used by the CTL lines. However, more CTL lines had polyclonal distribution patterns for sub-families -1, -2, -3, -6, -8, -9, -12, -13, -15, -17 and -22. A polyclonal distribution pattern was particularly enhanced for sub-family -9 (polyclonal: 77%(17/22); clonal: 18%(4/22); undetected: 4%(1/22)) (Figure 3.14). In contrast, the majority of CTL lines had clonal distribution patterns for sub-families -4, -11, -16, -21, -23 and -24 (Figure 3.14). Sub-families -18 and -25 were rarely used in

comparison to the other families (sub-family -18: polyclonal- 32%, clonal- 18%, undetected- 50%; sub-family -25: polyclonal- 0, clonal- 27%, undetected- 73%).

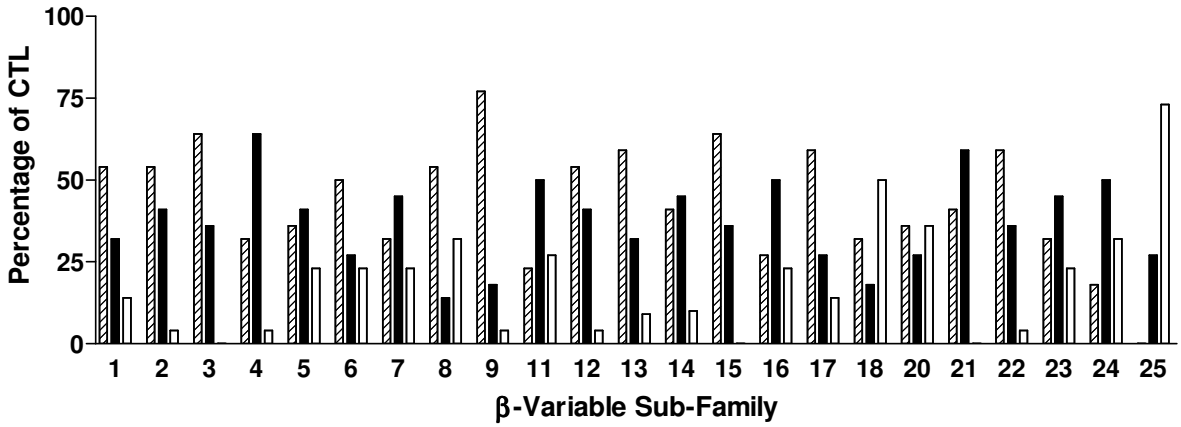


Figure 3.14: T-cell receptor β-variable sub-family usage of CTL.
Spectratyping analysis for 23 sub-families was performed. Each sub-family was analysed for clonal: black bar; polyclonal: hatched bar or undetected: white bar; distribution patterns. The percentage of CTL presenting with each pattern was estimated

3.11 Polyclonal TCR usage correlates with patient response

CTL TCR spectratyping data were available for 16 PTLD treatment responders and 11 non-responders. A significant difference was observed between the responder and non-responder groups for sub-family -2 with 75%(12/16) of responders and 27%(3/11) of non-responders presenting a polyclonal distribution; 19%(3/16) of responders and 73%(8/11) of non-responders a clonal distribution; and undetectable usage in 6%(1/16) of responders (p=0.01). Sub-families -3 and -9 also had an increase in polyclonal distribution within the responder group compared to the non-responder group (sub-family-3: 81%(13/16) responders and 45%(5/11) non-responders, p=0.05; sub-family-9: 94%(15/16) responders and 54%(6/11) non-responders, p=0.05) (Figure 3.15, Table 3.5).

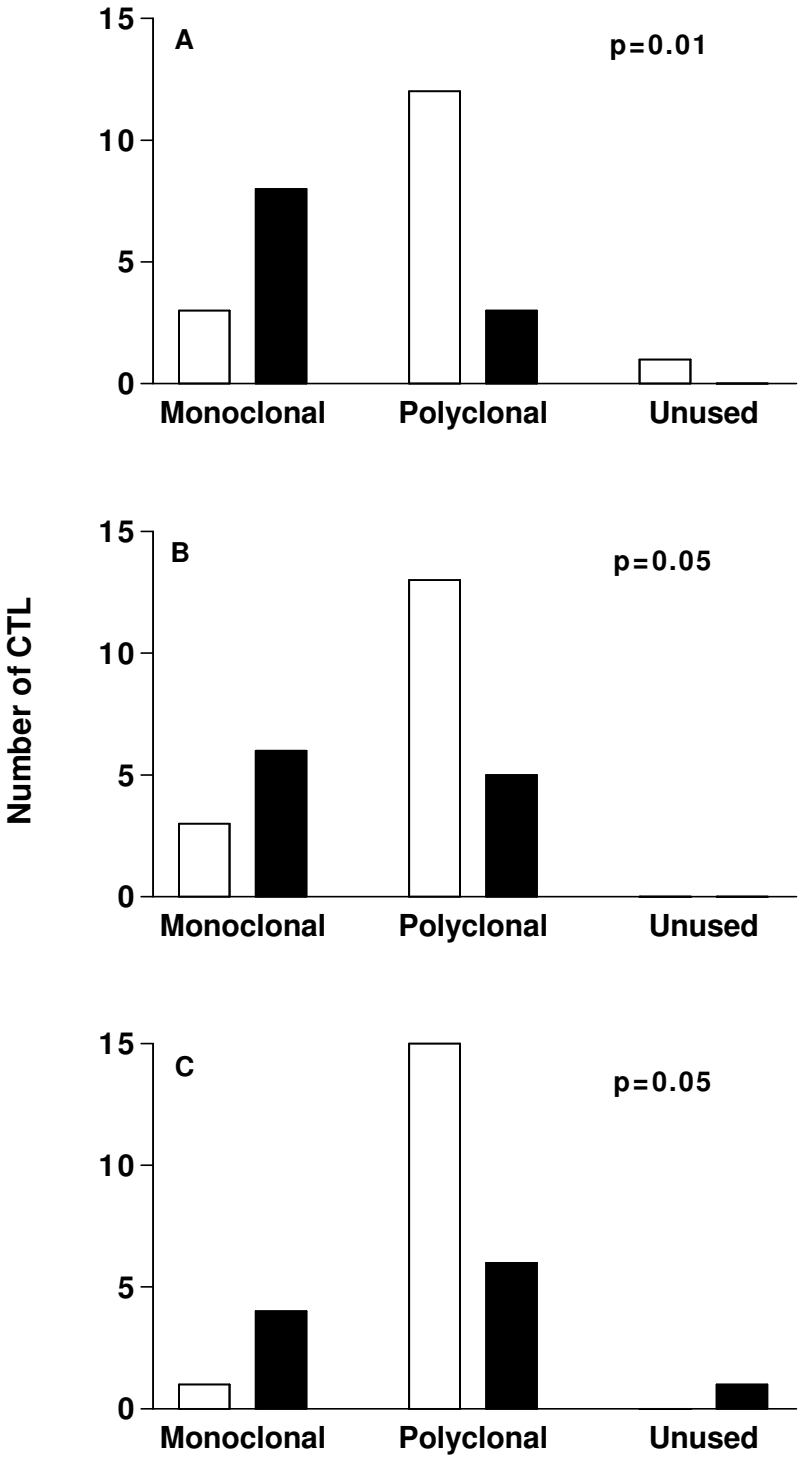


Figure 3.15: T cell receptor β-variable sub-family usage of CTL responder and non-responder groups.
Panel A: clonal, polyclonal and undetected distribution patterns for sub-family -2. Panel B: sub-family -3. Panel C: sub-family -9. Non-responder: black bar; responder: white bar.

A reduced polyclonal distribution for sub-families -5 and -16 was observed in the responder group compared to the non-responder group but this did not reach statistical significance (sub-family-5: 37%(6/16) of responders and 64%(7/11) of non-responders; p=0.4; sub-family-16: 19%(3/16) of responders and 45%(5/11) of non-responders; p=0.24) (Table 3.5). As with the overall CTL analysis, sub-families -18 and -25 were rarely used by CTL lines in both the responder (sub-family -18: 31%, 25%) and 44% for polyclonal, clonal and undetected use respectively; sub-family -25: 0, 31% and 69%, respectively) and non-responder groups (sub-family -18: 18%, 9% and 73% for polyclonal, clonal and undetected use; sub-family -25: 0, 18% and 82%, respectively) (Table 3.5).

	Monoclonal		Polyclonal		Undetected		
β-variable sub-family	**Responder**	**Non-responder**	**Responder**	**Non-responder**	**Responder**	**Non-responder**	**p-value[a]**
1	4 (25%)	4 (36%)	9 (56%)	5 (45%)	3 (19%)	2 (18%)	0.64
2	3 (19%)	8 (73%)	12 (75%)	3 (27%)	1 (6%)	0	**0.01**
3	3 (19%)	6 (54%)	13 (81%)	5 (45%)	0	0	**0.05**
4	8 (50%)	9 (82%)	7 (44%)	2 (18%)	1 (6%)	0	0.22
5	7 (44%)	3 (27%)	6 (37%)	7 (64%)	3 (19%)	1 (9%)	0.40
6	3 (19%)	3 (45%)	10 (62%)	6 (54%)	3 (19%)	2 (18%)	0.86
7	6 (37%)	5 (45%)	4 (25%)	4 (36%)	6 (37%)	2 (18%)	0.54
8	1 (6%)	2 (18%)	11 (69%)	5 (45%)	4 (25%)	4 (36%)	0.42
9	1 (6%)	4 (36%)	15 (94%)	6 (54%)	0	1 (9%)	**0.05**
11	9 (56%)	3 (27%)	5 (31%)	2 (18%)	2 (12%)	6 (54%)	0.06
12	6 (37%)	4 (36%)	9 (56%)	7 (64%)	1 (6%)	0	0.68
13	5 (31%)	3 (27%)	9 (56%)	7 (64%)	2 (12%)	1 (9%)	0.92
14	7 (44%)	5 (45%)	6 (37%)	6 (54%)	3 (19%)	0	0.28
15	5 (31%)	5 (45%)	11 (69%)	6 (54%)	0	0	0.45
16	9 (56%)	3 (27%)	3 (19%)	5 (45%)	4 (25%)	3 (27%)	0.24
17	4 (25%)	2 (18%)	9 (56%)	8 (73%)	3 (19%)	1 (9%)	0.66
18	4 (25%)	1 (9%)	5 (31%)	2 (18%)	7 (44%)	8 (73%)	0.31
20	4 (25%)	3 (27%)	6 (37%)	3 (27%)	6 (37%)	5 (45%0	0.85
21	8 (50%)	9 (82%)	8 (50%)	2 (18%)	0	0	0.09
22	7 (44%)	3 (27%)	7 (44%)	8 (73%)	2 (12%)	0	0.24
23	6 (37%)	6 (54%)	7 (44%)	2 (18%)	3 (19%)	3 (27%)	0.38
24	6 (37%)	8 (73%)	4 (25%)	1 (9%)	6 (37%)	2 (18%)	0.19
25	5 (31%)	2 (18%)	0	0	11 (69%)	9 (82%)	0.44

Table 3.5: CTL T cell receptor β-variable sub-families in responder and non-responder treatment groups
[a] *Chi Square*. Significant values are highlighted in bold font.

3.12 Discussion

In a recently reported phase II clinical trial using allogeneic, EBV-specific CTL lines to treat PTLD, we have shown that tumour response significantly increased with the number of CTL-recipient HLA allele matches (varying from 2 to 6) and the percentage of CD4 positive T-cells in the infused CTL (Haque *et al.*, 2007). In the present study we sought further correlates of tumour response in the trial participants by characterising the epitope specificity and TCR clonality of the infused CTL lines. Analysis of CTL protein specificity revealed that the majority (81%) were directed against 2 or more EBV latent proteins and this protein specificity was confirmed at the peptide level (HLA-restricted) in 8 of the CTLs. In line with previous studies on *ex vivo* grown, EBV-specific CTL, the lines predominantly recognised the EBNA-3 proteins, in particular EBNA-3C (Steven *et al.*, 1996;Whitney *et al.*, 2002). Since most PTLDs display full latent viral gene expression, CTLs with specificity for the EBNA 3 proteins would be expected to recognise and kill the tumour cells. However, when CTLs were chosen for specific recipients on HLA allele matching alone, without knowledge of the peptide specificity and HLA restriction of the CTL, mismatches clearly occurred (see Table 3.4, where recipient 3 was matched with the CTL donor for HLA-A*03, -B*07, and –DR*04, but the predominant peptide specificities of the CTL were both restricted through HLA-A*11). Thus prior knowledge of CTL specificity and HLA restriction at the peptide level would have enhanced the CTL-recipient matching process.

The complete resolution of Hodgkin's-type and Burkitt's-like PTLD tumours in patients which was sustained at 6 months in 5 of the 6 cases was unexpected since these tumours were all treated with CTLs with a predominant specificity for EBNA-3, a protein not normally expressed by these tumour cells. In one case (recipient 1) tumour cell killing could have been mediated by the subdominant clones within the CTLs with specificity

for EBNA-1, LMP-1 and LMP-2 proteins, but in the other cases no such activity could be detected using the methods and peptide panel available (Table 3.4). The modified chromium assay using recombinant vaccinia constructs may have missed subdominant clones expressed at low levels, whilst a more extensive peptide panel may have detected a larger array of subdominant responses. Uncharacterised minor HLA allele matching may also play a role in CTL recognition in these cases. During EBV transcription processes EBNA -2 is switched on prior to activation of the EBNA-3 proteins, therefore detection of EBNA-2 protein normally indicates that EBNA-3 is also present. In these cases, we demonstrated a latency type II (HD cases) phenotype by the absence of EBNA-2 expression in tumour cells, and a latency type I (BL case) phenotype by the absence of EBNA-2 and LMP-1 expression; the assumption being that no EBNA-3 proteins are expressed. Recently, rare Burkitt cell lines with mutated EBNA 2 genes that express EBNA-3A, -3B, -3C and –LP in the absence of EBNA-2 have been reported (Kelly *et al.*, 2005), and extrapolation of these findings to the *in vivo* situation could account for the response of the Burkitt's like tumour in our study. However, to our knowledge no such EBNA-2 mutants have been identified *in vivo*, therefore this scenario is unlikely. In two of the cases, CTL lines with subdominant reactivity directed against EBNA-1 were used. EBNA-1 has been identified as a target for HLA class II restricted, CD4 positive T-cell mediated killing (Mautner *et al.*, 2004;Leen *et al.*, 2001) and may therefore form a suitable target, along with the other antigens, for the CD4 positive T-cell population in the infused CTL if HLA matched to the recipient. Of the 2 EBNA-1 specific CTL lines only one was matched on the HLA-DR locus and corresponded to the non-responder HD case (Table 3); possible matching with HLA-DQ and -DP alleles was not assessed. Despite the significant relationship between HLA matching and a favourable patient outcome, it is possible that the infused allogeneic

CD4 and CD8 positive T-cells initiated a inflammatory response, either through activating endogenous EBV-specific CTL, through minor HLA mismatching, or by recruiting non-specific cytotoxic T-cells to the tumour site. Such a response would be similar to the beneficial graft-versus-leukaemia (GVL) response often seen following allogeneic bone marrow transplant where donor lymphocytes display anti-tumour effects primarily through T-cell recognition of mismatched minor histocompatability and/or tumour associated antigens (Bleakley M and Riddel SR, 2004). Recently donor natural-killer cells, antigen presenting cells and CD4 positive effector memory cells within infusions have been shown to facilitate this anti-tumour response in GVL (Parham and McQueen, 2003;Chakraverty and Sykes, 2007;Zheng et al., 2007). The small CD4 T-cell population within our CTL lines may contribute a similar effect. Indeed recognition of possible anti-tumour targets by CD4 T-cells has been reported (Gudgeon *et al.*, 2005). CD4 T-cells were found to recognise autologous LCL but not EBV negative B lymphoblasts, nor, EBV-specific latent and lytic antigens, suggesting that the CD4 T-cells recognise a tumour specific or an unidentified EBV-specific antigen.

TCR spectratyping analysis of the CTL lines found that no single TCR sub-family was preferentially used, a result that was not unexpected when considering the polyclonal nature of the lines, the fact that they were stimulated weekly with LCLs expressing all the latent viral antigens, and that they contained a mixed population of CD8 and CD4 positive T-lymphocytes (CD4+ percentage range: <1-60%) (Haque *et al.*, 2007). However it is interesting that for some TCR sub-families (notably 2, 3 and 9) a polyclonal distribution was significantly more likely to induce a tumour response than a monoclonal distribution (Figure 3.14; Table 3.5). This was probably because of the wider spectrum of epitope specificities inherent in the polyclonal distribution, and,

indeed, this was the reason for not attempting to clone the banked CTL lines grown for *in vivo* use.

In summary, the results of this study suggest that, in conjunction with donor and recipient HLA allele matching, mapping CTL peptide epitope specificity prior to CTL infusions would enhance patient responses by identifying those epitopes restricted through the recipient HLA alleles. These improved CTL selection criteria may now allow other EBV-associated tumours with restricted EBV latent gene expression to be treated effectively with CTLs. However, since we found no relationship between CTL protein specificity, tumour EBV antigen expression, and outcome in Hodgkin's-type and Burkitt's-like PTLD cases, other, perhaps non-specific, tumour cell killing mechanisms may be responsible for tumour regression in these cases.

Chapter 4:
Results II

Analysis of cytokine gene and receptor gene polymorphisms in EBV-associated post transplant lymphoproliferative disease (PTLD) and acute infectious mononucleosis (IM)

4.1 Introduction

Cytokine networks interact in a dynamic way to regulate the immune response, thus it is not surprising that variations in cytokine levels have been correlated with susceptibility to disease and disease progression. Cytokines play an important role in IM with the symptoms observed in IM attributed to the release of IFN-γ and IL-2 from activated T-cells. However studies on IM cytokine levels have shown varied and conflicting results. Increased levels of IFN-γ are routinely observed in most studies with reports of continued raised IFN-γ detected 6 months after development of symptoms (Attarbaschi *et al.*, 2003). Mixed results have been obtained for IL-2, TNF-α, IL-4, IL-6 and IL-10 with some studies reporting increased levels while others report a decrease (Foss *et al.*, 1994;Biglino *et al.*, 1996;Attarbaschi *et al.*, 2003). All of these studies have been performed on small numbers of subjects and this may account for the variations seen. However a fundamental issue of such studies is whether variation in the level of cytokine is a primary cause for the disease or a secondary downstream effect of the immune regulation process. Investigation of cytokine gene polymorphisms is one approach to unravelling the issue.

Polymorphisms within cytokine genes and cytokine receptor genes may be responsible for the differing cytokine levels and the immune response observed in many viral infections. For example, TNF-α gene polymorphisms have been associated with the progression of Hepatitis B infection and the hemorrhagic manifestations observed in Dengue virus infection, whilst specific haplotypes of IL-2 are associated with susceptibility to infection with HIV (Xu, Lu, and Tan, 2005;Niro *et al.*, 2005;Fernandez-Mestre *et al.*, 2004;Shrestha *et al.*, 2006). Cytokine gene studies on primary EBV infection and the development of IM have also been reported. A base-exchange polymorphism located at position -1082 (G/A) of IL-10 has been implicated

in primary EBV infection. A reduction in the frequency of the high IL-10 producing G allele in EBV seropositive and IM subjects has been reported, suggesting that high IL-10 production protects against EBV infection and, conversely, that low IL-10 production predisposes to EBV infection (Helminen, Lahdenpohja, and Hurme, 1999;Wu *et al.*, 2002). Another report by Hurme et al (1998)(Hurme and Helminen, 1998) indicates that a base exchange polymorphism in the IL-1 gene complex may also protect against EBV infection.

Similarly, polymorphisms in cytokine genes have been implicated in the risk of developing several EBV-associated tumours. IL-18 variants have been associated with more aggressive forms of NPC whilst, in contrast to the protective role in IM, the high producer IL-10 haplotype has been associated with EBV-positive gastric cancer (Pratesi *et al.*, 2006;Wu *et al.*, 2002). The low producer IFN-γ genotype has been implicated in EBV reactivation following stem cell transplantation and with the development of PTLD following renal and liver transplantation (Bogunia-Kubik *et al.*, 2006;Lee *et al.*, 2006;VanBuskirk *et al.*, 2001). However, we recently investigated the low producer IFN-γ genotype in EBV-positive PTLD following SOT and found no association with the development of disease (Thomas *et al.*, 2005). The reasons for this difference are unknown but may in part be due to the difference in the study groups; one type of organ transplant versus mixed organ transplant types. Similar to the cytokine gene polymorphisms studies in IM, small study cohorts may also account for the contradictory reports observed; our study was one of the larger cohorts with 37 PTLD patients enrolled.

Many cytokine polymorphism studies have been criticised for use of small cohorts, unmatched controls and investigation of single polymorphic alleles (Ollier, 2004). Therefore, to resolve the differences observed in the literature with regard to cytokine

gene polymorphisms in IM and PTLD we analysed several cytokine gene and cytokine receptor gene polymorphisms (alleles, genotypes and haplotypes) in a large cohort of IM, EBV-positive PTLD patients and appropriate controls to determine correlations, if any, with the development of disease.

4.2 Cytokine Polymorphism PCR

The cytokine polymorphism PCR conditions and primer sequences for all reactions were kindly provided by Professor Ken Welsh and Ms Anna Lagan, National Heart and Lung Institute, London (see section 2.6.6). Based on the current literature and in conjunction with Professor Welsh and Ms Lagan a panel of cytokine and cytokine receptor genes was chosen for investigation (see section 2.6.6). The pro-inflammatory cytokines TNF, LTα (also known as TNF-β), IL-1α, IL-6 and IL-10 were selected. Experimental conditions were tested upon transfer to our laboratory using DNA from a healthy volunteer (Figure 4.1). All primer sequences produced adequate amplicons for determination of genotypes

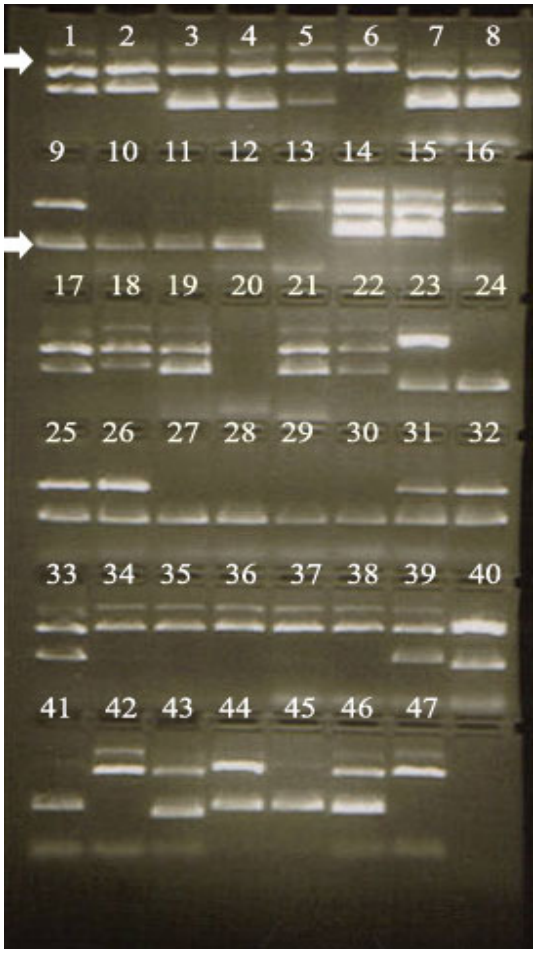


Figure 4.1: Cytokine Polymorphism PCR-SSP
A total of 47 reactions with sequence specific primers (SSP) were performed. Each reaction contained a set of control primers dependent on size of specific product; either a double band located above specific band (highlighted as in lanes 1) or as a single band below the specific band (as in lanes 9).
Specific primers were dispensed as follows: Lanes 1-2: TNFα-1031; lanes 3-4: TNFα-863; lanes 5-6: TNFα-857; lanes 7-8: IL-1RI-1339; lanes 9-12: TNFα haplotype; lanes 13-16: lymphotoxin-α haplotype; lanes17-18: TNFαRI-1663; lanes 19-20:TNFαRI-1668; lanes 21-22: TNFαRI-1690; lanes 23-24: TNFαRII-676; lanes 25-32: TNFαRI-promotor haplotype; lanes 33-36: TNFαRII-promotor haplotype; lanes 37-39: IL-10 haplotype; lanes 40-41: Il-6 intron 4; lanes 42-43: Il-6-174; lanes 44-45: Il-10R-241; lanes 46-47: IL-1α-889 (as detailed in section 2.6.6)

4.3 Analysis of cytokine gene and receptor gene polymorphisms in PTLD

Cytokine and cytokine receptor genotyping for TNF, LTα, IL-1α, IL-6 and IL-10 was performed on transplant patients with (n=45) or without (n=65) the development of EBV-associated post-transplant lymphoproliferative disease (designated PTLD and Control subject groups, respectively). Each polymorphism was assessed for Hardy-Weinberg equilibrium (HWE) in both study groups prior to statistical analysis. For

appropriate conclusions to be drawn from analysis polymorphisms are required to be in HWE; conclusions regarding association with disease cannot be drawn if not in HWE.

4.3.1 Increased frequency of the tumour necrosis factor -1031C and -863A alleles in PTLD subjects

A total of 5 polymorphisms within the TNF promoter region (nucleotide positions -1031, -863, -857, -307 and -237) were investigated. All five polymorphisms were in HWE for both subject groups. For the TNF promoter polymorphism at position -1031 we observed a significant increase in the frequency of the TNF -1031C allele in the PTLD subject group compared to the control group without PTLD (37% versus 19%; p=0.005); Table 4.1). A significant difference was also found in the genotype frequency of this polymorphism with homozygous CC and heterozygous TC frequencies increased while homozygous TT frequencies decreased (CC: 9% PTLD subjects versus 3% control subjects; TC: 38% versus 32%; TT: 44% versus 65%; p=0.01; Table 4.2). Likewise for position -863 we observed a significant increase in the TNF -863A allele frequency in the PTLD group compared to the control group (32% versus 11%; p=0.0001; Table 4.1). Comparison of the genotype frequencies within each group also revealed a significant difference for this polymorphism (CC: 44% PTLD subjects versus 82% control subjects; CA: 47% versus 15%; AA: 9% versus 3%; p=0.0003; Table 4.2). The remaining investigated TNF promoter polymorphisms, at nucleotide positions -857, -307 and -237, revealed no differences in allele or genotype frequency between transplant patients with PTLD and transplant patients without PTLD (Table 4.1).

Table 4.1: Allele frequencies of the TNF promoter polymorphisms in transplant patients with and without PTLD

Polymorphism	Allele	Transplant subjects without PTLD (n=65) *Frequency (%)*		Transplant subjects with PTLD (n=45) *Frequency (%)*	p-value[a]
-1031	T	81		63	0.005*
	C	19		37	
-863	C	89		68	0.0001*
	A	11		32	
-857	C	95		88	0.08
	T	5		12	
-307	G	74		80	1.0
	A	21		20	
-237	G	94		99	0.08
	A	6		1	

[a] Fisher's Exact 2-sided p-value
* Significant p-value, p<0.05

Table 4.2: Genotype frequencies of the TNF promoter polymorphisms in transplant patients with and without PTLD

Polymorphism	Genotype	Transplant subjects without PTLD (n=65) *Frequency (%)*		Transplant subjects with PTLD (n=45) *Frequency (%)*	p-value[a]
-1031	TT	65		44	0.01*
	TC	32		38	
	CC	3		9	
-863	CC	82		44	0.0003*
	CA	15		47	
	AA	3		9	
-857	CC	89		78	0.18
	CT	11		20	
	TT	0		20	
-307	GG	63		66	0.78
	GA	32		27	
	AA	5		7	
-237	GG	88		98	0.08
	GA	12		2	
	AA	0		0	

[a] Chi-square 3x2 contingency table
* Significant p-value, p<0.05

From the investigated TNF promoter polymorphisms it is possible to assign 1 of 6 TNF promoter haplotypes, detailed in Table 4.3 (Grutters *et al.*, 2002). Haplotype-1 (TCCGG) was under represented within the PTLD group compared to the control group (62% versus 83%, p=0.02; OR=2.6 (95%CI:1.2-7.29)) whereas haplotype-3 (CACGG) was over represented (50% versus 14%, p=0.0001; OR=0.16 (95%CI:0.06-0.4)) (Table 4.3). Haplotypes-2, -4, -5 and -6 were comparable between both groups (Table 4.3).

4.3.2 Altered frequency of the tumour necrosis factor receptor I and II promoter polymorphisms in PTLD subjects

Several polymorphisms within the TNF receptor II (exon-10 nucleotide position -1663, -1668, -1690 and exon 6, position -676) locus and TNF receptor I (nucleotide positions -201, -230, -845) and receptor II (nucleotide positions -839, -1135) promoter regions were investigated. All but one polymorphism was in HWE: TNF receptor I promoter nucleotide position -230 was not in HWE for the control subject group. Analysis of the TNF receptor I promoter at position -201 revealed a significant increase in the frequency of the -201T allele within the PTLD group compared to the control group (71% versus 53%, p=0.02) (Table 4.4). The TNF receptor II promoter-1135C allele was also significantly increased in the PTLD group compared to the control group (71% versus 57%, p=0.03) (Table 4.4). No differences were observed for TNF receptor II positions -1663, -1668, -1690, -676, TNF receptor I promoter positions -230, -845 and TNF receptor II promoter position -839 (Table 4.4).

Table 4.3: TNF promoter haplotypes in PTLD and control subjects

Haplotype	TNF Promoter Polymorphism					Transplant subjects without PTLD n=64 (freq %)	Transplant subjects with PTLD n=42 (freq %)	Odds ratio (95% CI)	p-value[a]
	-1031	-863	-857	-307	-237				
1	T	C	C	G	G	53 (83)	26 (62)	2.96 (1.2-7.29)	0.02*
2	T	C	C	A	G	23 (36)	13 (31)	1.25 (0.54-2.87)	0.67
3	C	A	C	G	G	9 (14)	21 (50)	0.16 (0.06-0.4)	0.0001*
4	T	C	T	G	G	6 (9)	8 (19)	0.43 (0.14-1.37)	0.23
5	C	C	C	G	A	9 (14)	1 (2)	6.70 (0.81-55.10)	0.08
6	C	C	C	G	G	2 (3)	3 (7)	0.41 (0.06-2.62)	0.38

Data are given as absolute numbers with percentages in parentheses
CI: confidence interval
[a] Fisher's Exact 2-sided p-value
* Significant p-value, $p<0.05$

Table 4.4: Allele frequencies of TNF receptor I and II polymorphisms

Polymorphism	Allele	Transplant subjects without PTLD *Frequency (%)*	Transplant subjects with PTLD *Frequency (%)*	p-value[a]
TNF receptor II				
Exon 10-1663	A	36	48	0.56
	G	48	52	
-1668	T	95	95	1
	G	5	5	
-1690	C	37	40	0.67
	T	63	60	
Exon 6-676	T	78	79	1
	G	22	21	
TNF receptor I promoter				
-201	G	55	71	0.02*
	T	45	29	
-230[†]	A	100	98	0.16
	G	0	2	
-845	A	62	49	0.07
	G	38	51	
TNF receptor II promoter				
-839	G	98	98	1
	A	2	2	
-1135	T	43	29	0.03*
	C	57	71	

[a] Fisher's Exact 2-sided p-value
* Significant p-value, $p<0.05$
[†] Not in Hardy-Weinberg equilibrium

Genotype frequencies of TNF receptor I and II polymorphisms were also compared between the PTLD and control groups. Analysis of the TNF receptor I promoter at position -201 revealed a significant increase in the frequency of the -201GG genotype and a decrease of the -201TT genotype in the PTLD group compared to the control group (GG: 47% versus 31%, TT: 4% versus 20%, p=0.03) (Table 4.5). Similar analysis of the TNF receptor II promoter position -1135 resulted in an observed decrease in the

frequency of the genotype -1135TT (4% versus 17%) and an increase in the frequency of genotype -1135CC (47% versus 31%) within the PTLD group compared to the control group, however, this did not reach significance (p=0.06) (Table 4.5). No genotypic differences were observed for TNF receptor II positions -1663, -1668, -1690, -676, TNF receptor I promoter positions -230, -845 and TNF receptor II promoter position -839 (Table 4.5).

Determination of the TNF receptor I promoter haplotypes from nucleotide positions -201, -230 and -845 results in 5 possible haplotypes, detailed in Table 4.6. An increase in the frequency of haplotype-1 (GAG) was observed in the PTLD group compared to the control group (80% versus 63%) and a decrease in the frequency of haplotype-3 (TAA: 53% versus 69%, Table 4.6). However these differences did not reach statistical significance (p=0.05 and p=0.11, respectively).

4.3.3 No difference in the frequency of the lymphotoxin-α polymorphisms in PTLD subjects

LTα polymorphisms at nucleotide positions -720, -365 and -249 were analysed in the PTLD and control groups. Within both groups each polymorphism was in HWE. For all 3 polymorphisms no difference in allele frequency (Table 4.7) or genotype frequency (Table 4.8) was observed. Haplotype determination, based on all 3 polymorphisms, gives 4 possible haplotypes: CCA, AGG, CGA and ACA. Further analysis of each haplotype was performed with no difference between the PTLD and control groups observed (CCA: 55% versus 71%, p=0.1; AGG: 52% versus 52%, p=1; CGA: 59% versus 49%, p=0.12; ACA: no sample in either group had the type 4 haplotype).

Table 4.5: Genotype frequencies of TNF receptor I and II polymorphisms

Polymorphism	Geneotype	Transplant subjects without PTLD *Frequency (%)*	Transplant subjects with PTLD *Frequency (%)*	p-value[a]
TNF receptor II				
Exon 10-1663	AA	18	20	0.39
	AG	35	55	
	GG	31	25	
-1668	TT	91	90	0.89
	TG	9	10	
	GG	0	0	
-1690	CC	11	16	0.25
	CT	52	48	
	TT	37	36	
Exon 6-676	TT	65	64	0.97
	TG	28	29	
	GG	8	7	
TNF receptor I promoter				
-201	GG	31	47	0.03*
	GT	48	49	
	TT	20	4	
-230	AA	100	96	0.08
	AG	0	4	
	GG	0	0	
-845	AA	38	20	0.12
	AG	48	58	
	GG	14	22	
TNF receptor II promoter				
-839	GG	95	96	0.96
	GA	5	4	
	AA	0	0	
-1135	TT	17	4	0.06
	TC	52	49	
	CC	31	47	

[a] Fisher's Exact 2-sided p-value
* Significant p-value, $p<0.05$

Table 4.6: TNF receptor I promoter haplotypes in PTLD

Haplotype	TNF receptor I promoter polymorphism			Transplant subjects without PTLD	Transplant subjects with PTLD	p-value[a]
	-201	-230	-845	*Frequency (%)*	*Frequency (%)*	
1	G	A	G	63	80	0.05
2	G	A	A	31	33	0.83
3	T	A	A	69	53	0.11
4	G	G	A	0	4	0.16
5	G	G	G	0	0	-

[a] Fisher's Exact 2-sided p-value

Table 4.7: Allele frequencies of IL-1, IL-6, IL-10 and LTα polymorphisms

Polymorphism	Allele	Transplant subjects without PTLD *Frequency (%)*	Transplant subjects with PTLD *Frequency (%)*	p-value[a]
IL-1α-889	C	66	67	
	T	34	33	0.88
IL-1 RI-1339	C	69	67	
	T	31	33	0.76
IL-6-174	C	32	40	
	G	68	60	0.25
IL-6 Intron 4	A	67	69	
	G	33	31	0.87
IL-10-1082	A	53	45	
	G	47	55	0.33
IL-10-819	C	71	80	
	T	29	20	0.15
IL-10-592	C	71	80	
	A	29	20	0.15
IL-10 RI-241	G	52	49	
	A	48	51	0.78
LTα-720	C	67	68	
	A	33	32	0.88
LTα-365	C	43	32	
	G	57	68	0.11
LTα-249	A	67	68	
	G	33	32	0.88

[a] Fisher's Exact 2-sided p-value

4.3.4 No difference in the frequency of interleukins -1, -6 and -10 polymorphisms in PTLD subjects

Polymorphisms within the IL-1α (nucleotide position -889), IL-1 receptor I (-1339), IL-6 (-174, intron 4-), IL-10 (-1082, -819, -592) and IL-10 receptor (-241) loci were analysed in both the PTLD and control groups. All polymorphisms were in HWE for both groups. No difference in allele frequency was observed for any of the polymorphisms studied between the PTLD and control groups (Table 4.7). Likewise no difference in genotype frequencies was seen between the two groups (Table 4.8).

IL-10 haplotypes were determined from nucleotide positions -1082, -819 and -592. No difference in the frequency of the 3 possible IL-10 haplotypes (ACC, GCC, ATA) was observed between the PTLD and control groups (ACC: 43% versus 40%, p=0.84; GCC: 75% versus 71%, p=0.66; ATA: 36% versus 51%, p=0.12).

Table 4.8: Genotype frequencies of IL-1, IL-6 and IL-10 polymorphisms

Polymorphism	Geneotype	Transplant subjects without PTLD *Frequency (%)*	Transplant subjects with PTLD *Frequency (%)*	p-value[a]
IL-1α-889	CC	42	44	0.88
	CT	49	47	
	CC	9	9	
IL-1 RI-1339	CC	45	40	0.89
	CT	49	53	
	TT	8	7	
IL-6-174	CC	9	18	0.39
	CG	45	44	
	GG	45	38	
IL-6 Intron 4	AA	43	45	0.97
	AG	49	48	
	GG	8	8	
IL-10-1082	AA	29	25	0.33
	AG	48	41	
	GG	23	34	
IL-10-819	CC	49	64	0.15
	CT	43	32	
	TT	8	5	
IL-10-592	CC	49	64	0.15
	CA	43	32	
	AA	8	5	
IL-10 RI-241	GG	32	22	0.28
	GA	38	53	
	AA	29	24	
LTα-720	CC	48	48	0.92
	CA	38	41	
	AA	14	11	
LTα-365	CC	15	9	0.19
	CG	55	45	
	GG	29	45	
LTα-249	AA	48	48	0.92
	AG	38	41	
	GG	14	11	

[a] Chi-square 3x2 contingency table

4.4 Analysis of cytokine gene and receptor gene polymorphisms in acute IM

Cytokine and cytokine receptor genotyping was performed on EBV seropositive individuals with or without symptoms of IM (IM and EBV seropositive subject groups, respectively) and EBV seronegative individuals (EBV seronegative group; see section 2.8 for cohort details). The study cohort included a total of 106 IM, 109 seronegative and 183 seropositive subjects (initial analysis was performed on two independent cohorts however these were combined to give greater statistical power). Each polymorphism was assessed for HWE in all 3 study groups prior to statistical analysis. Polymorphisms were analysed for differences in allele, genotype and haplotype frequency between the 3 subject groups.

4.4.1 Increased frequency of tumour necrosis factor receptor II -1663G allele in IM subjects

Polymorphisms within the TNF receptor II (exon-10 nucleotide position -1663, -1668, -1690) and exon 6-position 676) locus and the TNF receptor I (nucleotide positions -201, -230, -845) and receptor II (nucleotide positions -839, -1135) promoter regions were investigated. Polymorphisms at positions -1663, -1668, -1690, -201, -839 and -1135 were in HWE across all 3 subject groups. Polymorphisms at positions -676 and -230 were not in HWE for the seronegative group, and the polymorphism at position -845 was not in HWE for the seropositive group, therefore no conclusions can be drawn from the statistical analysis. For the TNF receptor II region we observed a significant increase in the frequency of the G allele at position -1663 within the IM group compared to the seronegative group (56% versus 42%, p=0.005; Table 4.9). A significant increase in the frequency of the G allele was also observed for the seropositive group when compared to the seronegative group (51% versus 42%, p=0.03, Table 4.9). Analysis of the

genotype across the subject groups revealed a significant increase in the GG genotype within the IM group compared to seronegatives (31% versus 16%, p=0.01; Table 4.10). Likewise the GG genotype was increased in the seropositive group compared to the seronegative group reaching borderline significance (29% versus 16%, p=0.05; Table 4.10).

Within the IM group we observed a significant increase in the frequency of the nucleotide position -1135 C allele compared to the seropositive group (63% versus 53%, p=0.02; Table 4.9). This increase was also apparent for the CC genotype reaching borderline significance (37% versus 25%, p=0.05; Table 4.10). At nucleotide position -1668 we observed a significant increase in the heterozygous genotype TG within the seropositive group when compared to the seronegative group (20% versus 10%, p=0.03; Table 4.10). This difference was not replicated when comparing allele frequency.

For nucleotide position -845 of the TNF receptor I promoter region we observed a significant decrease in the frequency of the GG genotype within the seropositive group compared to the seronegative group (9% versus 18%, p=0.03; Table 4.10) however this polymorphism was not in complete HWE across all 3 subject groups. No difference in allele or genotype frequencies was observed for polymorphisms at nucleotide positions -1690, -676, -201, -230 or -839 (Table 4.9 and 4.10). Likewise, analysis of the TNF receptor I promoter haplotypes revealed no significant differences in frequency across the subjects groups (Table 4.11)

Table 4.9: Allele frequencies of TNF receptor I and II polymorphisms in IM, seropositive and seronegative subjects

Polymorphism	Allele	IM *Frequency (%)*	EBV seropositive *Frequency (%)*	p-value[a]	EBV seronegative *Frequency (%)*	p-value[a]	p-value[a‡]
TNF receptor II							
Exon 10-1663	A	44	49	0.33	58	0.005*	0.03*
	G	56	51		42		
-1668	T	91	90	0.76	94	0.26	0.11
	G	9	10		8		
-1690	C	39	38	1	40	0.76	0.72
	T	62	62		60		
Exon 6-676[†]	T	74	71	0.62	74	1	0.56
	G	26	29		26		
TNF receptor I promoter							
-201	G	61	53	0.08	58	0.69	0.25
	T	39	47		42		
-230[†]	A	100	99	1	100	1	0.3
	G	0	1		0		
-845[†]	A	60	64	0.32	61	0.84	0.53
	G	40	36		39		
TNF receptor II promoter							
-839	G	97	98	0.37	99	0.5	1
	A	3	2		1		
-1135	T	38	47	0.02*	44	0.19	0.53
	C	63	53		56		

[a] Fisher's Exact 2-sided p-value
* Significant p-value, $p<0.05$
[†] Not in Hardy Weinberg equilibrium
[‡] p-value for comparison of seropositive and seronegative subjects groups

Table 4.10: Genotype frequencies of TNF receptor I and II polymorphisms

Polymorphism	Allele	IM *Freq (%)*	EBV seropositive *Freq (%)*	p-value[a]	EBV seronegative *Freq (%)*	p-value[a]	p-value[a‡]
TNF receptor II							
Exon 10-1663	AA	20	26	0.48	32	0.01*	0.05*
	AG	49	45		52		
	GG	31	29		16		
-1668	TT	83	80	0.62	89	0.16	0.03*
	TG	17	20		10		
	GG	0	0		1		
-1690	CC	17	18	0.96	20	0.86	0.89
	CT	43	41		41		
	TT	40	41		40		
Exon 6-676[†]	TT	55	52	0.83	59	0.29	0.21
	TG	37	38		28		
	GG	8	10		12		
TNF receptor I promoter							
-201	GG	35	26	0.18	35	0.6	0.3
	GT	52	54		47		
	TT	13	20		18		
-230[†]	AA	99	98	0.62	100	0.33	0.19
	AG	1	2		0		
	GG	0	0		0		
-845[†]	AA	32	37	0.56	41	0.08	0.03*
	AG	56	53		42		
	GG	12	9		18		
TNF receptor II promoter							
-839	GG	94	97	0.34	97	0.33	0.34
	GA	6	3		3		
	AA	0	0		0		
-1135	TT	12	20	0.05*	20	0.25	0.51
	TC	52	54		49		
	CC	37	25		32		

[a] Fisher's Exact 2-sided p-value
* Significant p-value, $p<0.05$
[†] Not in Hardy Weinberg equilibrium
[‡] p-value for comparison of seropositive and seronegative subjects groups

Table 4.11: TNF receptor I promoter haplotypes in IM, EBV seropositive and seronegative subjects

Haplotype	IM *Freq (%)*	EBV seropositive *Freq (%)*	p-value[a]	EBV seronegative *Freq (%)*	p-value[a]	p-value[a‡]
GAG	68	63	0.37	59	0.25	0.61
GAA	36	29	0.24	33	0.77	0.5
TAA	65	74	0.14	65	1.11	0.14
GGA	1	1	1	0	1	0.53
GGG	0	1	1	0		1

[a] Fisher's Exact 2-sided p-value

Investigation of the TNF promoter polymorphisms at nucleotide positions -1031, -865, -859, -307, -237 and the corresponding TNF haplotypes (1-6) revealed no significant differences between the IM and seropositive, the IM and seronegative or the seropositive and seronegative groups (Appendix III).

4.4.2 Increased frequency of lymphotoxin-α CCA haplotype in IM

LTα polymorphisms at nucleotide positions -720, -365 and -249 were assessed to be in Hardy-Weinberg equilibrium. Statistical analysis of the 3 subject groups revealed a decrease in the frequency of the LTα-365G allele in IM subjects compared to the seronegative group (57% versus 70%, p=0.01; Table 4.12). No difference in allele frequency was observed between the IM and seropositive groups or the seropositive and seronegative groups. The homozygous GG genotype was also decreased in IM compared to the seronegative group (34% versus 53%, p=0.03, Table 4.12). Analysis of the corresponding LTα haplotypes CCA, AGG, CGA and ACA revealed an increase in the frequency of the CCA haplotype in IM compared to the EBV seronegatives (66% versus 47%, p=0.01; Table 4.12). Again no further difference in haplotype frequency was observed between the IM and seropositive groups or the seropositive and seronegative groups.

Table 4.12: Analysis of Lymphotoxin-α polymorphisms in IM, seropositive and seronegative subjects

Polymorphism		IM *Frequency (%)*	EBV seropositive *Frequency (%)*	p-value[a]	EBV seronegative *Frequency (%)*	p-value[a]	p-value[a‡]
Allele							
-720	C	67	65	0.64	60	0.12	0.23
	A	33	35		40		
-365	C	43	37	0.21	30	0.01*	0.09
	G	57	63		70		
-249	A	67	65	0.54	60	0.12	0.23
	G	33	35		40		
Genotype							
-720	CC	42	44		36		
	CA	50	43	0.28	46	0.11	0.45
	AA	8	13		17		
-365	CC	20	16		13		
	CG	48	42	0.45	34	0.03*	0.2
	GG	34	42		53		
-249	AA	42	43		36		
	AG	50	44	0.28	46	0.11	0.45
	GG	8	13		17		
Haplotype							
1	CCA	66	58	0.25	47	0.01*	0.08
2	AGG	58	57	0.9	64	0.47	0.31
3	CGA	41	49	0.21	51	0.2	0.9
4	ACA	0	0		0		

[a] Fisher's Exact 2-sided p-value for allele and haplotype analysis; Chi Square for genotype analysis
* Significant p-value, $p<0.05$
[‡] p-value for comparison of seropositve and seronegative subjects groups

4.4.3 No difference in the frequency of interleukins -1, -6 and -10 polymorphisms in IM subjects

Polymorphisms within the IL-1α (nucleotide position -889), IL-1 receptor I (-1339), IL6 (-174, intron 4-), IL-10 (-1082, -819, -592) and IL10 receptor (-241) loci were analysed in IM, seropositive and seronegative subjects. Polymorphisms in IL-6 (-174), IL-1α (-889) and IL-10 (-1082) were assessed to be in HWE for all 3 subjects groups. The IL-6 (intron 4) and IL-1 receptor I (-1339) polymorphisms were not in HWE for the IM group whilst the IL-10 receptor (-241) and IL-10 (-819, -592) polymorphisms were assessed not to be in HWE for the seropositive group. An increase in the IL-10 -1082G allele was observed in seronegatives compared to the seropositive group (55% versus 45%, p=0.02; Appendix III) however no corresponding difference was noted for the genotype or in comparison to the IM group (IL-10-819 and -592 were not in HWE, however no differences were seen in these alleles). Likewise no frequency differences were noted in IL-10 haplotypes across all 3 subject groups (Appendix III).

Investigation of the IL-6 intron 4 genotype revealed a significant decrease in the frequency of the GG genotype in IM cases compared to seronegatives (8% versus 23%, p=0.006; Appendix III). However as not all subjects groups were in HWE this must be excluded from the disease association analysis. No further frequency differences were observed between the groups for the remaining polymorphisms IL-1α (-889), IL-1 receptor 1(-1339), IL-6 (-174), IL-10 receptor (-241) and IL-10 (-819, -592). The analysis of allele, genotype and haplotype frequency is summarised in Appendix III.

4.5 Disscussion

In this study we have assessed polymorphisms within the TNF, LTα, IL-1α, IL-6 and IL-10 loci and their corresponding receptor loci for evidence of an association with the

development of EBV-associated PTLD and acute IM. An increase in the rarer alleles of the TNF promoter polymorphisms at nucleotide positions -1031(C allele) and -863(A allele) were found to be significantly increased in EBV-positive PTLD cases compared to non-PTLD transplant controls. Likewise genotypes containing the rare allele were also significantly increased in the EBV-positive PTLD cases compared to the control group. Furthermore, these polymorphic differences within the TNF promoter region resulted in a significant increase of TNF haplotype-3 (CACGG) and a decrease of TNF haplotype-1 (TCCGG) in EBV-positive PTLD subjects compared to controls suggesting an important role for these haplotypes in determining susceptibility to EBV-positive lymphoma following transplantation.

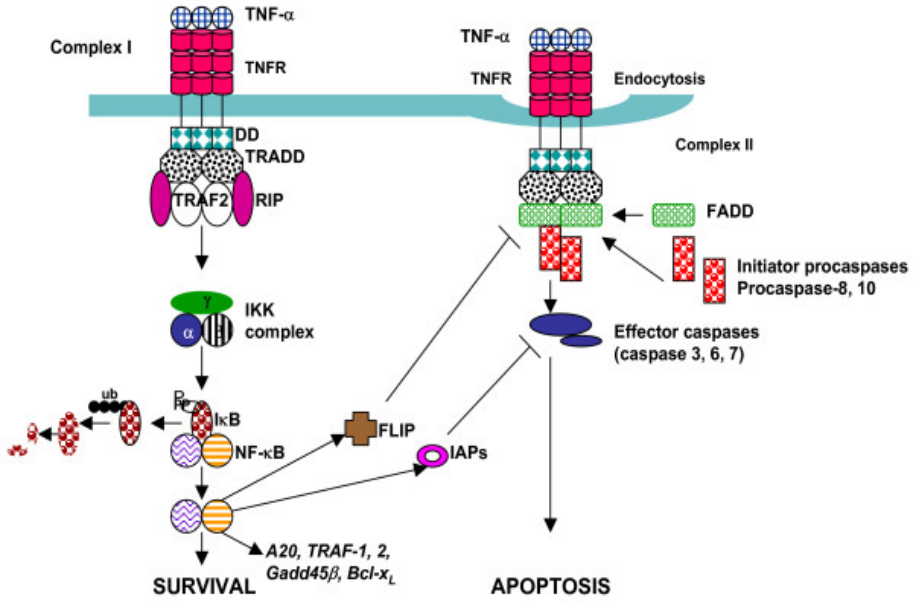
Similarly a significant increase in the frequency of the TNF RII-1663G allele and corresponding GG genotype in acute IM compared to seronegative subjects would suggest that possession of the G allele may be a risk factor for the development of symptoms following primary EBV infection. However a similar significant increase in the frequency of -1663G allele in EBV seropositive over seronegative individuals would perhaps suggest that the -1663G allele is a risk factor for EBV seropositivity in general or that the -1663A allele confers a degree of protection from primary EBV infection. Also the LTα-365G allele and GG genotype frequencies were significantly reduced in IM compared to the seronegative group resulting in an increased frequency of the LTα CCA haplotype. A similar reduction in comparison to seropositives was not observed, nor was a frequency reduction in the seropositive group compared to seronegative subjects. Therefore we are unable to conclude the protective role of the G allele or the susceptibility of the alternate C allele in primary EBV infection. Interestingly the frequency of the TNFRII promoter -1135C allele was significantly increased in both EBV-positive PTLD and acute IM (compared to seropositives). A corresponding

increase in the frequency of the homozygous CC genotype was also observed for the PTLD and IM groups (p=0.06 and p=0.05) respectively confirming the importance of the TNF family of cytokines and their receptors in the development of EBV-positive PTLD and acute IM.

TNF and LTα are both cytokines of the TNF family that function as potent mediators of immune regulation and inflammation. The TNF and LTα genes are located adjacent to each other in the HLA class III region on chromosome 6p21.3 and are closely linked to the polymorphic HLA-B and -DR regions (Nedwin *et al.*, 1985). Both cytokines have similar biological activities, share approximately 50% sequence homology, and bind to the same group of cellular receptors; TNFRI, which is widespread in many cells types and activated by soluble ligand, and the TNFRII that is primarily expressed on haemopoietic cells (Locksley, Killeen, and Lenardo, 2001;Chan, Siegel, and Lenardo, 2000). Both receptors are also shed and act as competitive soluble TNF binding proteins consequently affecting the levels of TNF production. In the case of TNF, ligand-receptor binding leads to recruitment of intracellular adaptor proteins that activate several signal transduction pathways including the activation of the transcription factor NF-κB and the apoptotic pathway through capsase 8 (Balkwill, 2006) (Figure 4.2). Many pathological situations are characterised by the balance between such survival and apoptotic signals and therefore gene polymorphisms that alter this signalling process either through the ligand or the receptor are important.

The function of the TNF-863A variant highlighted in our PTLD cohort has been widely investigated. The nucleotide change from C to A has been shown to have a clear effect on the binding of the NF-κB transcription complex to its DNA binding domain. In

Figure 4.2: TNF-TNF receptor signalling pathways



particular the affinity of the NFκB p50-p50 heterodimer, which acts as a transcriptional repressor when bound to the TNF promoter, is significantly decreased for the -863A variants (Udalova *et al.*, 2000). Decreased binding is thought to result in inadequate down-regulation of TNF gene expression and therefore increased TNF production. As yet, there is no comparable molecular data for the TNF-1031C allele (however there is some degree of linkage between the -1031 and -863 alleles) or for the TNFRII-1663G and promoter -1135C alleles. Interestingly, the presence of the TNF-863A allele has also been positively associated with susceptibility to another group of B-cell malignancies, the Non-Hodgkin's lymphomas (Spink *et al.*, 2006). Possession of specific alleles that act to increase TNF expression may therefore be central to the mechanisms of pathogenesis and susceptibility to lymphoid disease. Indeed TNF based mechanisms such as direct DNA damage, anti-apoptotic activity and induction of cytokines, have been implicated in several cancers (reviewed in (Balkwill, 2006).

It is also noteworthy that we were unable to replicate the association of IL-10 and susceptibility to primary EBV infection obtained by Helminen et al (1999). This is perhaps due to the differences in study size. The Helminen report (1999) recruited a small number of subjects (36 IM, 52 seropositive and 20 seronegative cases) and investigated only the -1082 polymorphism rather that the complete haplotype. We investigated 3 polymorphisms and the associated haplotypes in a larger cohort of subjects (106 IM, 183 seropositive, 109 seronegative). Testing of multiple SNPs within a gene and analysis based upon the SNP haplotype is important. During meiosis alleles on the same small block of the chromosome segment tend to be transmitted as a block or haplotype, therefore, any given allele may simply be a marker for another. Likewise, a SNP in one individual may be part of a haplotype with functional downstream effects whilst the same SNP in a second individual may form a haplotype with no functional effect.

In summary we have shown an association between variant alleles of the TNF promoter and the subsequent TNF promoter haplotype 3 (CACGG) with the development of EBV-positive PTLD. Likewise we have shown that the TNFRII -1663G allele may be a risk factor for susceptibility to EBV infection whilst the -1663A allele confers some protection. However, there remains a group of both PTLD and IM subjects who do not carry these alleles, genotypes or haplotypes, perhaps indicating that these polymorphisms are not completely functional and that other, as yet unidentified variants, are in linkage disequlibrium with these loci. These data also require confirmation in a second, similar sized cohort to be certain of an association and further analysis of soluble TNF and LTα levels may offer some information on the functional activity of the polymorphic alleles. Nevertheless, the genotypic evidence for the

involvement of TNF in both PTLD and IM is promising and may provide further information in identifying those most at risk particularly in the post-transplant situation.

Chapter 5:
Results III

Analysis of HLA microsatellite and single nucleotide polymorphisms in acute infectious mononucleosis (IM) and post transplant lymphoproliferative disease (PTLD)

5.1 Introduction

Persistent EBV infection is aetiologically linked to a number of lymphoid and epithelial tumours including Hodgkin's lymphoma (HL) and nasopharyngeal carcinoma (NPC). HL is one of the commonest tumours in young adults in the West where its incidence is increasing (Swerdlow, 2003). Approximately 1500 new cases occur each year in the UK, and HL now accounts for one in eight of all lymphomas diagnosed. In approximately 25-50% of Western HL cases the malignant Reed-Sternberg (RS) cells carry the EBV genome (Andersson, 2006) and express viral antigens. The aetiological link between EBV and HL is further substantiated by the finding that a previous history of IM is a significant risk factor for EBV-associated HL with around one in 1000 cases of IM later developing HL (Jarrett *et al.*, 2005;Hjalgrim *et al.*, 2003). Further studies by Hjalgrim et al show that the risk of developing EBV-positive HL varies with time since IM with a median of 2.9 years ((Hjalgrim *et al.*, 2007).

The factors which determine the development of IM as opposed to silent primary EBV infection are unknown. Genetic differences in the HLA locus are of interest since HLA class 1 alleles may affect the efficiency of viral peptide presentation to T-cells, with resultant differences in the effectiveness of the immune response. Clearance of Hepatitis C virus, for example, has been associated with HLA-A*03 and B*27 alleles whilst CTL expressing different, but closely related HLA molecules, have shown significant functional differences when targeting identical HIV epitopes (McKiernan *et al.*, 2004;Frahm *et al.*, 2005;Leslie *et al.*, 2006). Recent studies have highlighted HLA class I associations with both EBV-positive HL and NPC. Diepstra et al identified alleles of two microsatellite markers (D6S265: 126bp allele and D6S510: 284bp allele) which are significantly associated with EBV-positive HL and a class III microsatellite D6S273 which correlated with EBV negative HL (Diepstra *et al.*, 2005). Further work

by the same group found Single-Nucleotide Polymorphisms (SNPs) within an 80kb region, located near the HLA-A and HcG9 genes, that are also associated with EBV-positive HL (Niens *et al.*, 2006), (Figure 5.1). Interestingly, the region between the D6S510 and D6S211 markers of the HLA-A locus is also associated with the development of NPC (Cheng-chan Lu *et al.*, 2005), (Figure 5.1). Microsatellite markers show the highest degree of linkage disequilibrium with the HLA locus that is located nearest in the genome. In haplotype prediction studies both the D6S510 and D6S265 microsatellite markers have been shown to have strong linkage disequilibrium with the HLA-A locus, with D6S510 associated with HLA*A1 subtype and D6S265 with HLA-A*03 subtype (Malkki *et al.*, 2005). Furthermore, the HLA-A1 subtype has recently been associated with an increased risk of developing EBV-positive HL whilst the HLA*A2 subtype appears to confer a reduced risk (Niens *et al.*, 2007).

Due to the well substantiated association between IM and EBV-positive HL, we speculated that the development of IM during primary EBV infection may also be associated with HLA class I polymorphisms. Likewise, since similar HLA class I polymorphisms have been associated with 2 EBV-associated tumours (HL and NPC) we speculated that these polymorphic variations may also have a role to play in the development of EBV-positive PTLD. We therefore analysed two microsatellite markers from the HLA class I region (D6S510 and D6S265) previously associated with EBV-positive HL, and two SNPs (rs2530388 and rs6457110) situated within the 80kb region of interest, to identify links between IM or EBV-positive PTLD and allele frequency. One further marker from the class III region (D6S273), associated with EBV negative HL, was analysed as a control.

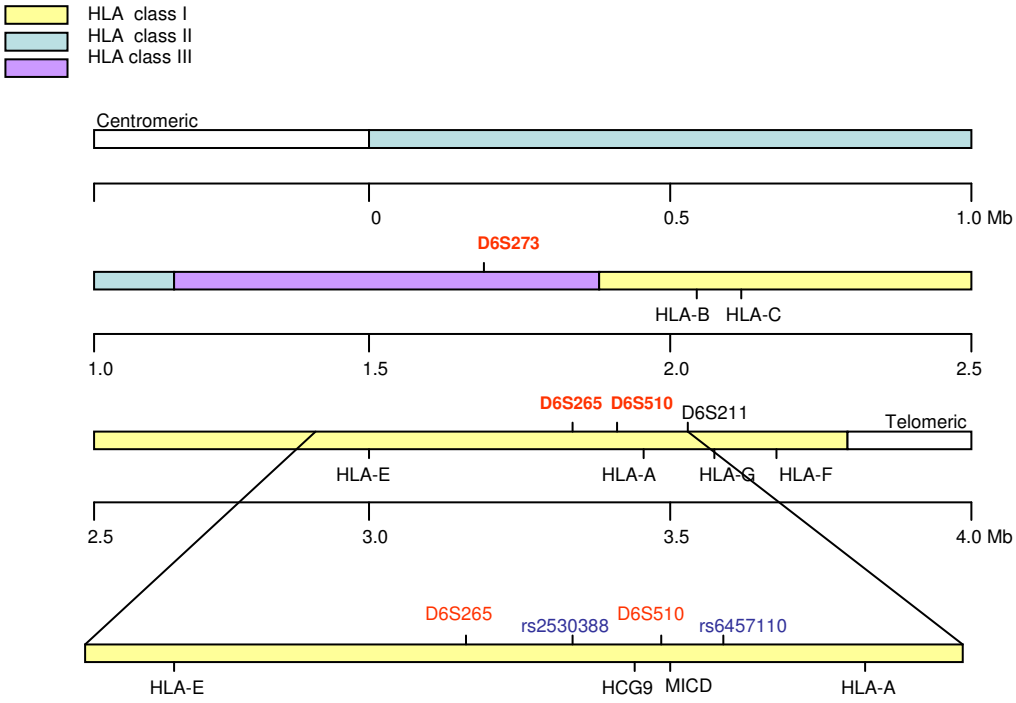


Figure 5.1: Map of markers and genes in the HLA region
Major genes are indicated in black below the bar. Microsatellite markers are depicted above the bar (markers studied are highlighted in red). SNP's studied are indicated in blue above the bar. [Adapted from 'Association with HLA class I in Epstein-Barr-positive and with HLA class III in Epstein-Barr negative Hodgkin's lymphoma', Diepstra *et al* (2005), The Lancet. By kind permission of Elsevier Health Sciences]

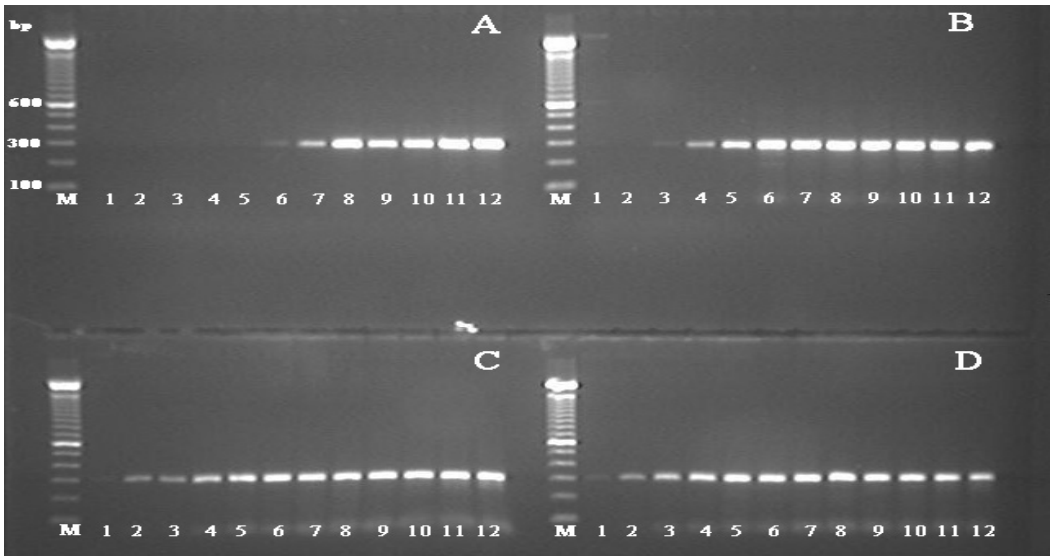
5.2 Optimisation of HLA-PCR

PCR conditions (magnesium concentration, primer concentration, annealing temperature and DNA concentration) and dilutions for analysis by Genemapper software were optimised for each microsatellite marker: D6S265, D6S510 and D6S273.

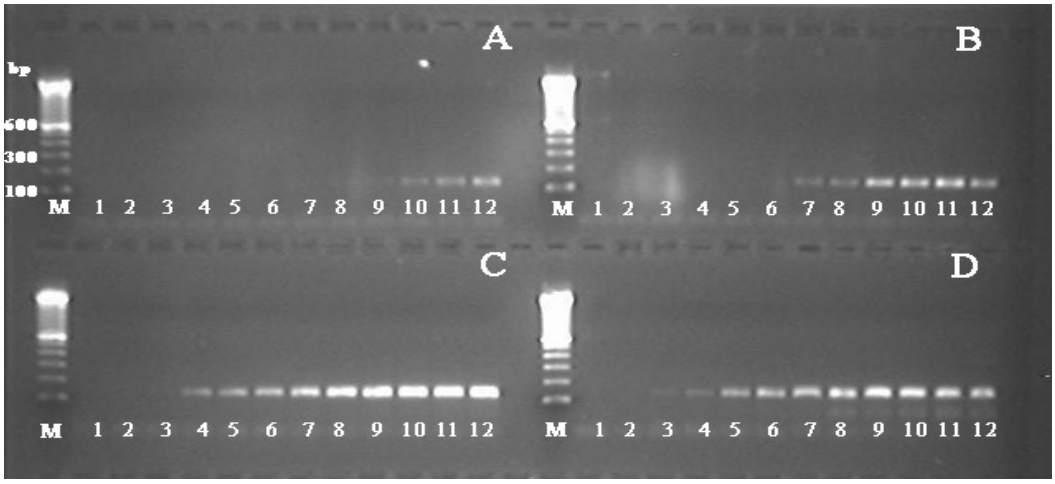
5.2.1 Optimisation of magnesium concentration and annealing temperature

A chequerboard of magnesium concentration (range: 1mM, 1.5mM, 2mM, 2.5mM) and annealing temperature (range 53˚C-64˚C) was performed for each marker. A primer concentration of 0.5μM for both the forward and reverse primers and 50ng of DNA, extracted from the peripheral blood of a healthy volunteer, were chosen for the initial experiment. Cycling conditions were selected based upon the size of the amplified products: initial denaturation at 94˚C for 5mins followed by 35 cycles of 94˚C for 30secs, 53-64˚C for 30secs, 72˚C for 45secs and a final extension at 72˚C for 5mins. For all 3 markers a magnesium concentration of between 2 and 2.5mM was optimal (Figure 5.2). A magnesium concentration of 2.5mM was selected for all further experiments. Amplified products declined with increasing temperature range for all 3 markers (Figure 5.2). For markers D6S265 and D6S273 a temperature of 55˚C was chosen and for marker D6S510 a temperature of 62˚C was selected.

Panel-1



Panel-2



Panel-3

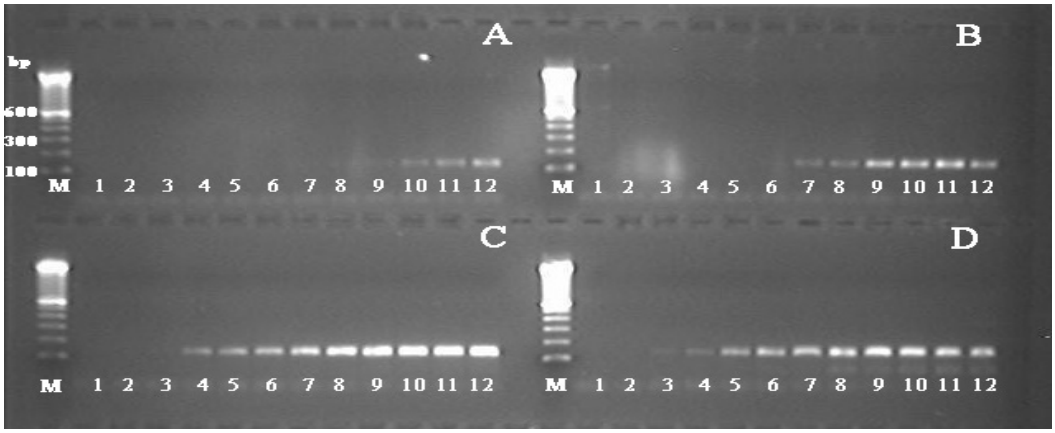


Figure 5.2: Optimisation of magnesium concentration and annealing temperature for markers D6S510, D6S265 and D6S273.

Panel-1: marker D6S510; panel-2: marker D6S265; panel-3: marker D6S273. Magnesium concentrations of 1mM (A), 1.5mM (B), 2mM (C) and 2.5mM (D) are shown. Lanes 1-12 represent an annealing temperature range of 64˚C-53˚C.
M: 100bp marker.

5.2.2 Optimisation of primer and DNA concentrations

Further optimisation was performed for all 3 markers to reduce, if possible, the amount of primer and DNA required. The primer concentration was reduced from 0.5µM to 0.25µM and the DNA from 50ng to 25ng. The selected magnesium concentration, annealing temperature and cycling conditions from the previous optimisation experiment were used. For all 3 markers the reduced primer and DNA concentrations were adequate and therefore selected for all future experiments (Figure 5.3).

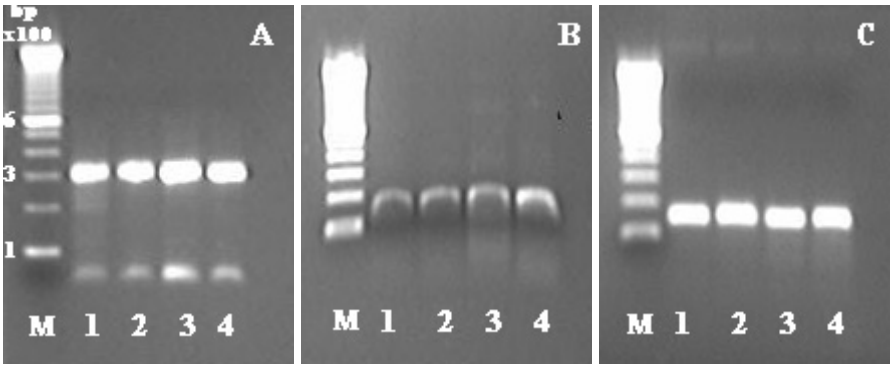


Figure 5.3: Optimisation of primer and DNA concentration for markers D6S510, D6S265 and D6S273
A: Marker D6S510, B: marker D6S265, C; marker D6S273. Lanes 1 and 2: 0.25µM forward and reverse primer, lanes 3 and 4: 0.5µM forward and reverse primer. Lanes 1 and 3: 50ng DNA, lanes 2 and 4: 25ng DNA, M: 100bp marker.

5.2.3 Optimisation of PCR product dilution for Genemapper analysis

Analysis of PCR products on the ABI 3730 automated sequencer and Genemapper software is often quite sensitive and it is therefore recommended to try various dilutions of the PCR product to obtain a peak height similar to that of the size standards used. We opted to use the Genescan 500LIZ size standard (ABI): 2µl diluted in 1ml of Hi-Di formamide (ABI) as per manufactures instructions. PCR products were diluted in nuclease free water to give 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280 dilutions. One microlitre of each dilution was then further diluted in 10µl of Hi-Di/size standard mix prior to running on an automated sequencer. Figure 5.4 shows the

spectratype obtained for the D6S265 marker. Dilutions of 1/1280 and 1/640 had the lowest relative fluorescent intensities whereas dilution 1/10 had the highest relative fluorescent intensity. A dilution of 1/50 was selected as optimal, giving a relative fluorescent intensity similar to the size standard. Similar spectratypes were obtained for markers D6S510 and D6S273, therefore, a dilution of 1/50 was chosen for all three markers (Table 5.1).

Table 5.1: Optimisation of dilution factor for markers D6S510, D6S265 and D6S273

	RELATIVE FLUORESCENT INTENSITY OF ALLELE					
	D6S510		**D6S265**		**D6S273**	
Dilution	**294bp**	**298bp**	**122bp**	**132bp**	**138bp**	**140bp**
1/10	26279	20262	16195	11020	21790	12570
1/20	13945	10801	1768	1208	14576	8373
1/40	9277	7403	5197	3589	8567	4672
1/80	4785	3773	4892	3330	5533	3170
1/160	3477	2743	3038	2049	4378	2485
1/320	2024	1595	1775	1232	2575	1465
1/640	59	66	1103	728	727	415
1/1280	352	283	601	390	958	502

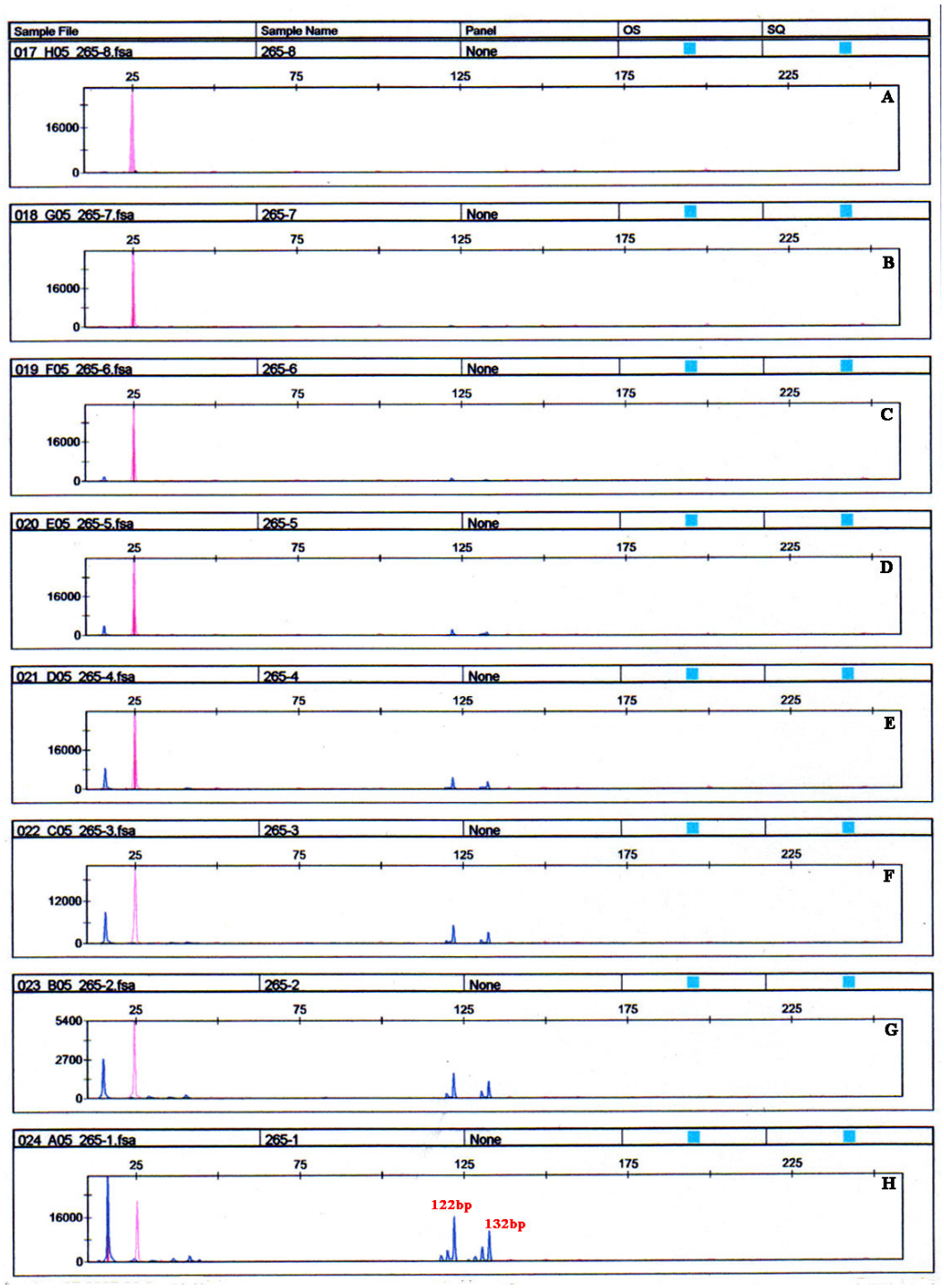


Figure 5.4: Spectratype of marker D6S265 dilution series
Panel A: 1/1280; panel B: 1/640; panel C: 1/320; panel D: 1/160; panel E: 1/80; panel F: 1/40; panel G: 1/20; panel H: 1/10. Allele base pair size is indicated (122 and 132bp).

5.3 Analysis of HLA class I microsatellite markers D6S510 and D6S265 in Infectious Mononucleosis

HLA genotyping was performed on EBV seropositive (n=146), EBV seronegative (n=50) and IM (n=98) subject groups (recruitment details are outlined in section 2.7.3). We identified 10 alleles (range 284-306bp) for the D6S510 marker, 13 alleles (range 122-142bp) for the D6S265 marker and 9 alleles (range 128-144bp) for the D6S273 marker (Table 5.2). All three markers were in Hardy Weinberg equilibrium for each group. Among EBV-positive subjects, a significant difference between those with symptoms of IM and those without symptoms was observed for allele 1 of marker D6S510 (41.8% and 30.5% respectively, p=0.01) and allele 3 of D6S265 (45.9% and 35.2%, p=0.02) (Table 5.2). Significant differences were also found between EBV seronegative subjects and IM patients for the same two alleles (allele 1: 28.0% versus 41.8%, P=0.02; allele 3: 31.6% versus 45.9%, P=0.02) as well as for allele 8 of D6S510 (24.0% versus 13.3%, P=0.02) (Table 5.2).

Comparison of IM and EBV seropositive groups revealed that individuals homozygous for allele 1 of the D6S510 marker and allele 3 of the D6S265 marker had odds ratios for development of IM of 2.7 (95% CI: 1.1-8.5) and 2.7 (95% CI: 1.1-6.4) respectively. The corresponding odds ratios for heterozygotes were 1.6 (95% CI: 0.9-2.9) and 1.4 (95% CI: 0.7-2.5) (Table 5.3).

Table 5.2: Allele frequency of microsatellite markers D6S510, D6S265 and D6S273 in EBV seropositive, seronegative and IM subjects

Locus	Allele	Base Pair Size	EBV-positive subjects with symptoms of IM (n= 196 alleles) Freq (%)		EBV-positive subjects without symptoms of IM (n=292 alleles) Freq (%)	p-value[A]		EBV-negative subjects (n=100 alleles) Freq (%)	p-value[A]
D6S510	**1**	**284**	**41.8**		**30.5**	**0.01***		**28.0**	**0.02***
D6S510	2	290	0.0		0.0	1.00		1.0	0.33
D6S510	3	292	10.2		13.7	0.26		11.0	0.84
D6S510	4	294	9.2		7.9	0.62		7.0	0.66
D6S510	5	296	6.6		3.8	0.20		2.0	0.10
D6S510	6	298	17.9		24.3	0.09		26.0	0.13
D6S510	7	300	0.5		1.7	0.41		1.0	1.00
D6S510	**8**	**302**	**13.3**		**17.5**	**0.25**		**24.0**	**0.02***
D6S510	9	304	0.0		0.3	1.00		0.0	1.00
D6S510	10	306	0.5		0.3	1.00		0.0	1.00
D6S265	1	122	10.2		13.1	0.40		11.2	0.84
D6S265	2	124	1.5		0.3	0.31		0.0	0.55
D6S265	**3**	**126**	**45.9**		**35.2**	**0.02***		**31.6**	**0.02***

Continued on next page…..

D6S265	4	128	9.2		10.1	0.88		11.2	0.68
D6S265	5	130	17.9		23.2	0.18		25.5	0.13
D6S265	6	132	13.8		13.8	1.00		19.4	0.24
D6S265	7	134	0.5		1.7	0.41		1.0	1.00
D6S265	8	136	0.0		0.3	1.00		0.0	1.00
D6S265	9	138	0.5		0.7	1.00		0.0	1.00
D6S265	10	140	0.0		0.3	1.00		0.0	1.00
D6S265	11	142	0.5		0.0	0.40		0.0	1.00
D6S265	12	144	0.0		0.7	0.52		0.0	1.00
D6S265	13	146	0.0		0.7	0.52		0.0	1.00
D6S273	1	128	3.6		4.7	0.65		4.0	1.00
D6S273	2	130	5.7		5.4	1.00		6.0	1.00
D6S273	3	132	12.4		12.4	1.00		13.0	0.86
D6S273	4	134	27.3		25.2	0.60		23.0	0.48
D6S273	5	136	33.0		34.6	0.77		33.0	1.00
D6S273	6	138	1.6		4.4	0.12		2.0	1.00
D6S273	7	140	16.0		11.1	0.13		19.0	0.52
D6S273	8	142	0.5		1.7	0.41		0.0	1.00
D6S273	9	144	0.0		0.7	0.52		0.0	1.00

[A] Fisher's-exact 2-sided p-value; * significant value <0.05. Alleles highlighted in bold were analysed further.

Odds ratios were also different upon comparison of IM and EBV seronegative groups for allele 3 (D6S265 marker) with odds ratios of 3.3 (95% CI: 1.0-4.1) for homozygotes and 1.8 (95% CI: 0.8-4.1) for heterozygotes (Table 5.3). The increased odds ratio in IM subjects homozygous for these alleles indicates a co-dominant effect. No significant frequency differences were observed between IM patients and either of the other two groups (EBV seropositive without IM symptoms, or EBV seronegative) for alleles of the control marker D6S273.

Table 5.3: Genotype frequency and odds ratios of D6S510 allele 1 and allele 8, and D6S265 allele 3 in EBV seropositive, seronegative and IM subjects

Locus and genotype	EBV-positive subjects with symptoms of IM (n=98)	EBV-positive subjects without symptoms of IM (n=149)	Odds Ratio (95%CI)	p-value[A]		EBV-negative subjects (n=49)	Odds Ratio (95%CI)	p-value[A]
D6S510								
Allele 1 heterozygotes	46 (47%)	61 (42%)	1.6 (0.9-2.9)	0.12		18 (36%)	2.0 (0.9-4.6)	0.07
Allele 1 homozygotes	18 (18%)	14 (10%)	2.7 (1.1-8.5)	0.02		5 (10%)	2.9 (0.9-11)	0.08
Allele 1 negative	34 (35%)	71 (49%)				27 (54%)		
Allele 8 heterozygotes	20 (20%)	49 (34%)	0.5 (0.3-1.0)	0.04		18 (36%)	0.4 (0.2-0.8)	0.04
Allele 8 homozygotes	3 (3%)	1 (1%)	3.8 (0.3-203.6)	0.33		3 (6%)	0.4 (0.1-3.1)	0.35
Allele 8 negative	75 (77%)	96 (66%)				29 (58%)		
D6S265								
Allele 3 heterozygotes	48 (49%)	73 (49%)	1.4 (0.7-2.5)	0.09		21 (43%)	1.8 (0.8-4.1)	0.13
Allele 3 homozygotes	21 (21%)	16 (11%)	2.7 (1.1-6.4)	0.02		5 (10%)	3.3 (1.0-4.1)	0.04
Allele 1 negative	29 (30%)	60 (40%)				23 (47%)		

[A] Fisher's-exact 2-sided p-value

5.4 Analysis of single nucleotide polymorphisms rs253088 and rs6457110 in Infectious Mononucleosis

DNA from all three subject groups was sent to the LRF Virus Centre, Institute of Comparative Medicine, University of Glasgow, for Taqman PCR of two SNP's (rs2530388 and rs6457110). Allele determination was performed by Ms. Annette Lake and Professor Ruth Jarrett. Analysis of SNP rs2530388 (T/A) and rs6457110 (T/A) was performed on 97 EBV IM, 49 EBV seronegative and 140 EBV seropositive subjects. Both SNPs were in Hardy-Weinberg equilibrium for all three subject groups. A significant difference in frequency was found between EBV seronegative subjects and IM patients for allele-A of SNP rs2530388 (30% versus 45%; p=0.011) and for allele-T of SNP rs 6457110 (57% versus 70%; p=0.038) (Table 5.4). Genotype analysis between EBV seronegative subjects and IM patients was also performed. For the SNP rs2530388 the frequency of the A/A genotype was higher in the IM group compared to seronegative subjects (10% versus 23%) whilst the T/T genotype was lower in the IM group (51% versus 32%; p=0.049) (Table 5.4). Comparison between EBV seropositive subjects without symptoms of IM and IM patients was of borderline significance for allele-A of rs2530388 (37% versus 45%; p=0.07) with no significant difference observed for allele-T of rs6457110 (p=0.42). Further comparison between these groups for the genotype A/A of SNP rs2530388 revealed an increase in IM subjects carrying the A/A genotype (23%) compared to EBV seropositive subjects without IM (12%) although this did not reach statistical significance (p=0.098) (Table 5.4). No significant differences were observed between EBV seronegative and EBV seropositive individuals for any of the alleles or genotypes.

Table 5.4: Allele and genotype frequency of rs2530388 and rs6547110 SNPs in EBV seropositive, seronegative and IM subjects

Locus	Allele	EBV-positive subjects with symptoms of IM (n= 97 subjects: 194 alleles) Freq (%)		EBV-positive subjects without symptoms of IM (n=140 subjects: 280 alleles) Freq (%)	p-value[A]		EBV-negative subjects (n=49 subjects: 98 alleles) Freq (%)	p-value[A]
	A	45		37			30	
	T	56		63	0.07		70	0.011
	AA	23		12			10	
rs2530388	**AT**	45		49			39	
	TT	32		39	0.098		51	0.049
	A	30		34			43	
	T	70		66	0.427		57	0.038
	AA	10		13			20	
rs6457110	**AT**	40		42			45	
	TT	50		45	0.748		35	0.122

[A] Fisher's-exact 2-sided p-value

5.5 Correlation between clinical data and HLA/SNP polymorphisms

Clinical data and viral load estimations were available for 48 IM patients. For each allele studied, IM patients were grouped according to the presence or absence of each allele and counts of total lymphocytes, neutrophils, and monocytes, as well as EB viral loads were assessed in relation to these groupings.

5.5.1 Decreased total lymphocyte counts in IM patients positive for allele 1 (D6S510 marker) and allele 3 (D6S265 marker)

The median total lymphocyte count was significantly lower (p=0.03) among patients positive for allele 1 of marker D6S510 than among those who were negative for the allele (Figure 5.5-A; Table 5.5). The association was even stronger (p=0.001) with

respect to allele 3 of marker D6S265. Those positive for the allele had a median total lymphocyte count of 3.15 compared to a count of 6.80 among those who were negative for the allele (Figure 5.5-D; Table 5.5). Individual CD3 T-cell counts for 18, CD4 T-cell counts for 15 and CD8 T-cell counts for 19 IM cases were available for analysis. The CD3, CD8 and CD4 T-cell counts were reduced in IM cases that were positive for allele 1 of D6S510 and allele 3 of D6S265 compared to those that were negative although these did not reach significance (Table 5.6). All subjects who were positive for allele 1 of marker D6S510 were also positive for allele 3 of marker D6S265, so the apparent association observed for allele 1 of marker D6S510 may be due to the linkage with the other allele. In contrast, the small number of subjects who were positive for allele 3 of marker D6S265 but negative for allele 1 of D6S510 still had significantly lower ($p=0.003$) lymphocyte counts indicating the importance of the latter allele even in the absence of the former (Table 5.5).

Table 5.5: Analysis of D6S510 and D6S265 microsatellite markers in IM patients; comparison of total lymphocyte count, neutrophil count and viral load.

Locus	Allele	Status	Total Number	Total lymphocytes *Median (range)* [B]	Neutrophils *Median (range)* [B]	Viral load *Median (range)* [C]
D6S510	**1**	Negative	19	5.8 (1.92 - 9.55)	2.36 (0.56 – 6.03)	2563 (0 – 16840)
		Positive	29	3.22 (1.32 – 8.97)	3.16 (1.52 – 5.47)	10466 (59 – 48283)
				p-value = 0.03	***p-value = 0.03***	***p-value = 0.05***
D6S265	**3**	Negative	14	6.80 (4.13 – 9.55)	1.45 (0.56 – 5.97)	2409 (0 – 16840)
		Positive	34	3.15 (1.32 – 8.97)	3.16 (1.52 – 6.03)	8387 (26 – 48283)
				p-value = 0.001	***p-value = 0.004***	***p-value = 0.12***
		Positive [A]	5	3.01 (1.92 – 3.41)	3.69 (2.36 – 6.03)	3766 (26 – 7800)
				p-value = 0.003	***p-value = 0.07***	***p-value = 0.84***

[A] Positive for Allele 3 D6S265 but negative for Allele 1 of D6S510
[B] $x10^6$ cells/ml
[C] EBV copies per million cells

Table 5.6: Analysis of D6S510 and D6S265 microsatellite markers in IM patients; comparison of CD3, CD4 and CD8 count.

Locus	Allele	Status	CD3 *Median (range)*[A]	CD4 *Median (range)* [A]	CD8 *Median (range)*[A]
D6S510	1	Negative	5.38 (2.55-8.6)	0.86 (0.35-1.02)	4.2 (1.13-6.78)
		Positive	3.33 (0.93-8.32)	0.52 (0.18-0.95)	2.34 (0.36-7.36)
			P=0.23	*P=0.18*	*P=0.38*
D6S265	3	Negative	5.68 (2.71-8.6)	0.86 (0.35-1.02)	4.4 (1.98-6.78)
		Positive	2.55 (0.93-8.32)	0.52 (0.18-0.95)	2.14 (0.36-7.36)
			P=0.12	*P=0.18*	*P=0.19*

[A] $x10^6$ cells/ml

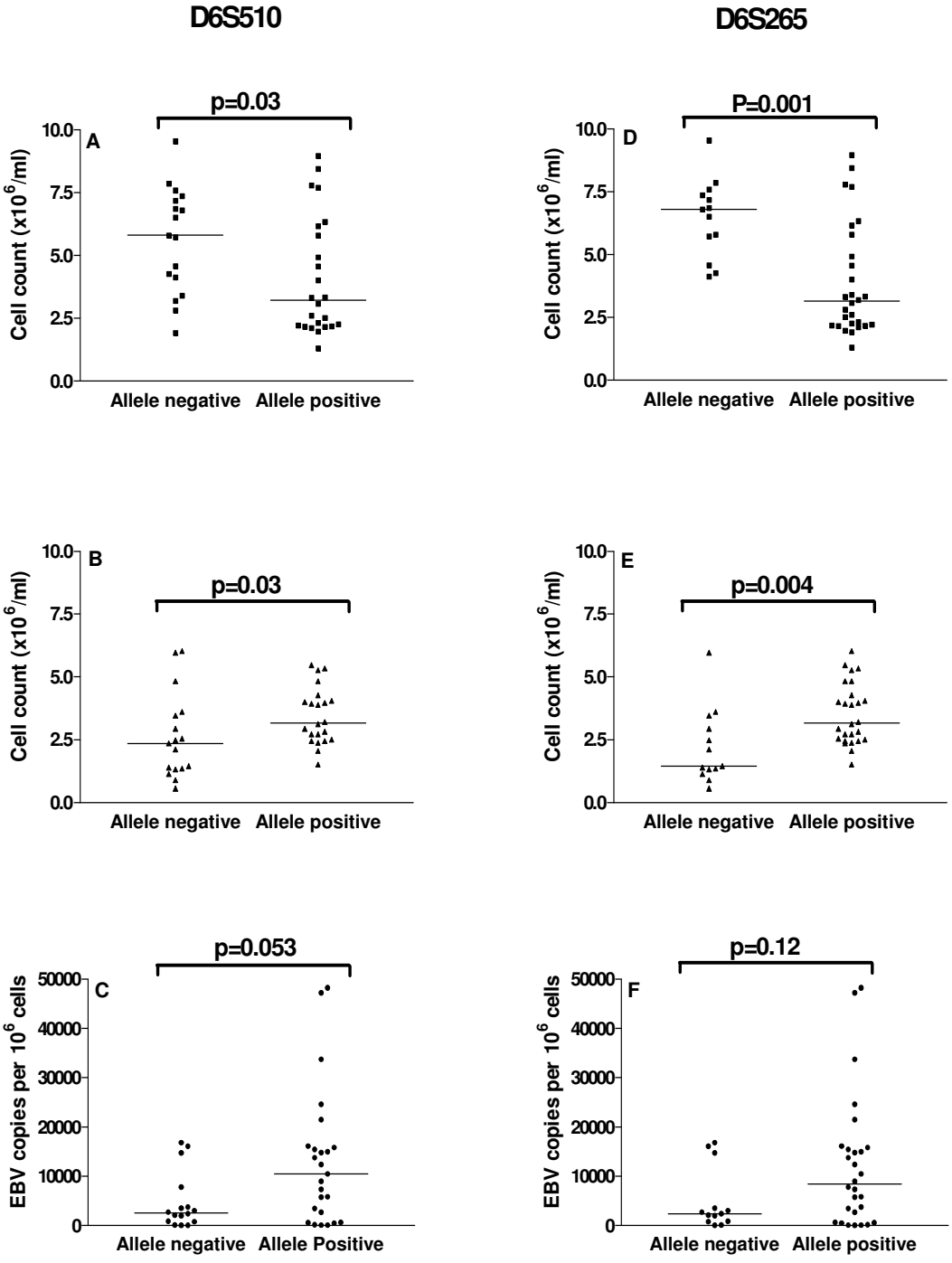


Figure 5.5: Analysis of microsatellite markers with clinical features in IM subjects.
Panels A, B and C refer to subjects positive and negative for the microsatellite marker D6S510-allele 1; panels D, E and F to those subjects positive and negative for microsatellite marker D6S265-allele 3. Total lymphocyte counts (x10^6/ml): panels A and D; neutrophil counts (x10^6/ml): panels B and E; viral load (EBV genome copies per million cells): panels C and F

Similar analyses were performed for the SNP alleles and genotypes. Allele-A (rs2530388) and allele-T (rs6457110) positive subjects had lower median total lymphocyte counts when compared to allele negative subjects however this did not reach statistical significance (rs2530388: p=0.31, rs6457110: p=0.67) (Table 5.7). Individuals homozygous for each of these alleles also displayed lower median total lymphocyte counts when genotypes were compared.

Table 5.7: Analysis of rs2530388 and rs6457110 SNP's in IM patients; comparison of total lymphocyte count, neutrophil count and viral load.

Locus	Allele	Status	Total Number	Total lymphocytes[A] *Median (range)*	Neutrophils[A] *Median (range)*	Viral load[B] *Median (range)*
rs2530388	**A**	Neg	29	4.58 (1.92 – 8.97)	2.94 (0.56 – 6.03)	3633 (26 – 48283)
		Pos	25	3.35 (1.32 – 9.55)	3.03 (1.4 – 5.47)	9720 (0 – 48283)
				p-value = 0.31	***p-value = 0.31***	***p-value = 0.38***
	AA	-	11	4.01 (1.32 – 9.55)	2.72 (1.4 – 5.97)	7366 (0 – 21466)
	AT	-	15	3.34 (2.16 – 8.97)	2.36 (0.56 – 6.03)	12400 (82 – 48283)
	TT	-	15	5.8 (1.92 – 7.86)	2.36 (0.56 – 6.03)	2716 (26 – 16840)
				p-value=0.34	*p-value=0.32*	*p-value=0.14*
rs6457110	**T**	Neg	19	4.58 (2.16 – 7.7)	2.74 (0.56 – 6.03)	3633 (26 – 24650)
		Pos	37	4.13 (1.32 – 9.55)	2.82 (0.9 – 6.03)	5841 (0 – 48283)
				p-value = 0.67	***p-value = 0.99***	***p-value = 0.54***
	AA	-	4	6.69 (2.33 – 7.6)	2.2 (0.56 – 3.89)	2142 (59 – 16840)
	AT	-	15	3.41 (2.16 – 7.7)	3.12 (0.9 – 6.03)	4770 (26 –24650)
	TT	-	22	4.2 (1.32 – 9.55)	2.63 (1.32 – 5.97)	7366 (0 – 48283)
				p-value=0.59	*p-value=0.39*	*p-value=0.65*

[A] $x10^6$ cells/ml
[B] EBV copies per million cells

5.5.2 Altered neutrophil counts in IM patients positive for allele 1 (D6S510 marker) and allele 3 (D6S265 marker)

Although within the normal range for the Coulter counter, significantly higher neutrohil counts were observed among subjects positive for allele 1 of D6S510 compared to those who were negative for the allele (p=0.03). However, as with total lymphocyte counts, a much stronger association (p=0.004) was observed for allele 3 of D6S265, in which the median count among those who were negative for the allele was below the normal range for the Coulter counter (Figure 5.5-B and E; Table 5.5). Again, the much stronger association for the latter allele suggests its relative importance. Similar analyses for the two SNPs revealed no significant differences (Table 5.7).

5.5.3 Increased EB viral load in IM patients positive for allele 1 (D6S510 marker) and allele 3 (D6S265 marker)

There was a suggestion that viral loads were raised among subjects positive for allele 1 of D6S510 compared to those negative for the allele but the association was of borderline significance (p=0.053). Similarly, the viral load was raised among those who were positive for allele 3 of marker D6S265 but the association was not significant (p=0.12) (Figure 5.5-C and F; Table 5.5). This trend was also observed with allele-A (rs2530388) and allele-T (rs6457110) and for the homozygous genotype of both alleles (Table 5.7).

5.5.4 IM patients positive for allele 1 (D6S510 marker) and allele 3 (D6S265) marker present with milder symptoms

We analysed the IM groupings in relation to the severity of clinical symptoms. Data on sore throat were available for 39 of the patients and was assessed as mild (able to swallow a normal diet) (24 subjects) or severe (unable to swallow a normal diet) (15 subjects). Among the 21 patients positive for allele 1 of the D6S510 marker, six (29%)

reported that their sore throat was severe, compared to nine (50%) of the 18 cases negative for the allele (p=0.20). Of the 25 patients positive for allele 3 of the D6S265 marker, eight (32%) reported a severe sore throat compared to seven (50%) of the fourteen cases negative for the allele (p=0.32) (Figure 5.6).

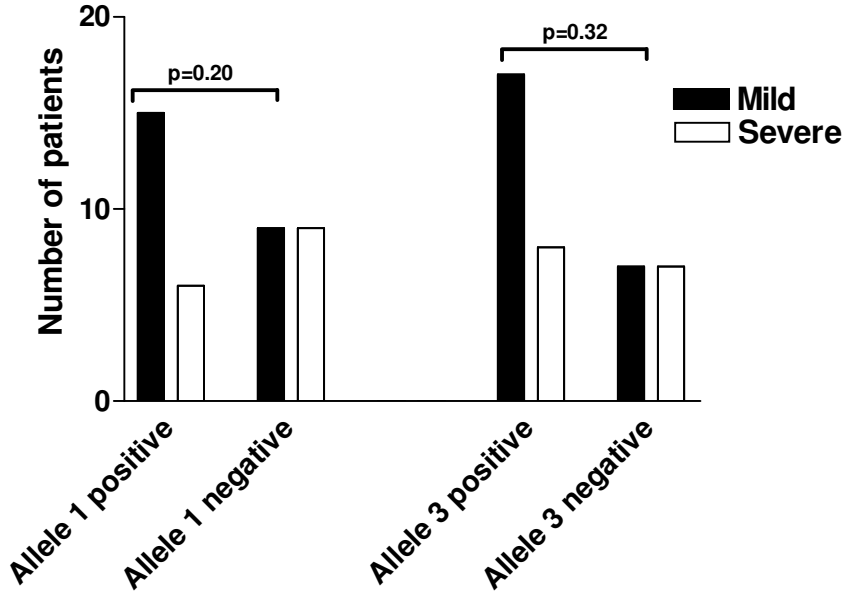


Figure 5.6: Comparison of severe and mild sore throat in IM subjects.
IM subjects positive and negative for marker D6S510-allele 1 and marker D6S265-allele 3 were compared for differences in presentation of sore throat. Mild: black bar; severe: white bar. The p-value for comparison of mild symptoms between allele positive and negative subjects is indicated.

5.6 Analysis of HLA class I microsatellite markers D6S510 and D6S265 in EBV-associated post transplant lymphoproliferative disease

HLA genotyping for all 3 microsatellite markers (D6S510, D6S265 and D6S273) was performed on transplant patients with (n=34) or without (n=49) the development of EBV-associated post transplant lymphoproliferative disease (PTLD). All three markers were in Hardy Weinberg equilibrium for each group. No significant differences were

observed between the two groups for any of the markers tested and therefore no further analyses were undertaken (Table 5.8).

Table 5.8: Allele frequency of microsatellite markers D6S510, D6S265 and D6S273 in transplant subjects with and without

Locus	Allele	Base Pair Size	Transplant subjects with PTLD Freq (%)		Transplant subjects without PTLD Freq (%)	p-value[A]
D6S510	1	284	29		29	1
D6S510	2	290	1		1	1
D6S510	3	292	9		4	0.32
D6S510	4	294	12		10	0.79
D6S510	5	296	6		9	0.76
D6S510	6	298	19		28	0.44
D6S510	7	300	6		1	0.16
D6S510	8	302	16		16	1
D6S510	9	304	0		1	1
D6S510	10	306	0		2	0.5
D6S265	1	122	14		21	0.3
D6S265	2	124	0		0	1
D6S265	3	126	38		32	0.5
D6S265	4	128	9		6	0.54
D6S265	5	130	27		22	0.57
D6S265	6	132	13		10	0.79
D6S265	7	134	0		1	1
D6S265	8	136	0		1	1
D6S265	9	138	0		0	1
D6S265	10	140	0		2	0.51
D6S265	11	142	0		0	
D6S265	12	144	0		2	0.51
D6S265	13	146	0		2	0.51
D6S273	1	128	4		4	1
D6S273	2	130	7		5	0.74
D6S273	3	132	13		17	0.52
D6S273	4	134	21		19	0.84
D6S273	5	136	41		41	1
D6S273	6	138	4		4	1
D6S273	7	140	9		9	1
D6S273	8	142	0		0	1
D6S273	9	144	0		0	1

[A] Fisher's-exact 2-sided p-value

5.7 Discussion

In this study we have assessed two HLA class I and one HLA class III microsatellite markers for evidence of an association with acute IM and EBV-associated PTLD. Both HLA class I markers were found to be significantly associated with the development of IM, with allele 1 (284bp) of marker D6S510 and allele 3 (126bp) of marker D6S265 showing the strongest effects. Furthermore a co-dominant effect was observed in IM subjects homozygous for these alleles. An unexpected observation was that the frequency of marker D6S510-allele 8 (302bp) was significantly reduced in the IM group when compared to the seronegative group but not when compared to the seropositive group, perhaps indicating a role for this allele in protection from IM. However the lack of any significant difference in allele frequency between the EBV seropositive subjects without symptoms of IM and the seronegative subjects for all three alleles is an indication that these alleles do not confer protection from sub-clinical EBV infection. No association was observed between the subject groups and the control HLA class III marker D6S273. These findings are similar to recent reports associating the HLA class I region, in particular the D6S510 (284bp allele) and D6S265 (126bp allele) microsatellite markers, with EBV-positive HL (Diepstra *et al.*, 2005) where the frequency of both markers in EBV-positive HL was almost double that of the controls. Our findings provide further evidence for an aetiological link between IM and EBV-positive HL.

Also in line with recent reports (Niens *et al.*, 2006) showing an association between several SNPs from the HLA class I region and EBV-positive HL is our finding that allele-A of SNP rs2530388 and allele-T of rs6457110 are more frequent in the IM group compared to EBV seronegative individuals. The A/A genotype for SNP rs2530388 is also more frequent in IM compared to our EBV seronegative group. The

results suggest a genetic link between IM and EBV-associated HL, although the association between IM and the markers analysed in this study is slightly weaker than the reported association with EBV-positive HL.

The markers we have identified as conferring a predisposition to IM are known to be in linkage disequilibrium with the HLA-A locus; marker D6S510 with HLA-A*01 and marker D6S265 with HLA-A*03 (Malkki *et al.*, 2005). A number of studies have shown that specific EBV peptides are presented in the context of specific HLA class I alleles (Rickinson and Moss, 1997;Moss *et al.*, 2001). Interestingly, the number of EBV specific epitopes restricted through HLA-A*01 and -A*03, so far identified, is low; one HLA-A*01 restricted LMP-2 epitope and two HLA-A*03 restricted epitopes (EBNA-3A and BRLF1 proteins) (Hislop *et al.*, 2007). This association of HLA class I markers, and subsequent linkage with HLA-A alleles, with IM suggests that the genetic makeup of an individual's HLA class I locus dictates the efficiency of viral peptide presentation and the recruitment of T-cells during the immune response to primary EBV infection. Thus, as IM is assumed to be immunopathological in nature, caused by T-cell derived cytokines, possession of these markers might be expected to result in an exuberant T-cell response and severe IM. However the results show that in individuals with these alleles lymphocyte counts are significantly lower than in those without. The reduction in total lymphocyte count was accounted for by a reduction in individual median CD3, CD4 and CD8+ T-cell counts, and was not due to variations in the duration of IM symptoms at the time of bleeding (allele 1 positive and negative: median 9 days and 10 days respectively post onset of symptoms (p=0.87); allele 3 positive and negative: median 8 days and 12 days respectively (p=0.25)). Allele positive patients also had higher viral loads than the allele negative group. This increase was not accounted for by a higher proportion of B-cells within the lymphocyte population in allele positive

subjects (allele 1 and 3 positive: 5%; allele 1 and 3 negative: 7%). This suggests that perhaps the virus specific T-cell response itself was insufficient to control the virus infection effectively, either through a reduction in the number of T-cells recruited or through T-cells undergoing more rapid and early apoptosis. Linkage of the markers with HLA-A*01 and -A*03 alleles may contribute to the insufficient T-cell response observed through presentation of low affinity peptides. Moreover, the HLA-A*01 allele is associated with an increased risk, and the HLA-A*02 allele a decreased risk, of developing EBV-positive HL (Niens *et al.*, 2007): possibily as a result of low versus high immunogenic peptide presentation. In addition there is a suggestion that IM patients with the microsatellite alleles had milder IM symptoms than those without perhaps resulting from reduced levels of cytokine production by the virus-specific T-cells present – less T-cells, less cytokine released. Thus possession of either of the alleles we have identified appears to result in a weakened or aberrant immune response to EBV which predisposes to a mild form of IM. It is also possible that other, as yet unidentified, alleles from microsatellite markers not investigated in this study may predispose to a more severe form of IM.

The HLA-A gene locus is but one located within the vicinity of the microsatellite risk alleles. The region of interest also contains 9 psuedogenes, including MHC class I polypeptide-related sequence D (MICD), which are unlikely to be causative, and the protein-coding HLA complex gene 9 (HCG9). HCG9 belongs to a multigene family that associates with the MIC multigene family; both of which are dispersed throughout the HLA class I region. The MIC and HCG genes tend to form clusters within the HLA class I region; the function of which is unknown. Both MICA and MICB are recognised by NK-cells and CD8 T-cells expressing the NKG2D receptor and therefore have the potential to affect both innate and adaptive immune responses (Moretta *et al.*,

2001;Pende *et al.*, 2002). Due to its association with the MIC family, HCG9 may also be involved in activation of NK- and T-cells, therefore the reduced T-cell response observed in allele positive IM could also result from an association between the microsatellite markers and the HCG9 gene locus.

The significantly higher neutrophil counts observed in IM patients positive for the alleles is more difficult to explain since there is no known direct link between the HLA locus and neutrophil function. As part of the innate immune response neutrophils, together with NK-cells and monocytes, are thought to form the main effector cells early in primary EBV infection. By releasing cytokines and chemokines they attract other immune cells to the site of infection and are thereafter depleted, mainly by apoptotic cell death (Savard and Gosselin, 2006). Recent reports suggest that EBV directly infects neutophils in the early phase of IM altering their cytokine production. Infection is believed to occur in approximately 30% of neutrophils via a receptor other than CD21. *In-vitro* infection studies identified the secretion of the pro-inflammatory cytokines IL-1α, IL-1β, the IL-1 receptor antagonist IL-Ra, IL-8 and macrophage inflammatory protein-1α. Secretion of these molecules is thought to attract other leucocytes to the infection site, thereby allowing EBV direct access to B-cells (Savard and Gosselin, 2006). However, it seems more likely that the low neutrophil counts observed in allele negative subjects are a direct result of neutrophil apoptosis following recruitment of T-cells to sites of infection. In a recent case control study on IM the median neutrophil count in IM subjects at time of diagnosis was significantly lower than that of age matched controls, with 26% of IM cases having neutrophil counts below the lower limit of the normal range (p=0·015, K Macsween, personal communication). Thus finding a significantly higher median neutrophil count (although still within the normal range) in

allele positive subjects in the present study may just reflect the milder IM symptoms in these subjects.

The classic RS cells of HL represent post germinal centre B-cells which contain non-functional immunoglobulin genes (Kuppers and Rajewsky, 1998;Tamaru *et al.*, 1994). In EBV-positive HL these abnormal cells are thought to have been rescued from apoptosis in germinal centres by the expression of EBV latent membrane proteins 1 and 2 which provide crucial survival signals (Brauninger *et al.*, 2006). We postulate that the HLA class I markers, D6S510 and D6S265, predispose to EBV-associated HL by inducing a suboptimal T-cell immune response to the virus during IM which in turn would result in poor virus clearance allowing a high level of virus persistence in B-cells. Indeed increased viral load in pre-treatment blood samples from EBV-positive HL patients has been reported (Khan *et al.*, 2005). Elevated levels of viral persistence may increase the chance of EBV infection and survival of abnormal B-cells with malignant potential providing an explanation for the link between HL and IM.

In contrast the development of EBV-positive PTLD following solid organ transplantation, which has the same suggested aetiology, does not appear to be associated with any of the HLA class I microsatellite markers tested. However, a recent study reports a negative association for the development of PTLD with the HLA-A*03 and HLA-DR*07 alleles and a positive association with the HLA-B*18 and HLA-B*21 alleles (Subklewe *et al.*, 2006). In this context we may have expected to see an association with the D6S265 marker which is known to be in linkage disequilibrium with the HLA-A*03 allele (Malkki *et al.*, 2005) however we did not find this to be the case. Differences in the numbers tested (34 versus 115 PTLD and 49 versus 1995 controls) may account for the differing observations as the HLA-A*03 allele is only

present in approximately 25% of the population. Also we did not analyse markers from the HLA-B or HLA-DR loci which may have provided further interesting correlations. In summary, we have demonstrated an association between polymorphisms in the HLA class I region and IM, and show evidence suggestive of reduced T-cell control of EBV during primary infection in allele positive cases. We observed no associations between HLA class I polymorphisms and the development of EBV-associated PTLD.

Chapter 6:
Concluding Remarks

6. Concluding Remarks

Understanding the viral and host immuno-regulatory mechanisms involved in the development of EBV-assciated disease is pivotal in devising new and improved treatments and in identifying those who are most susceptible to disease. The findings of this thesis cast some light on both of these aspects.

Firstly, analysis of the specific characteristics of CTL used to treat PTLD patients revealed that patient response was enhanced when donor CTLs were matched to the recipient via HLA specificity and peptide epitope specificity rather than HLA specificity alone. Such an improved selection process may allow more restricted EBV-associated tumours, such as HL and BL, to be treated effectively. As stated previously (introduction; section 1.5) new methods, such as peptide loading and transducing the LCL target used in the CTL culturing process, are being employed to generate epitope specific CTL. More recent approaches are aimed at engineering the TCR in order to redirect CTL to alternative antigens. In this case TCR genes specific for a particular antigen are derived from T cell clones and transferred to naïve T-cells, thereby redirecting the cells to an alternative epitope. This approach has been shown to produce EBV-specific CTL that are fully functional, home to target sites and have some anti-tumour effect (Kessels *et al.*, 2001;Schaft *et al.*, 2006). More robust matching criteria based upon HLA and peptide specificity mapping may be of benefit in the application of these treatment options.

However, HLA restriction of CTL presents a continued barrier to main-streaming CTL therapy in the clinic as it dictates individualised patient treatment. Strategies have now been developed to bypass the HLA recognition process by creating antibody based chimeric receptors or ‘T-bodies’. These constructs when cloned into T-cells replace the TCR element and enable T-cells to recognise antigen via the non-HLA-restricted

antibody recognition site, but still retain the down stream cytolytic function of the TCR (Gross and Eshhar, 1992). T-bodies that target the CD19 molecule on B lymphocytes have been developed and shown to have some anti-tumour effect (Cheadle *et al.*, 2005). The development of such 'T-bodies' specific for LMP-1 and and -2 antigens is currently being investigated in our laboratory and may provide an alternative HLA-independent treatment option for EBV-associated disease.

Secondly, vaccination trials to prevent the development of IM have recently been reported and show promising results (Elliott *et al.*, 2007;Moutschen *et al.*, 2007). Therefore, identifying those most at risk of developing IM is crucial for the implementation of future vaccination strategies. The results of this thesis indicate that genetic variation within cytokine and HLA genes may render some individuals more susceptible to the development of IM, however, more detailed investigation of these genes is required to further enhance this observation. Similarly, genetic variation in cytokine genes may help to identify those with the highest risk of developing B-cell lymphomas following transplant. Time constraints in growing EBV-specific CTL restrict their use, especially with more aggressive tumours, therefore identification of risk groups would allow autologous EBV-specific CTLs to be established prior to transplant and development of tumour.

The identification of such risk groups may also provide an environment for investigating the immune response in EBV-associated disease. In this thesis we observed that the T-cell response was reduced in the specific genetic group with a predisposition to IM (HLA-class I alleles). The reasons for this reduction are unclear and require further investigation. It would be interesting to investigate differences in EBV-specific T-cell response from risk and non-risk genetic groups, especially as the alleles identified are located in the region of the genome involved with antigen

presentation to T-cells. We hope to investigate these differences in future studies by focusing on the quantitative and qualitative differences in T-cell recognition, binding, and killing of EBV targets. We aim to measure cytotoxic responses against EBV proteins using a modified chromium release assay and estimate the production of IFN-γ by peptide-stimulated PBMCs in ELISPOT assays. Binding of T-cells will also be investigated by flow cytometry using HLA-A2 and A3 restricted latent and lytic peptide-specific tetramers. Differences in T-cell response identified may provide further explanation for the link between IM and EBV-positive HL.

Chapter 7:
Appendices

Appendix I: Patient information and 6 month outcome of CTL infusions

Patient number	Sex	Age (years)	Transplant type	PTLD histology	Infused CTL number	Outcome 6-months
1	F	1	Liver	Monomorphic	67	R
2	F	76	Liver	Hodgkin	86	R
3	M	51	Heart	Monomorphic	91	R
4	F	30	Stem cell	Polymorphic	12	R
5	M	27	Bone marrow	Polymorphic	28	R
6	F	68	Kidney	Burkitt	86	R
7	F	67	Kidney	Hodgkin	95	R
8	F	13	Liver	Hyperplastic	68	R
9	F	50	Liver	Hodgkin	48	R
10	M	3	Liver, small bowel	Hyperplastic	47	R
11	F	5	Liver	Hyperplastic	30	R
12	M	2	Liver, small bowel	Hodgkin	7	R
13	F	19	Kidney	Polymorphic	24	R
14	F	35	Kidney	Monomorphic	40 & 15	R
15	M	41	Kidney	Monomorphic	57	R
16	M	64	Kidney	Monomorphic	68	R
17	M	60	Liver	Polymorphic	8	R
18	M	11	Kidney	Polymorphic	18	NR
19	M	33	Heart	Hyperplastic	9	NR
20	M	14	Liver	Hodgkin	50	NR
21	F	51	Heart, lung	Monomorphic	117	NR
22	M	61	Kidney	Monomorphic	44	NR
23	M	49	Liver	Polymorphic	58	NR
24	M	48	Kidney	Monomorphic	55	NR
25	M	51	Kidney	Monomorphic	13	NR
26	M	19	Kidney	Polymorphic	117	NR
27	M	8	Lung	Polymorphic	55	NR
28	M	61	Liver	Monomorphic	30	NR

F: female, M: male, R: response, NR: No response

Appendix II: Protein and Peptide Specificity of Donor CTL

CTL Number	Dominant Response	Subdominant Response	Peptide Response
7	EBNA-1	EBNA-3C LMP-1, -2	-
8	EBNA-3A	EBNA-1, -LP, -3C	-
9	EBNA-3C	EBNA-LP	-
12	ND	ND	-
13	EBNA-3C	-	-
15	EBNA-3C	-	LLD (EBNA-3C)
18	LMP-1	EBNA-3C, -LP LMP-2	-
24	EBNA-3B	-	-
28	ND	ND	-
30	EBNA-3C	EBNA-2	-
40	EBNA-3C	LMP-1	LLD (EBNA-3C) LLW (LMP-2)
44	EBNA-3A	EBNA-3C LMP-2	-
47	EBNA-3C	LMP-1	-
48	EBNA-3A	EBNA-LP	QAK (EBNA-3A)
50	EBNA-3B	EBNA-1, -3C	IVT (EBNA-3B) AVF (EBNA-3B)
55	EBNA-3C	EBNA-LP	LLD (EBNA-3C)
57	ND	ND	-
58	EBNA-3B	EBNA-1, -3A	IVT (EBNA-3B)
67	LMP-2	EBNA-3B	GQG (EBNA-3B) LLW (LMP-2)
68	EBNA-3C	EBNA-3B	-
86	EBNA-3A	-	-
91	EBNA-3B, -3C	LMP-1, -2	VEI (EBNA-3B) GQG (EBNA-3B) LLD (EBNA-3C)
95	EBNA-3A,	EBNA-3C	LLD (EBNA-3C)
117	EBNA-2	LMP-1, -2	-

ND: not determined
EBNA: Epstein-Barr nuclear antigen
LMP: Latent membrane protein

Appendix III: Cytokine and cytokine receptor polymorphisms in IM, seropositive and seronegative subjects

Polymorphism		IM *Freq (%)*	EBV sero-positive *Freq (%)*	p-value[a]	EBV sero-negative *Freq (%)*	p-value[a]	p-value[a‡]
Allele							
TNF-1031	T	75	77	0.76	74	0.82	0.48
	C	26	23		26		
TNF-863[†]	C	85	85	1	83	0.59	0.55
	A	15	15		17		
TNF-857[†]	C	89	91	0.66	91	0.74	1
	T	10	9		9		
TNF-307	G	83	80	0.32	77	0.13	0.51
	A	17	20		23		
TNF-237	G	97	95	0.28	95	0.21	0.84
	A	3	5		5		
IL-1-889	C	69	71	0.63	71	0.74	1
	T	32	29		30		
IL-1RI-1339[†]	C	71	71	0.92	72	0.74	0.84
	T	29	29		28		
IL-6-174[†]	C	38	41	0.47	46	0.15	0.36
	G	62	59		54		
IL-6 intron 4	A	65	63	0.78	57	0.1	0.14
	G	35	37		43		
IL-10-1082	A	49	55	0.21	45	0.48	0.02*
	G	51	45		55		
IL-10-819[†]	C	76	73	0.46	79	0.55	0.15
	T	24	27		21		
IL-10-592[†]	C	76	73	0.46	79	0.55	0.15
	A	24	27		21		
IL-10RI-241[†]	G	59	51	0.06	56	0.54	0.29
	A	41	49		44		
Genotype							
TNF-1031	TT	56	57	0.11	53	0.84	0.70
	TC	38	40		42		
	CC	6	3		5		
TNF-863	CC	71	73	0.24	66	0.46	0.11
	CA	29	25		34		
	AA	0	2		0		
TNF-857	CC	79	84	0.42	82	0.59	0.21
	CT	19	12		18		
	TT	1	2		0		
TNF-307	GG	70	64	0.55	60	0.26	0.73
	GA	27	32		34		
	AA	3	4		6		
TNF-237	GG	94	90	0.21	89	0.17	0.77
	GA	6	10		11		

	AA	0	0		0		
IL-1α-889	CC	46	49		51		
	CT	45	44	0.86	39	0.69	0.86
	CC	9	7		10		
IL-1 RI-1339	CC	45	48		50		
	CT	51	46	0.67	44	0.33	0.72
	TT	4	5		8		
IL-6-174	CC	11	17		18		
	CG	55	49	0.32	55	0.24	0.42
	GG	34	34		27		
IL-6 Intron 4	AA	38	39		37		
	AG	55	48	0.36	40	0.006*	0.06
	GG	8	13		23		
IL-10-1082	AA	27	28		22		
	AG	44	52	0.15	46	0.72	0.04*
	GG	29	19		32		
IL-10-819	CC	60	57		62		
	CT	32	33	0.77	33	0.65	0.35
	TT	8	10		5		
IL-10-592	CC	60	57		62		
	CA	32	33	0.77	33	0.65	0.35
	AA	8	10		5		
IL-10 RI-241	GG	35	22		35		
	GA	49	59	0.05	42	0.42	0.05
	AA	17	19		23		
Haplotype							
TNF	TCCGG	80	76	0.45	70	0.13	0.39
	TCCAG	28	37	0.14	38	0.17	0.89
	CACGG	23	26	0.66	33	0.14	0.26
	TCTGG	19	15	0.49	19	1	0.39
	CCCGA	5	9	0.34	9	0.4	1
	CCCGG	14	8	0.09	9	0.27	0.82
IL-10	ATA	40	43	0.61	38	0.77	0.38
	ACC	42	48	0.38	40	0.77	0.21
	GCC	73	72	0.88	78	0.51	0.32

[a] Fisher's Exact 2-sided p-value for allele and haplotype analysis; Chi Square for genotype analysis
* Significant p-value, $p<0.05$
[†] Not in Hardy Weinberg equilibrium
[‡] p-value for comparison of seropositve and seronegative subjects groups

Chapter 8:
References

Reference List

Adams,A. (1987) Replication of latent Epstein-Barr virus genomes in Raji cells *J.Virol.* **61**: 1743-1746.

Adler,B., Schaadt,E., Kempkes,B., Zimber-Strobl,U., Baier,B., and Bornkamm,G.W. (2002) Control of Epstein-Barr virus reactivation by activated CD40 and viral latent membrane protein 1 *PNAS* **99**: 437-442.

Alfieri,C., Birkenbach,M., and Kieff,E. (1991) Early events in Epstein-Barr virus infection of human B lymphocytes *Virology* **181**: 595-608.

Allan,G.J., Inman,G.J., Parker,B.D., Rowe,D.T., and Farrell,P.J. (1992) Cell growth effects of Epstein--Barr virus leader protein *J Gen Virol* **73**: 1547-1551.

Allday,M.J., Crawford,D.H., and Griffin,B.E. (1989) Epstein-Barr Virus Latent Gene Expression during the Initiation of B Cell Immortalization *J Gen Virol* **70**: 1755-1764.

Allday,M.J., Crawford,D.H., and Thomas,J.A. (1993) Epstein--Barr virus (EBV) nuclear antigen 6 induces expression of the EBV latent membrane protein and an activated phenotype in Raji cells *J Gen Virol* **74**: 361-369.

Allday,M., Crawford,D. (1988) Role of epithelium in EBV persistence and pathogenesis of B-cell tumours *Lancet* **1**: 855-857.

Aman,P., von Gabain,A. (1990) An Epstein-Barr virus immortilization associated gene segment interferes specifically with the IFN-induced anti-proliferative response in human B-lymphoid cell lines *EMBO* **9**: 147-152.

Amyes,E., Hatton,C., Montamat-Sicotte,D., Gudgeon,N., Rickinson,A.B., McMichael,A.J., and Callan,M.F.C. (2003) Characterization of the CD4+ T Cell Response to Epstein-Barr Virus during Primary and Persistent Infection *J.Exp.Med.* **198**: 903-911.

Anagnostopoulos,I., Herbst,H., Niedobitek G., and Stein,H. (1989) Demonstartion of monoclonal EBV genomes in Hodgkin's disease and Ki-1-positive anaplastic large cell lymphoma by combined southern blot and in situ hybridization *Blood* **74**: 810-816.

Andersson,J. (2006) Epstein-Barr Virus and Hodgkin's Lymphoma *Herpes* **13**: 12-16.

Annels,N.E., Callan,M.F.C., Tan,L., and Rickinson,A.B. (2000) Changing Patterns of Dominant TCR Usage with Maturation of an EBV-Specific Cytotoxic T Cell Response *J.Immunol* **165**: 4831-4841.

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

Armitage,J., Kormos,R., Stuart,R., Fricker,F., Griffith,B., Nalesnik,M., Hardestry,R., and Dummer,J. (1991) Posttransplant lymphoproliferative disease in thoracic organ transplant patients: ten years of cyclosporine-based immunosuppession *Journal of Heart and Lung Transplantation* **10**: 877-886.

Attarbaschi,T., Willheim,M., Ramharter,M., Hofmann,A., Wahl,K., Winkler,H., Graninger,W., and Winkler,S. (2003) T cell cytokine profile during primary Epstein-Barr virus infection (infectious mononucleosis) *European Cytokine network* **14**: 34-39.

Ayako Demachi-Okamura, Yoshinori Ito, Yoshiki Akatsuka, Kunio Tsujimura, Yasuo Morishima, Toshitada Takahashi, and Kiyotaka Kuzushima (2006) Epstein-Barr virus (EBV) latent membrane protein-1-specific cytotoxic Tlymphocytes targeting EBV-carrying natural killer cell malignancies *European journal of Immunology* **36**: 593-602.

Babcock,G.J., Hochberg,D., and Thorley-Lawson,D.A. (2000) The Expression Pattern of Epstein-Barr Virus Latent Genes In Vivo Is Dependent upon the Differentiation Stage of the Infected B Cell *Immunity* **13**: 497-506.

Babcock,G.J., Thorley-Lawson,D.A. (2000) Tonsillar memory B cells, latently infected with Epstein-Barr virus, express the restricted pattern of latent genes previously found only in Epstein-Barr virus-associated tumors *PNAS* **97**: 12250-12255.

Baer,R., Bankier,A.T., Biggin,M.D., Deininger,P.L., Farrell,P.J., Gibson,T.J., Hatfull,G., Hudson,G.S., Satchwell,S.C., Seguin,C., Tuffnell,P.S., and Barrell,B.G. (1984) DNA sequence and expression of the B95-8 Epstein-Barr virus genome *Nature* **310**: 207-211.

Bain,M., Watson,R.J., Farrell,P.J., and Allday,M.J. (1996) Epstein-Barr virus nuclear antigen 3C is a powerful repressor of transcription when tethered to DNA *J.Virol.* **70**: 2481-2489.

Balkwill,F. (2006) TNF-+¦ in promotion and progression of cancer *Cancer and Metastasis Reviews* **25**: 409-416.

Beaufils,P., Choquet,D., Mamoun,R., and Malissen,B. (1993) The (YXXL/1)2 signalling motif found in the cytoplasmic segments of the bovine leukaemia virus envelope protein and Epstein-Barr virus latent mambrane protein 2A can elicit early and late lymphocyte activation events *EMBO* **12**: 5105-5112.

Berger,C., Hug,M., Gysin,C., Molinari,L., Frei,M., Bossart,W., and Nadal,D. (2007) Distribution patterns of β- and γ-Herpesviruses within Waldeyer's ring organs *Journal of Medical Virology* **79**: 1147-1152.

Bharadwaj,M., Burrows,S.R., Burrows,J.M., Moss,D.J., Catalina,M., and Khanna,R. (2001) Longitudinal dynamics of antigen-specific CD8+ cytotoxic T lymphocytes following primary Epstein-Barr virus infection *Blood* **98**: 2588-2589.

Bhat,R.A., Thimmappaya,B. (1983) Two Small RNAs Encoded by Epstein-Barr Virus Can Functionally Substitute for the Virus-Associated RNAs in the Lytic Growth of Adenovirus 5 *PNAS* **80**: 4789-4793.

Bickham,K., Goodman,K., Paludan,C., Nikiforow,S., Tsang,M.L., Steinman,R.M., and Munz,C. (2003) Dendritic Cells Initiate Immune Control of Epstein-Barr Virus Transformation of B Lymphocytes In Vitro *J.Exp.Med.* **198**: 1653-1663.

Bickham,K., Munz,C. (2003) Contrasting roles of dendritic cells and B cells in the immune control of Epstein-Barr virus *Current Topics in Microbiology and Immunology* **276**: 55-76.

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

Biglino,A., Sinicco,A., Forno,B., Pollono,A.M., Sciandra,M., Martini,C., Pich,P., and Gioannini,P. (1996) Serum Cytokine Profiles in Acute Primary HIV-1 Infection and in Infectious Mononucleosis *Clinical Immunology and Immunopathology* **78**: 61-69.

Biron,C.A., Nguyen,K.B., Pien,G.C., Cousens,L.P., and Salazar-Mather,T.P. (1999) Natural killer cells in antiviral defence: Function and regulation by innate cytokines *Ann Rev Immunol* **17**: 220.

Biron,C.A., Sen,G.C. (2001) Interferons and other cytokines. In *Fundamental Virology*. Knipe,D.M., Howley,P.M. (eds). Lippencot, Williams and Wilkins, pp. 321-351.

Blake,N., Haigh,T., Shaka'a,G., Croom-Carter,D., and Rickinson,A. (2000) The Importance of Exogenous Antigen in Priming the Human CD8+ T Cell Response: Lessons from the EBV Nuclear Antigen EBNA1 *J Immunol* **165**: 7078-7087.

Blake,N., Lee,S., Redchenko,I., Thomas,W., Steven,N., Leese,A., Steigerwald-Mullen,P., Kurilla,M.G., Frappier,L., and Rickinson,A. (1997) Human CD8+ T Cell Responses to EBV EBNA1: HLA Class I Presentation of the (Gly-Ala)-Containing Protein Requires Exogenous Processing *Immunity* **7**: 791-802.

Bleakley M, Riddel SR (2004) Molecules and mechanisms of graft-versus-leukaemia effect *Nature Reviews* **4**: 371-380.

Bogunia-Kubik,K., Mlynarczewska,A., Jaskula,E., and Lange,A. (2006) The presence of IFNG 3/3 genotype in the recipient associates with increased risk for Epstein-Barr virus reactivation after allogeneic haematopoietic stem cell transplantation *Br.J.Haematol* **132**: 326-332.

Bollard,C.M., Aguilar,L., Straathof,K.C., Gahn,B., Huls,M.H., Rousseau,A., Sixbey,J., Gresik,M.V., Carrum,G., Hudson,M., Dilloo,D., Gee,A., Brenner,M.K., Rooney,C.M., and Heslop,H.E. (2004) Cytotoxic T Lymphocyte Therapy for Epstein-Barr Virus+ Hodgkin's Disease *J.Exp.Med.* **200**: 1623-1633.

Bornkamm,G.W. (2001) Molecular virology of EpsteinΓÇôBarr virus *Philosophical Transactions of the Royal Society B: Biological Sciences* **356**: 437-459.

Borza,C.M., Hutt-Fletcher,L.M. (2002) Alternate replication in B cells and epithelial cells switches tropism of Epstein-Barr virus *Nat Med* **8**: 594-599.

Borza,C.M., Morgan,A.J., Turk,S.M., and Hutt-Fletcher,L.M. (2004) Use of gHgL for Attachment of Epstein-Barr Virus to Epithelial Cells Compromises Infection *J.Virol.* **78**: 5007-5014.

Boyer,J.L., Swaminathan,S., and Silverstein,S.J. (2002) The Epstein-Barr Virus SM Protein Is Functionally Similar to ICP27 from Herpes Simplex Virus in Viral Infections *J.Virol.* **76**: 9420-9433.

Brauninger,A., Schmitz,R., Bechtel,D., Renne,C., Hansmann,M.L., and Kuppers,R. (2006) Molecular biology of Hodgkin's and Reed/Sternberg cells in Hodgkin's lymphoma *Int J Cancer* **118**: 1853-1861.

Brooks,L., Yao,Q.Y., Rickinson,A.B., and Young,L.S. (1992b) Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts *J.Virol.* **66**: 2689-2697.

Brooks,L., Yao,Q.Y., Rickinson,A.B., and Young,L.S. (1992a) Epstein-Barr virus latent gene transcription in nasopharyngeal carcinoma cells: coexpression of EBNA1, LMP1, and LMP2 transcripts *J.Virol.* **66**: 2689-2697.

Brooks,L.A., Lear,A.L., Young,L.S., and Rickinson,A.B. (1993) Transcripts from the Epstein-Barr virus BamHI A fragment are detectable in all three forms of virus latency *J.Virol.* **67**: 3182-3190.

Brown,M.H., Boles,K., Anton van der Merwe,P., Kumar,V., Mathew,P.A., and Neil Barclay,A. (1998) 2B4, the Natural Killer and T Cell Immunoglobulin Superfamily Surface Protein, Is a Ligand for CD48 *J.Exp.Med.* **188**: 2083-2090.

Brumbaugh,J., Baldwin,J., Stinson,E., Oyer,P., Jamieson,S., Bieber,C., Henle,W., and Shumway,N. (1985) Quantitative analysis of immunosuppression in cyclosporine-treated heart transplant patients with lymphoma *Journal of Heart and Lung Transplantation* **4**: 307-311.

Burkhardt,A.L., Bolen,J.B., Kieff,E., and Longnecker,R. (1992) An Epstein-Barr virus transformation-associated membrane protein interacts with src family tyrosine kinases *J.Virol.* **66**: 5161-5167.

Burkitt,D.P. (1958) A sarcoma involving the jaws of African children *British Journal of Surgery* **46**: 218-223.

Burkitt,D.P. (1983) The discovery of Burkitt's lymphoma *Cancer* **51**: 1777-1786.

Burkitt,D.P., O'conor,G.T. (1961) Malignant lymphoma in African children. I. A clinical syndrome *Cancer* **14**: 258-259.

Burns,D.M., Crawford,D.H. (2004) Epstein-Barr virus-specific cytotoxic T-lymphocytes for adoptive immunotherapy of post-transplant lymphoproliferative disease *Blood Reviews* **18**: 193-209.

Burrows,S.R., Sculley,T.B., Misko,I.S., Schmidt,C., and Moss,D.J. (1990) An Epstein-Barr virus-specific cytotoxic T cell epitopes in EBV nuclear antigen 3 (EBNA 3) *Journal of Experimental Medicine* **171**: 345-349.

Cahir-McFarland,E., Kieff,E. (2005) Epstein-Barr virus latent infection membrane protein one. In *Epstein-Barr Virus*. Robertson,E.S. (ed). Caister Academic Press, pp. 553-570.

Cahir-McFarland,E.D., Carter,K., Rosenwald,A., Giltnane,J.M., Henrickson,S.E., Staudt,L.M., and Kieff,E. (2004) Role of NF-{kappa}B in Cell Survival and Transcription of Latent Membrane Protein 1-Expressing or Epstein-Barr Virus Latency III-Infected Cells *J.Virol.* **78**: 4108-4119.

Cahir-McFarland,E.D., Davidson,D.M., Schauer,S.L., Duong,J., and Kieff,E. (2000) NF-kappa B inhibition causes spontaneous apoptosis in Epstein-Barr virus-transformed lymphoblastoid cells *PNAS* **97**: 6055-6060.

Caldwell,R.G., Wilson,J.B., Anderson,S.J., and Longnecker,R. (1998) Epstein-Barr Virus LMP2A Drives B Cell Development and Survival in the Absence of Normal B Cell Receptor Signals *Immunity* **9**: 405-411.

Calender,A., Billaud,M., Aubry,J.P., Banchereau,J., Vuillaume,M., and Lenoir,G.M. (1987) Epstein-Barr Virus (EBV) Induces Expression of B-Cell Activation Markers on in vitro Infection of EBV-Negative B-Lymphoma Cells *PNAS* **84**: 8060-8064.

Callan,M.F.C., Tan,L., Annels,N., Ogg,G.S., Wilson,J.D.K., O'Callaghan,C.A., Steven,N., McMichael,A.J., and Rickinson,A.B. (1998a) Direct Visualization of Antigen-specific CD8+ T Cells during the Primary Immune Response to Epstein-Barr Virus In Vivo *J.Exp.Med.* **187**: 1395-1402.

Callan,M.F.C., Annels,N.E., Steven,N., Tan,L., Wilson,J., McMichael,A.J., and Rickinson,A.B. (1998b) T cell selection during the evolution of CD8+ T cell memory i*n vivo European journal of Immunology* **28**: 4382-4390.

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

Callan,M., Steven,N., and Krausa,P. (1996) Large clonal expansions of CD8+ T cells in acute infectious mononucleosis *Nat Med* **2**: 906-911.

Carbone,A., Gaidano,G., Gloghini,A., Larocca,L.M., Capello,D., Canzonieri,V., Antinori,A., Tirelli,U., Falini,B., and Dalla-Favera,R. (1998) Differential Expression of BCL-6, CD138/Syndecan-1, and Epstein-Barr Virus-Encoded Latent Membrane Protein-1 Identifies Distinct Histogenetic Subsets of Acquired Immunodeficiency Syndrome-Related Non-Hodgkin's Lymphomas *Blood* **91**: 747-755.

Cen,H., Williams,P.A., McWilliams,H.P., Breinig,M.C., Ho,M., and McKnight,J.L. (1993) Evidence for restricted Epstein-Barr virus latent gene expression and anti-EBNA antibody response in solid organ transplant recipients with posttransplant lymphoproliferative disorders *Blood* **81**: 1393-1403.

Chadburn,A., Cesarman,E., and Knowles,D. (1997) Molecular pathology of posttransplantation lymphoproliferative disorders *Seminars in Diagnostic Pathology* **14**: 15-26.

Chakraverty,R., Sykes,M. (2007) The role of antigen-presenting cells in triggering graft-versus-host disease and graft-versus-leukemia *Blood* **110**: 9-17.

Chan,F.K.-M., Siegel,R.M., and Lenardo,M.J. (2000) Signaling by the TNF Receptor Superfamily and T Cell Homeostasis *Immunity* **13**: 419-422.

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

Chaplin,D.D., Fu,Y. (1998) Cytokine regulation of secondary lymphoid organ development *Current Opinion in Immunology* **10**: 289-297.

Cheadle,E.J., Gilham,D.E., Thistlethwaite,F.C., Radford,J.A., and Hawkins,R.E. (2005) Killing of non-Hodgkin lymphoma cells by autologous CD19 engineered T cells *Br.J.Haematol* **129**: 322-332.

Cheng-chan Lu, Jung-Chin Chen, Sen-Tien Tsai, Ying-Tai Jin, Jui-ChenTsai, Shih-Huang Chan, and Ih-Jen Su (2005) Nasopharyngeal carcinoma-susceptibility locus is localised to a 132 kb segment containing HLA-A using high-resolution microsatellite mapping *International Journal of Cancer* **115**: 742-746.

Chiang,A.K., T, Tao,Q., Srivastava,G., and Ho,F.C. (1996) Nasal NK- and T-celllymphomas share the same type of Epstein-Barr virus latency as nasopharangeal carcinoma and Hodgkin's disease *Int J Cancer* **68**: 285-290.

Choquet,S., Leblond,V., Herbrecht,R., Socie,G., Stoppa,A.M., Vandenberghe,P., Fischer,A., Morschhauser,F., Salles,G., Feremans,W., Vilmer,E., Peraldi,M.N., Lang,P., Lebranchu,Y., Oksenhendler,E., Garnier,J.L., Lamy,T., Jaccard,A., Ferrant,A., Offner,F., Hermine,O., Moreau,A., Fafi-Kremer,S., Morand,P., Chatenoud,L., Berriot-Varoqueaux,N., Bergougnoux,L., and Milpied,N. (2006) Efficacy and safety of rituximab in B-cell post-transplantation lymphoproliferative disorders: results of a prospective multicenter phase 2 study *Blood* **107**: 3053-3057.

Coates,P., Slavin,G., and D'Ardenne,A. (1991) Persistence of Epstein-Barr virus in Reed-Sternberg cells throughout the course of Hodgkin's disease *Journal of Pathology* **164**: 291-297.

Cockfield,S.M. (2001) Identifying the patient at risk for post-transplant lymphoproliferative disorder *Transplant Infectious Disease* **3**: 70-78.

Coffey,A.J., Brooksbank,R.A., Brandau,O., Oohashi,T., Howell,G.R., Bye,J.M., Cahn,A.P., Durham,J., Heath,P., Wray,P., Pavitt,R., Wilkinson,J., Leversha,M., Huckle,E., Shaw-Smith,C.J., Dunham,A., Rhodes,S., Schuster,V., Porta,G., Yin,L., Serafini,P., Sylla,B., Zollo,M., Franco,B., Bolino,A., Seri,M., Lanyi,A., Davis,J.R., Webster,D., Harris,A., Lenoir,G., St Basile,G., Jones,A., Behloradsky,B.H., Achatz,H., Murken,J., Fassler,R., Sumegi,J., Romeo,G., Vaudin,M., Ross,M.T., Meindl,A., and Bentley,D.R. (1998) Host response to EBV infection in X-linked lymphoproliferative disease results from mutations in an SH2-domain encoding gene *Nat Genet* **20**: 129-135.

Cohen,J.I. (2005) Clinical aspects of Epstein-Barr virus infection. In *Epstein-Barr Virus*. Robertson,E.S. (ed). Caister Academic Press, pp. 35-54.

Cohen,J.I., Lekstrom,K. (1999) Epstein-Barr Virus BARF1 Protein Is Dispensable for B-Cell Transformation and Inhibits Alpha Interferon Secretion from Mononuclear Cells *J.Virol.* **73**: 7627-7632.

Cohen,J. (2006) Epstein-Barr Virus Infection *N Engl J Med* **343**: 481-492.

Comoli,P., De Palma,R., Siena,S., Nocera,A., Basso,S., Del Galdo,F., Schiavo,R., Carminati,O., Tagliamacco,A., Abbate,G.F., Locatelli,F., Maccario,R., and Pedrazzoli,P. (2004) Adoptive transfer of allogeneic Epstein-Barr virus (EBV)-specific cytotoxic T cells with in vitro antitumor activity boosts LMP2-specific immune response in a patient with EBV-related nasopharyngeal carcinoma *Ann Oncol* **15**: 113-117.

Comoli,P., Maccario,R., Locatelli,F., Valente,U., Basso,S., Garaventa,A., Toma,P., Botti,G., Melioli,G., Baldanti,F., Nocera,A., Perfumo,F., and Ginevri,F. (2005a) Treatment of EBV-Related Post-Renal Transplant Lymphoproliferative Disease with a Tailored Regimen Including EBV-Specific T Cells *American Journal of Transplantation* **5**: 1415-1422.

Comoli,P., Pedrazzoli,P., Maccario,R., Basso,S., Carminati,O., Labirio,M., Schiavo,R., Secondino,S., Frasson,C., Perotti,C., Moroni,M., Locatelli,F., and Siena,S. (2005b) Cell Therapy of Stage IV Nasopharyngeal Carcinoma With Autologous Epstein-Barr Virus-Targeted Cytotoxic T Lymphocytes *J Clin Oncol* **23**: 8942-8949.

Cooper,M.A., Fehniger,T.A., and Caligiuri,M.A. (2001) The biology of human natural killer-cell subsets *Trends in Immunology* **22**: 633-640.

Cousens,L.P., Peterson,R., Hsu,S., Dorner,A., Altman,J.D., Ahmed,R., and Biron,C.A. (1999) Two Roads Diverged: Interferon alpha /beta - and Interleukin 12-mediated Pathways in Promoting T Cell Interferon gamma áResponses during Viral Infection *J.Exp.Med.* **189**: 1315-1328.

Cox,M., Leahy,J., and Hardwick,M. (1990) An enhancer within the divergent promoter of Epstein-Barr virus responds synergistically to the R and Z transactivators *J.Virol* **64**: 313-323.

Crawford,D.H. (2001) Biology and disease associations of Epstein-Barr virus *Philosophical Transactions of the Royal Society B: Biological Sciences* **356**: 461-473.

Crawford,D.H., Ando,I. (1986) EB induction is associated with B-cell maturation *Immunology* **59**: 405-409.

Crawford,D.H., Macsween,K.F., Higgins,C.D., Thomas,R., McAulay,K., Williams,H., Harrison,N., Reid,S., Conacher,M., Douglas,J., and Swerdlow,A.J. (2006) A Cohort Study among University Students: Identification of Risk Factors for Epstein-Barr Virus Seroconversion and Infectious Mononucleosis *Clin Infect Dis* **43**: 276-282.

Crawford,D.H., Swerdlow,A.J., Higgins,C.D., McAulay,K., Harrison,N., Williams,H., Britton,K., and Macsween,K.F. (2002) Sexual History and Epstein-Barr Virus Infection *J Infect Dis* **186**: 731-736.

Crotzer,V.L., Christian,R.E., Brooks,J.M., Shabanowitz,J., Settlage,R.E., Marto,J.A., White,F.M., Rickinson,A.B., Hunt,D.F., and Engelhard,V.H. (2000) Immunodominance Among EBV-Derived Epitopes Restricted by HLA-B27 Does Not Correlate with Epitope Abundance in EBV-Transformed B-Lymphoblastoid Cell Lines *J Immunol* **164**: 6120-6129.

Davenport,M.G., Pagano,J.S. (1999) Expression of EBNA-1 mRNA Is Regulated by Cell Cycle during Epstein-Barr Virus Type I Latency *J.Virol.* **73**: 3154-3161.

Davison,A.J. (2007) Overview of classification. In *Human herpesviruses: biology, therapy, and immunoprophylaxis*. Arvin,A., Campadelli-Fiume,G., Mocarski,E., Moore,P.S., Roizman,B., Whitley,R., and Yamanishi,K. (eds). Cambridge University Press, pp. 3-9.

de Jesus,O., Smith,P.R., Spender,L.C., Elgueta Karstegl,C., Niller,H.H., Huang,D., and Farrell,P.J. (2003) Updated Epstein-Barr virus (EBV) DNA sequence and analysis of a promoter for the BART (CST, BARF0) RNAs of EBV *J Gen Virol* **84**: 1443-1450.

de-The,G. (1977) Is Burkitt's lymphoma related to perinatal infection by Epstein-Barr virus *Lancet* **1**: 335-338.

de-The,G., Geser,A., Day,N.E., Tukei,P.H., Williams,E.H., Beri,D.P., Smith,P.G., Dean,A.G., Bronkamm,G.W., Feorino,P., and Henle,W. (1978) Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphoma from Ugandan prospective study *Nature* **274**: 756-761.

Deacon,E., Pallesen,G., Niedobitek,G., Crocker,J., Brooks,L., Rickenson,A., and Young,L. (1993) Epstein-Barr virus and Hodgkin's disease: transcriptional analysis of virus latency in the malignant cells *J exp med* **177**: 339-349.

Delcayre,A.X., Lotz,M., and Lernhardt,W. (1993) Inhibition of Epstein-Barr virus-mediated capping of CD21/CR2 by alpha interferon (IFN-alpha): immediate antiviral activity of IFN-alpha during the early phase of infection *J.Virol.* **67**: 2918-2921.

Diepstra,A., Niens,M., Vellenga,E., van Imhoff,G.W., Nolte,I.M., Schaapveld,M., van der Steege,G., van den Berg,A., Kibbelaar,R.E., te Meerman,G.J., and Poppema,S. (2005) Association with HLA class I in Epstein-Barr-virus-positive and with HLA class III in Epstein-Barr-virus-negative Hodgkin's lymphoma *The Lancet* **365**: 2216-2224.

Dinand,V., Dawar,R., Arya,L.S., Unni,R., Mohanty,B., and Singh,R. (2007) Hodgkin's lymphoma in Indian children: Prevalence and significance of Epstein-Barr virus detection in Hodgkin's and Reed-Sternberg cells *European Journal of Cancer* **43**: 161-168.

Dirmeier,U., Hoffmann,R., Kilger,E., Schultheiss,U., Briseno,C., Gires,O., Kieser,A., Eick,D., Sugden,B., and Hammerschmidt,W. (2005) Latent membrane protein 1 of Epstein-Barr virus coordinately regulates proliferation with control of apoptosis *Oncogene* **24**: 1711-1717.

Dirmeier,U., Neuhierl,B., Kilger,E., Reisbach,G., Sandberg,M.L., and Hammerschmidt,W. (2003) Latent Membrane Protein 1 Is Critical for Efficient Growth Transformation of Human B Cells by Epstein-Barr Virus *Cancer Res* **63**: 2982-2989.

Elia,A., Vyas,J., Laing,K.G., and Clemens,M.J. (2004) Ribosomal protein L22 inhibits regulation of cellular activities by the Epstein-Barr virus small RNA EBER-1 *European Journal of Biochemistry* **271**: 1895-1905.

Elliott,S.L., Suhrbier,A., Miles,J.J., Lawrence,G., Pye,S.J., Le,T.T., Rosenstengel,A., Nguyen,T., Allworth,A., Burrows,S.R., Cox,J., Pye,D., Moss,D.J., and Bharadwaj,M. (2007) A Phase I trial of a CD8+ T Cell Peptide Epitope-based Vaccine for Infectious Mononucleosis *J.Virol.*: JVI.

Epstein,M.A., Achong,B.G., and Barr,Y.M. (1964) Virus particles in cultured lymphoblasts from Burkitt's lymphoma *Lancet* **i**: 702-703.

Epstein,M.A., Barr,Y.M. (1964) Cultivation *in vitro* of human lymphoblasts from Burkitt's malignant lymphoma *Lancet* **i**: 252-253.

Epstein,M.A., Henle,G., Achong,B.G., and Barr,Y.M. (1965) Morphological and iological studies on a virus in cultured lymphoblasts from Burkitt's lymphoma *Journal of Experimental Medicine* **121**: 761-770.

Evans,A.S., Niederman,J.C., and McCollum,R.W. (1968) Seroepidemiological studies of infectious mononucleosis with EB virus *New England Journal of Medicine* **279**: 1121-1127.

Fafi-Kremer,S., Morand,P., Brion,J.–P., Pavese,P., Baccard,M., Germi,R., Genoulaz,O., Nicod,S., Jolivet,M., Ruigrok,R., Stahl,J.–P., and Seigneurin,J.–M. (2005a) Long–Term Shedding of Infectious Epstein–Barr Virus after Infectious Mononucleosis *The Journal of Infectious Diseases* **191**: 985-989.

Fafi-Kremer,S., Morand,P., Germi,R., Ballout,M., Brion,J.–P., Genoulaz,O., Nicod,S., Stahl,J.–P., Ruigrok,R., and Seigneurin,J.–M. (2005b) A Prospective Follow–Up of Epstein–Barr Virus LMP1 Genotypes in Saliva and Blood during Infectious Mononucleosis *The Journal of Infectious Diseases* **192**: 2108-2111.

Fahmi,H., Cochet,C., Hmama,Z., Opolon,P., and Joab,I. (2000) Transforming Growth Factor Beta 1 Stimulates Expression of the Epstein-Barr Virus BZLF1 Immediate-Early Gene Product ZEBRA by an Indirect Mechanism Which Requires the MAPK Kinase Pathway *J.Virol.* **74**: 5810-5818.

Fanning,G.C., Bunce,M., Black,C.M., and Welsh,K.I. (1997) Polymerase chain reaction haplotyping using 3' mismatches in the forward and reverse primers: application to the biallelic polymorphisms of tumor necrosis factor and lymphotoxin alpha *Tissue Antigens* **50**: 23-31.

Faulkner,G.C., Burrows,S.R., Khanna,R., Moss,D.J., Bird,A.G., and Crawford,D.H. (1999) X-Linked Agammaglobulinemia Patients Are Not Infected with Epstein-Barr Virus: Implications for the Biology of the Virus *J.Virol.* **73**: 1555-1564.

Faulkner,G.C., Krajewski,A.S., and Crawford,D.H. (2000) The ins and outs of EBV infection *Trends in Microbiology* **8**: 185-189.

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

Fennewald,S., van Santen,V., and Kieff,E. (1984) Nucleotide sequence of an mRNA transcribed in latent growth-transforming virus infection indicates that it may encode a membrane protein *J.Virol.* **51**: 411-419.

Ferlazzo,G., Munz,C. (2004) NK Cell Compartments and Their Activation by Dendritic Cells *J Immunol* **172**: 1333-1339.

Ferlazzo,G., Pack,M., Thomas,D., Paludan,C., Schmid,D., Strowig,T., Bougras,G., Muller,W.A., Moretta,L., and Munz,C. (2004a) Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs *PNAS* **101**: 16606-16611.

Ferlazzo,G., Thomas,D., Lin,S.L., Goodman,K., Morandi,B., Muller,W.A., Moretta,A., and Munz,C. (2004b) The Abundant NK Cells in Human Secondary Lymphoid Tissues Require Activation to Express Killer Cell Ig-Like Receptors and Become Cytolytic *J Immunol* **172**: 1455-1462.

Ferlazzo,G., Tsang,M.L., Moretta,L., Melioli,G., Steinman,R.M., and Munz,C. (2002) Human Dendritic Cells Activate Resting Natural Killer (NK) Cells and Are Recognized via the NKp30 Receptor by Activated NK Cells *J.Exp.Med.* **195**: 343-351.

Fernandez-Mestre,M.T., Gendzekhadze,K., Rivas-Vetencourt,P., and Layrisse,Z. (2004) TNF-alpha-308A allele, a possible severity risk factor of hemorrhagic manifestation in dengue fever patients *Tissue Antigens* **64**: 469-472.

Feuillard,J., Schuhmacher,M., Kohanna,S., Asso-Bonnet,M., Ledeur,F., Joubert-Caron,R., Bissieres,P., Polack,A., Bornkamm,G.W., and Raphael,M. (2000) Inducible loss of NF-kappa B activity is associated with apoptosis and Bcl-2 down-regulation in Epstein-Barr virus-transformed B lymphocytes *Blood* **95**: 2068-2075.

Fingeroth,J.D., Weis,J.J., Tedder,T.F., Strominger,J.L., Biro,P.A., and Fearon,D.T. (1984) Epstein--Barr Virus Receptor of Human B Lymphocytes is the C3d Receptor CR2 *PNAS* **81**: 4510-4514.

Flemington,E.K., Goldfeld,A.E., and Speck,S.H. (1991) Efficient transcription of the Epstein-Barr virus immediate-early BZLF1 and BRLF1 genes requires protein synthesis *J.Virol.* **65**: 7073-7077.

Foss,H.D., Herbst,H., Hummel,M., Araujo,I., Latza,U., Rancso,C., Dallenbach,F., and Stein,H. (1994) Patterns of cytokine gene expression in infectious mononucleosis *Blood* **83**: 707-712.

Foster,A.E., Marangolo,M., Sartor,M.M., Alexander,S.I., Hu,M., Bradstock,K.F., and Gottlieb,D.J. (2004) Human CD62L- memory T cells are less responsive to alloantigen

stimulation than CD62L+ naive T cells: potential for adoptive immunotherapy and allodepletion *Blood* **104**: 2403-2409.

Frade,R., Barel,M., Ehlin-Henriksson,B., and Klein,G. (1985) Gp140, the C3d Receptor of Human B Lymphocytes, is Also the Epstein--Barr Virus Receptor *PNAS* **82**: 1490-1493.

Frahm,N., Adams,S., Kiepiela,P., Linde,C., Hewitt,H., Lichterfeld,M., Sango,K., Brown,N., Pae,E., Wureel,A., Altfeld,M., Feeney,M., Allen,T., Roach,T., St John,M., Daar,E., Rosenberg,E., Korber,B., Marincola,F., Walker,B., and Goulder,P. (2005) HLA-B63 presents HLA-B57/B58-restricted cytotxic T-lymphocyte epitopes and is associated with low human immunodeficiency virus load *J.Virol* **79**: 10218-10225.

Fu,T., Voo,K.S., and Wang,R.F. (2004) Critical role of EBNA1-specific CD4+ T cells in the control of mouse Burkitt lymphoma in vivo *J.Clin.Invest.* **114**: 542-550.

Fu,Z., Cannon,M.J. (2000) Functional Analysis of the CD4+ T-Cell Response to Epstein-Barr Virus: T-Cell-Mediated Activation of Resting B Cells and Induction of Viral BZLF1 Expression *J.Virol.* **74**: 6675-6679.

Gandhi,M.K., Wilkie,G.M., Dua,U., Mollee,P.N., Grimmett,K., Williams,T., Whitaker,N., Gill,D., and Crawford,D.H. (2007) Immunity, Homing and Efficacy of Allogeneic Adoptive Immunotherapy for Posttransplant Lymphoproliferative Disorders *American Journal of Transplantation* **7**: 1293-1299.

Gao,Y., Xue,S.a., and Griffin,B.E. (1999) Sensitivity of an Epstein-Barr Virus-Positive Tumor Line, Daudi, to Alpha Interferon Correlates with Expression of a GC-Rich Viral Transcript *Mol.Cell.Biol.* **19**: 7305-7313.

Gerber,P., Lucas,S., Nonoyama,M., Oerlin,E., and Goldstein,L. (1972) Oral excretion of Epstein-Barr virus by healthy subjects and patients with infectious mononucleosis *Lancet* **2**: 988-989.

Gerosa,F., Baldani-Guerra,B., Nisii,C., Marchesini,V., Carra,G., and Trinchieri,G. (2002) Reciprocal Activating Interaction between Natural Killer Cells and Dendritic Cells *J.Exp.Med.* **195**: 327-333.

Gill,M.B., Roecklein-Canfield,J., Sage,D.R., Zambela-Soediono,M., Longtine,N., Uknis,M., and Fingeroth,J.D. (2004) EBV attachment stimulates FHOS/FHOD1 redistribution and co-aggregation with CD21: formin interactions with the cytoplasmic domain of human CD21 *J Cell Sci* **117**: 2709-2720.

Gires,O., Zimber-Strobl,U., Gonnella,R., Ueffing,M., Marschall,G., Zeidler,R., Pich,D., and Hammerschmidt,W. (1997) Latent membrane protein 1 of Epstein-Barr virus mimics a constitutively active receptor molecule *EMBO* **16**: 6131-6140.

Glaser,S., Lin,R., Stewart,S., Ambinder,R., Jarrett,R., Brousset,P., Pallesen,G., Gulley,M., Khan,G., O'Grady,J., Hummel,M., Preclado,M., Knecht,H., Chan,J., and Claviez,A. (1997) Epstein-Barr virus-associated Hodgkin's disease: epidemiologic characteristics in international data. *Int J Cancer* **70**: 375-382.

Gottschalk,S., Heslop,H., and Rooney,C. (2005) Adoptive Immunotherapy for EBV-associated Malignancies *Leukemia & Lymphoma* **46**: 1-10.

Gottschalk,S., Edwards,O.L., Sili,U., Huls,M.H., Goltsova,T., Davis,A.R., Heslop,H.E., and Rooney,C.M. (2003) Generating CTLs against the subdominant Epstein-Barr virus LMP1

antigen for the adoptive immunotherapy of EBV-associated malignancies *Blood* **101**: 1905-1912.

Gottschalk,S., Ng,C.Y.C., Perez,M., Smith,C.A., Sample,C., Brenner,M.K., Heslop,H.E., and Rooney,C.M. (2001) An Epstein-Barr virus deletion mutant associated with fatal lymphoproliferative disease unresponsive to therapy with virus-specific CTLs *Blood* **97**: 835-843.

Grasser,F.A., Murray,P.G., Kremmer,E., Klein,K., Remberger,K., Feiden,W., Reynolds,G., Niedobitek,G., Young,L.S., and Mueller-Lantzsch,N. (1994) Monoclonal antibodies directed against the Epstein-Barr virus-encoded nuclear antigen 1 (EBNA1): immunohistologic detection of EBNA1 in the malignant cells of Hodgkin's disease *Blood* **84**: 3792-3798.

Gratama,J.W., Ernberg,I. (1995) Molecular epidemiology of Epstein-Barr infection *Adv Cancer Res* **67**: 197-255.

Gratama,J.W., Oosterveer,M.A.P., Zwaan,F.E., Lepoutre,J., Klein,G., and Ernberg,I. (1988) Eradication of Epstein-Barr Virus by Allogeneic Bone Marrow Transplantation: Implications for Sites of Viral Latency *PNAS* **85**: 8693-8696.

Greenspan,J.S., Greenspan,D., Lennette,E.T., Abrams,D.I., Conant,M.A., Petersen,V., and Freese,U.K. (1985) Replication of Epstein-Barr virus within the epithelial cells of oral 'hairy' leukoplakia, an AIDS-associated lesion *New England Journal of Medicine* **313**: 1564-1571.

Gregory,C.D., Tursz,T., Edwards,C.F., Tetaud,C., Talbot,M., Caillou,B., Rickinson,A.B., and Lipinski,M. (1987) Identification of a subset of normal B cells with a Burkitt's lymphoma (BL)-like phenotype *J Immunol* **139**: 313-318.

Griffin,B.E., Rochford,R. (2005) Endemic Burkitt's lymphoma. In *Epstein-Barr Virus*. Robertson,E.S. (ed). Caister Academic Press, pp. 113-138.

Gross,G., Eshhar,Z. (1992) Endowing T cells with antibody specificity using chimeric T cell receptors *FASEB J.* **6**: 3370-3378.

Grossman,S.R., Johannsen,E., Tong,X., Yalamanchili,R., and Kieff,E. (1994) The Epstein-Barr Virus Nuclear Antigen 2 Transactivator is Directed to Response Elements by the J{kappa} Recombination Signal Binding Protein *PNAS* **91**: 7568-7572.

Grutters,J.C., Sato,H., Pantelidis,P., Ruven,H.J., McGrath,D.S., Wells,A.U., van den Bosch,J.M., Welsh,K.I., and du Bois,R.M. (2003) Analysis of IL6 and IL1A gene polymorphisms in UK and Dutch patients with sarcoidosis. *Sarcoidosis Vasc Diffuse Lung Dis.* **20**: 20-27.

Grutters,J.C., Sato,H., Pantelidis,P., Lagan,A.L., McGrath,D.S., Lammers,J.W., van den Bosch,J.M.M., Wells,A.U., du Bois,R.M., and Welsh,K.I. (2002) Increased Frequency of the Uncommon Tumor Necrosis Factor -857T Allele in British and Dutch Patients with Sarcoidosis *Am.J.Respir.Crit.Care Med.* **165**: 1119-1124.

Gudgeon,N.H., Taylor,G.S., Long,H.M., Haigh,T.A., and Rickinson,A.B. (2005) Regression of Epstein-Barr Virus-Induced B-Cell Transformation In Vitro Involves Virus-Specific CD8+ T Cells as the Principal Effectors and a Novel CD4+ T-Cell Reactivity *J.Virol.* **79**: 5477-5488.

Gutensohn,N., Cole,P. (1980) Epidemiology of Hodgkin's disease *Seminars in Oncology* **7**: 92-102.

Haan,K.M., Kwok,W.W., Longnecker,R., and Speck,P. (2000) Epstein-Barr Virus Entry Utilizing HLA-DP or HLA-DQ as a Coreceptor *J.Virol.* **74**: 2451-2454.

Haddad,R.S., Hutt-Fletcher,L.M. (1989) Depletion of glycoprotein gp85 from virosomes made with Epstein-Barr virus proteins abolishes their ability to fuse with virus receptor-bearing cells *J.Virol.* **63**: 4998-5005.

Hallee,T., Evans,A., Niederman,J., Brooks,C., and Voegtly,J. (1974) Infectious mononucleosis at the US Military Academy: a prospective study of a single class over four years *Yale J Biol Med* **47**: 182-195.

Hammerschmidt,W., Sugden,B. (1989) Genetic analysis of immortalizing functions of Epstein-ûBarr virus in human B lymphocytes *Nature* **340**: 393-397.

Haque,T., Wilkie,G.M., Taylor,C., Amlot,P.L., Munrad,P., Iley,A., Dombagoda,D., Britton,K.M., Swerdlow,A.J., and Crawford,D.H. (2002) Treatment of Epstein-Barr-virus-positive post-transplantation lymphoproliferative disease with partly HLA-matched allogeneic cytotoxic T cells *The Lancet* **360**: 436-442.

Haque,T., Wilkie,G.M., Jones,M.M., Higgins,C.D., Urquhart,G., Wingate,P., Burns,D., McAulay,K., Turner,M., Bellamy,C., Amlot,P.L., Kelly,D., MacGilchrist,A., Gandhi,M.K., Swerdlow,A.J., and Crawford,D.H. (2007) Allogeneic cytotoxic T-cell therapy for EBV-positive posttransplantation lymphoproliferative disease: results of a phase 2 multicenter clinical trial *Blood* **110**: 1123-1131.

Harris,N.L., Jaffe,E.S., Stein,H., Banks,P.M., Chan,J.K., Cleary,M.L., Delsol,G., De,W., Falini,B., and Gatter,K.C. (1994) A revised European-American classification of lymphoid neoplasms: a proposal from the International Lymphoma Study Group [see comments] *Blood* **84**: 1361-1392.

Hatzivassiliou,E., Miller,W.E., Raab-Traub,N., Kieff,E., and Mosialos,G. (1998) A Fusion of the EBV Latent Membrane Protein-1 (LMP1) Transmembrane Domains to the CD40 Cytoplasmic Domain Is Similar to LMP1 in Constitutive Activation of Epidermal Growth Factor Receptor Expression, Nuclear Factor-{kappa}B, and Stress-Activated Protein Kinase *J Immunol* **160**: 1116-1121.

Hayward,S.D. (2004) Viral interactions with the Notch pathway *Seminars in Cancer Biology* **14**: 387-396.

Heath,C.W., Jr., Brodsky,A.L., and Potolosky,A.I. (1972) Infectious mononucleosis in a general population *Am.J.Epidemiol.* **95**: 46-52.

Hecht,J.L., Aster,J.C. (2000) Molecular Biology of Burkitt's Lymphoma *J Clin Oncol* **18**: 3707-3721.

Helminen,M., Lahdenpohja,N., and Hurme,M. (1999) Polymorphism of the Interleukin-10 gene is associated with susceptibility to Epstein-Barr virus infection. *Journal of Infectious Disease* **180**: 496-499.

Henderson,S., Huen,D., Rowe,M., Dawson,C., Johnson,G., and Rickinson,A. (1993) Epstein-Barr Virus-Coded BHRF1 Protein, a Viral Homologue of Bcl-2, Protects Human B Cells from Programmed Cell Death *PNAS* **90**: 8479-8483.

Henderson,S., Rowe,M., Gregory,C., Croom-Carter,D., Wang,F., Longnecker,R., Kieff,E., and Rickinson,A. (1991) Induction of bcl-2 expression by epstein-barr virus latent membrane protein 1 protects infected B cells from programmed cell death *Cell* **65**: 1107-1115.

Henle,G., Henle,W. (1976) Epstein-Barr virus-specific IgA serum antibodies as an outstanding feature of nasopharangeal carcinoma *Int J Cancer* **17**: 1-7.

Henle,G., Henle,W., and Diehl,V. (1968) Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis *PNAS* **59**: 94-101.

Henle,W., Henle,G., Andersson,J., Ernberg,I., Klein,G., Horwitz,C.A., Marklund,G., Rymo,L., Wellinder,C., and Straus,S.E. (1987) Antibody Responses to Epstein--Barr Virus-Determined Nuclear Antigen (EBNA)-1 and EBNA-2 in Acute and Chronic Epstein--Barr Virus Infection *PNAS* **84**: 570-574.

Herbst,H., Foss,H.D., Samol,J., Araujo,I., Klotzbach,H., Krause,H., Agathanggelou,A., Niedobitek,G., and Stein,H. (1996) Frequent expression of interleukin-10 by Epstein-Barr virus-harboring tumor cells of Hodgkin's disease *Blood* **87**: 2918-2929.

Heslop,H., Ng CY, Li C, Smith CA, Loftin S, Krance,R., Brenner,M., and Rooney,C. (1996) Long term restoration of immunity against Epstein-Barr virus infections by adoptive transfer of gene-modified virus specific T lymphocytes *Nat Med* **2**: 551-555.

Hesse,J., Ibsen,K.K., Krabbe,S., and Uldall,P. (1983) Prevalence of antibodies to Epstein-Barr virus (EBV) in childhood and adolescence in Denmark *Scand J Infect Dis* **15**: 335-358.

Higgins,C.D., Swerdlow,A.J., Macsween,K.F., Harrison,N., Williams,H., McAulay,K., Thomas,R., Reid,S., Conacher,M., Britton,K., and Crawford,D.H. (2007) A Study of Risk Factors for Acquisition of Epstein-Barr Virus and Its Subtypes *The Journal of Infectious Diseases* **195**: 474-482.

Hill,A.B., Lee,S.P., Haurum,J.S., Murray,N., Yao,Q.Y., Rowe,M., Signoret,N., Rickinson,A.B., and McMichael,A.J. (1995) Class I major histocompatability complex-restricted cytotoxic T lymphocytes specific for Epstein-Barr virus (EBV)-transformed B lymphoblastoid cell lines against which they were raised *Journal of Experimental Medicine* **181**: 2221-2228.

Hislop,A.D., Annels,N.E., Gudgeon,N.H., Leese,A.M., and Rickinson,A.B. (2002) Epitope-specific Evolution of Human CD8+ T Cell Responses from Primary to Persistent Phases of Epstein-Barr Virus Infection *J.Exp.Med.* **195**: 893-905.

Hislop,A.D., Gudgeon,N.H., Callan,M.F.C., Fazou,C., Hasegawa,H., Salmon,M., and Rickinson,A.B. (2001) EBV-Specific CD8+ T Cell Memory: Relationships Between Epitope Specificity, Cell Phenotype, and Immediate Effector Function *J Immunol* **167**: 2019-2029.

Hislop,A.D., Taylor,G.S., Sauce,D., and Rickinson,A.B. (2007) Cellular Responses to Viral Infection in Humans: Lessons from Epstein-Barr Virus *Ann Rev Immunol* **25**: 587-617.

Hjalgrim,H., Askling,J., Rostgaard,K., Hamilton-Dutoit,S., Frisch,M., Zhang,J.S., Madsen,M., Rosdahl,N., Konradsen,H.B., Storm,H.H., and Melbye,M. (2003) Characteristics of Hodgkin's Lymphoma after Infectious Mononucleosis *N Engl J Med* **349**: 1324-1332.

Hjalgrim,H., Askling,J., Sorensen,P., Madsen,M., Rosdahl,N., Storm,H.H., Hamilton-Dutoit,S., Eriksen,L.S., Frisch,M., Ekbom,A., and Melbye,M. (2000) Risk of Hodgkin's Disease and Other Cancers After Infectious Mononucleosis *J Natl Cancer Inst* **92**: 1522-1528.

Hjalgrim,H., Ekstrom Smedby,K., Rostgaard,K., Molin,D., Hamilton-Dutoit,S., Chang,E.T., Ralfkiaer,E., Sundstrom,C., Adami,H.O., Glimelius,B., and Melbye,M. (2007) Infectious Mononucleosis, Childhood Social Environment, and Risk of Hodgkin Lymphoma *Cancer Res* **67**: 2382-2388.

Ho,M., Miller,G., Atchison,R., Breinig,M., Dummer,J., Andiman,W., Starzl,T., Eastman,R., Griffith,B., and Hardestry,R. (1985) Epstein-Barr virus infections and DNA hybridization studies in posttransplantation lymphoma and lymphoproliferative lesions: the role of primary infection *Journal of Infectious Disease* **152**: 876-886.

Ho,M., Schollaardt,T., Snape,S., Looareesuwan,S., Suntharasamai,P., and Whitr,N. (1998) Endogenous interleukin-10 modulates proinflammatory response in Plasmodium falciparum malaria *J Infect Dis* **1**: 2-520.

Hoagland,R.J. (1964) The incubation period of infectious mononucleus *American Journal of Public Health* **54**: 1705.

Hoffmann,G.J., Lazarowitz,S.G., and Hayward,D.S. (1980) Monoclonal antibody against a 250,000-dalton glycoprotein of Epstein-Barr virus identifies a membrane antigen and a neutralizing antigen *PNAS* **77**: 2979-2983.

Holley-Guthrie,E., Seaman,W., Bhende,P., Merchant,J., and Kenney,S. (2005) The Epstein-Barr virus protein BMRF1 activates gastrin transcription *J.Virol* **79**: 745-755.

Hopwood,P.A., Brooks,L., Parratt,R., Hunt,B.J., Maria,B., Alero,T.J., Magdi,Y., and Crawford,D.H. (2002) Persistent epstein-barr virus infection: unrestricted latent and lytic viral gene expression in healthy immunosuppressed transplant recipients1 *Transplantation* **74**: 194-202.

Hurley,E.A., Thorley-Lawson,D.A. (1988) B cell activation an the establishment of Epstein-Barr virus latency *Journal of Experimental Medicine* **168**: 2059-2075.

Hurme,M., Helminen,M. (1998) Polymorphism of the IL-1 Gene Complex in Epstein-Barr Virus Seronegative and Seropositive Adult Blood Donors *Scand J Immunol* **48**: 219-222.

Hutt-Fletcher,L.M. (2007) Epstein-Barr Virus Entry *J.Virol.* **81**: 7825-7832.

Hviid,L., Jakobsen,P.H., Abu-Zeid,Y.A., and Theander,T.G. (1992) T cell responses in malaria *APMIS* **100**: 95-106.

Inman,G.J., Binne,U.K., Parker,G.A., Farrell,P.J., and Allday,M.J. (2001) Activators of the Epstein-Barr Virus Lytic Program Concomitantly Induce Apoptosis, but Lytic Gene Expression Protects from Cell Death *J.Virol.* **75**: 2400-2410.

Israele,V., Shirley,P., and Sixbey,J.W. (1991) Excretion of the Epstein-Barr virus from the genital tract of men *Journal of Infectious Disease* **163**: 1341-1343.

Iwakiri,D., Eizuru,Y., Tokunaga,M., and Takada,K. (2003) Autocrine Growth of Epstein-Barr Virus-Positive Gastric Carcinoma Cells Mediated by an Epstein-Barr Virus-Encoded Small RNA *Cancer Res* **63**: 7062-7067.

Janz,A., Oezel,M., Kurzeder,C., Mautner,J., Pich,D., Kost,M., Hammerschmidt,W., and Delecluse,H.J. (2000) Infectious Epstein-Barr Virus Lacking Major Glycoprotein BLLF1 (gp350/220) Demonstrates the Existence of Additional Viral Ligands *J.Virol.* **74**: 10142-10152.

Jarrett,R.F. (2002) Viruses and Hodgkin's lymphoma *Ann Oncol* **13**: 23-29.

Jarrett,R.F., Krajewski,A.S., Angus,B., Freeland,J., Taylor,P.R., Taylor,G.M., and Alexander,F.E. (2003) The Scotland and Newcastle epidemiological study of Hodgkin's disease: impact of histopathological review and EBV status on incidence estimates *J Clin Pathol* **56**: 811-816.

Jarrett,R.F., Stark,G.L., White,J., Angus,B., Alexander,F.E., Krajewski,A.S., Freeland,J., Taylor,G.M., Taylor,P.R.A., and for the Scotland and Newcastle Epidemiology of Hodgkin Disease Study Group (2005) Impact of tumor Epstein-Barr virus status on presenting features and outcome in age-defined subgroups of patients with classic Hodgkin lymphoma: a population-based study *Blood* **106**: 2444-2451.

Jiang,W., Szekely,L., Wendel-Hansen,V., Ringertz,N., Klein,G., and Rosen,A. (1991) Co-localization of the retinoblastoma protein and the Epstein-Barr virus-encoded nuclear antigen EBNA-5 *Exp Cell Res* **197**: 314-318.

Johannessen,I., Asghar,M., and Crawford,D.H. (2000) Essential role for T cells in human B-cell lymphoproliferative disease development in severe combined immunodeficient mice *Br.J.Haematol* **109**: 600-610.

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

Johannsen,E., Miller,C.L., Grossman,S.R., and Kieff,E. (1996) EBNA-2 and EBNA-3C extensively and mutually exclusively associate with RBPJkappa in Epstein-Barr virus-transformed B lymphocytes *J.Virol.* **70**: 4179-4183.

Joseph,A.M., Babcock,G.J., and Thorley-Lawson,D.A. (2000) Cells Expressing the Epstein-Barr Virus Growth Program Are Present in and Restricted to the Naive B-Cell Subset of Healthy Tonsils *J.Virol.* **74**: 9964-9971.

Kanangat,S., Babu,J.S., Knipe,D.M., and Rouse,B.T. (1996) HSV-1-mediated modulation of ctyokine gene expression in a permissive cell line: selective upregulation of IL-6 gene expression *Virology* **219**: 295-300.

Kelly,G.L., Milner,A.E., Tierney,R.J., Croom-Carter,D.S.G., Altmann,M., Hammerschmidt,W., Bell,A.I., and Rickinson,A.B. (2005) Epstein-Barr Virus Nuclear Antigen 2 (EBNA2) Gene Deletion Is Consistently Linked with EBNA3A, -3B, and -3C Expression in Burkitt's Lymphoma Cells and with Increased Resistance to Apoptosis *J.Virol.* **79**: 10709-10717.

Kessels,H.W.H.G., Wolkers,M.C., van den Boom,M.D., van den Valk,M.A., and Schumacher,T.N.M. (2001) Immunotherapy through TCR gene transfer *Nat Immunol* **2**: 957-961.

Khan,G., Lake,A., Shield,L., Freeland,J., Andrew,L., Alexander,F.E., Jackson,R., Taylor,P.R.A., McCruden,E.A.B., and Jarrett,R.F. (2005) Phenotype and frequency of Epstein-Barr virus-infected cells in pretreatment blood samples from patients with Hodgkin lymphoma *Br.J.Haematol* **129**: 511-519.

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

Khanna,R., Burrows,S., Nicholls,J., and Poulsen,L. (1998) Identification of cytotoxic T cell epitopes within Epstein-Barr virus (EBV) oncogene latent membrane protein 1 (LMP1): evidence for HLA A2 supertype-restricted immune recognition of EBV-infected cells by LMP1-specific cytotoxic T lymphocytes *European journal of Immunology* **28**: 451-458.

Khanna,R., Bell,S., Sherritt,M., Galbraith,A., Burrows,S.R., Rafter,L., Clarke,B., Slaughter,R., Falk,M.C., Douglass,J., Williams,T., Elliott,S.L., and Moss,D.J. (1999) Activation and adoptive transfer of Epstein-Barr virus-specific cytotoxic T cells in solid organ transplant patients with posttransplant lymphoproliferative disease *PNAS* **96**: 10391-10396.

Kieff,E.D., Rickinson,A.B. (2006) Epstein-Barr virus and its replication. In *Field's Virology*. Snipe,D.M., Howley,P.M. (eds). Lippincroft, Williams & Wilkins, pp. 2604-2655.

Kikuta,H., Mizuno,F., Yano,S., and Osato,T. (1984) Interferon Production by Epstein--Barr Virus in Human Mononuclear Leukocytes *J Gen Virol* **65**: 837-841.

Kilger,E., Kieser,A., Baumann,M., and Hammerschmidt,W. (1998) Epstein-Barr vvirus-mediated B-cell proliferationis dependent upon latent membrane protein 1, which simulates an activated CD40 receptor *EMBO* **17**: 1700-1709.

Kim,O.J., Yates,J.L. (1993) Mutants of Epstein-Barr virus with a selective marker disrupting the TP gene transform B cells and replicate normally in culture *J.Virol.* **67**: 7634-7640.

Kiss,C., Nishikawa,J., Takada,K., Klein,G., and Szekely,L. (2003) T cell leukemia I oncogene expression depends on the presence of Epstein-Barr virus in the virus-carrying Burkitt lymphoma lines *PNAS* **100**: 4813-4818.

Kitagawa,N., Goto,M., Kurozumi,K., Maruo,S., Fukayama,M., Naoe,T., Yasukawa,M., Hino,K., Suzuki,T., Todo,S., and Takada,K. (2000) Epstein-Barr virus-encoded poly(A)-RNA supports Burkitt's lymphoma growth through interleukin-10 induction *EMBO* **19**: 6742-6750.

Kitay,M.K., Rowe,D.T. (1996) Cell cycle stage-specific phosphorylation of the Epstein-Barr virus immortalization protein EBNA-LP *J.Virol.* **70**: 7885-7893.

Knowles,D.M., Cesarman,E., Chadburn,A., Frizzera,G., Chen,J., Rose,E.A., and Michler,R.E. (1995) Correlative morphologic and molecular genetic analysis demonstrates three distinct categories of posttransplantation lymphoproliferative disorders *Blood* **85**: 552-565.

Komano,J., Maruo,S., Kurozumi,K., Oda,T., and Takada,K. (1999) Oncogenic Role of Epstein-Barr Virus-Encoded RNAs in Burkitt's Lymphoma Cell Line Akata *J.Virol.* **73**: 9827-9831.

Koss,K., Satsangi,J., Fanning,G.C., Welsh,K.I., and Jewell,D.P. (2000) Cytokine (TNFa, LTa and IL-10) polymorphisms in inflammatory bowel diseases and normal controls: differential effects on production and allele frequencies *Genes and Immunity* **1**: 185-190.

Kuppers,R. (1999) Identifying the precursors of Hodgkin and Reed-Sternberg cells in Hodgkin's disease: role of the germinal center in B-cell lymphogenesis *Journal of Acquired Immunodefficiency Syndrome* **21**: 574-579.

Kuppers,R., Hansmann,M.L. (2005) The Hodgkin and Reed/Sternberg cell *The International Journal of Biochemistry & Cell Biology* **37**: 511-517.

Kuppers,R., Rajewsky,K. (1998) The origin of Hodgkin and Reed/Sternberg cells in Hodgkin's disease *Ann Rev Immunol* **16**: 471-493.

Labrecque,L., Lampert,I., Kazembe,P., Philips,J., and Griffen,B. (1994) Correlation between cytopathological results and in situ hybridisation on needle aspiration biopsies of suspected African Burkitt's lymphomas *Int J Cancer* **59**: 591-596.

Laichalk,L.L., Thorley-Lawson,D.A. (2005) Terminal Differentiation into Plasma Cells Initiates the Replicative Cycle of Epstein-Barr Virus In Vivo *J.Virol.* **79**: 1296-1307.

Lam,K.M., Syed,N., Whittle,H., and Crawford,D.H. (1991) Circulating Epstein-Barr virus-carrying B cells in acute malaria *Lancet* **337**: 876-878.

Landais,E., Saulquin,X., Scotet,E., Trautmann,L., Peyrat,M.A., Yates,J.L., Kwok,W.W., Bonneville,M., and Houssaint,E. (2004) Direct killing of Epstein-Barr virus (EBV)-infected B cells by CD4 T cells directed against the EBV lytic protein BHRF1 *Blood* **103**: 1408-1416.

Larratt,L.M., Hamilton,M., Coupland,R., and Preiksaitis,J.K. (2001) Recurrent Epstein-Barr virus associated disease in a cardiac transplant patient: evolution from plasmacytic hyperplasia to diffuse large cell lymphoma *Transplant Infectious Disease* **3**: 119-123.

Laux,G., Adam,B., Strobl,L., and Moreau-Gachelin,F. (1994a) The Spi-1/PU.1 and Spi-B ets family transcriptionfactors and the recombination signal binding protein RBP-Jκ interact with anEpstein-Barr virus nuclear antigen 2 responsive *cis*-element *EMBO* **13**: 5624-5632.

Laux,G., Dugrillon,F., Eckert,C., Adam,B., Zimber-Strobl,U., and Bornkamm,G.W. (1994b) Identification and characterization of an Epstein-Barr virus nuclear antigen 2-responsive cis element in the bidirectional promoter region of latent membrane protein and terminal protein 2 genes *J.Virol.* **68**: 6947-6958.

Leblond,V., Davi,F., Charlotte,F., Dorent,R., Bitker,M., Sutton,L., Gandjbakhch,I., Binet,J.-L., and Raphael,M. (1998) Posttransplant lymphoproliferative disorders not associated with Epstein-Bar virus: a distinct entity *J Clin Oncol* **16**: 2052-2059.

Lee,S.P., Tierney,R.J., Thomas,W.A., Brooks,J.M., and Rickinson,A.B. (1997) Conserved CTL epitopes within EBV latent membrane protein 2: a potential target for CTL-based tumor therapy *J Immunol* **158**: 3325-3334.

Lee,S.P., Brooks,J.M., Al Jarrah,H., Thomas,W.A., Haigh,T.A., Taylor,G.S., Humme,S., Schepers,A., Hammerschmidt,W., Yates,J.L., Rickinson,A.B., and Blake,N.W. (2004) CD8 T Cell Recognition of Endogenously Expressed Epstein-Barr Virus Nuclear Antigen 1 *J.Exp.Med.* **199**: 1409-1420.

Lee,T.C., Savoldo,B., Barshes,N.R., Rooney,C.M., Heslop,H.E., Gee,A.P., Caldwell,Y., Scott,J.D., and Goss,J.A. (2006) Use of cytokine polymorphisms and Epstein-Barr virus viral load to predict development of post-transplant lymphoproliferative disorder in paediatric liver transplant recipients *Clinical Transplantation* **20**: 389-393.

Leen,A., Meij,P., Redchenko,I., Middeldorp,J., Bloemena,E., Rickinson,A., and Blake,N. (2001) Differential Immunogenicity of Epstein-Barr Virus Latent-Cycle Proteins for Human CD4+ T-Helper 1 Responses *J.Virol.* **75**: 8649-8659.

Leslie,A., Price,D., Mkhize,P., Bishop,K., Rathod,A., Day,C., Crawford,H., Honeyborne,I., Asher,T., Luzzi,G., Edwards,A., Rousseau,C., Mullins,J., Tudor-Williams,G., Novelli,V., Brander,C., Douek,D., Kiepiela,P., Walker,B., and Goulder,P. (2006) Differential selection presure exerted on HIV by CTL targeting identical epitopes but restricted by distinct HLA alleles from the same HLA supertype *J.Immunol* **177**: 4699-4708.

Levine,P.H., Ablashi,D.V., Berard,C.W., Carbone,P.P., Waggoner,D.E., and Malan,L. (1971) Elevated antibody titers to Epstein-Barr virus in Hodgkin's disease *Cancer* **27**: 416-421.

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

Levitskaya,J., Sharipo,A., Leonchiks,A., Ciechanover,A., and Masucci,M.G. (1997) Inhibition of ubiquitin/proteasome-dependent protein degradation by the Gly-Ala repeat domain of the Epstein-Barr virus nuclear antigená1 *PNAS* **94**: 12616-12621.

Li,Q., Spriggs,M.K., Kovats,S., Turk,S.M., Comeau,M.R., Nepom,B., and Hutt-Fletcher,L.M. (1997) Epstein-Barr virus uses HLA class II as a cofactor for infection of B lymphocytes *J.Virol.* **71**: 4657-4662.

Li,Q.X., Young,L.S., Niedobitek,G., Dawson,C.W., Birkenbach,M., Wang,F., and Rickinson,A.B. (1992) Epstein-Barr virus infection and replication in a human epithelial cell system *Nature* **356**: 347-350.

Lindstrom,M.S., Wiman,K.G. (2002) Role of genetic and epigenetic changes in Burkitt lymphoma *Seminars in Cancer Biology* **12**: 381-387.

Ling,P.D., Rawlins,D.R., and Hayward,S.D. (1993) The Epstein-Barr Virus Immortalizing Protein EBNA-2 is Targeted to DNA by a Cellular Enhancer-Binding Protein *PNAS* **90**: 9237-9241.

Locksley,R.M., Killeen,N., and Lenardo,M.J. (2001) The TNF and TNF Receptor Superfamilies: Integrating Mammalian Biology *Cell* **104**: 487-501.

Lotz,M., Tsoukas,C., Fong,S., Carson,D., and Vaughan,J. (1985) Regulation of Epstein-Barr virus infection by recombinant interferons. Selected sensitivity to infereon-gamme *European journal of Immunology* **15**: 520-525.

Lotz,M., Tsoukas,C.D., Fong,S., Dinarello,C.A., Carson,D.A., and Vaughan,J.H. (1986) Release of lymphokines after Epstein Barr virus infection in vitro. I. Sources of and kinetics of production of interferons and interleukins in normal humans *J Immunol* **136**: 3636-3642.

Lozzio,C.B., Lozzio,B.B. (1975) Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome *Blood* **45**: 321-334.

LU,X.L., LIANG,Z.H., Zhang,C.E., LU,S.J., WENG,X.F., and Wu,X.W. (2006) Induction of the Epstein-Barr Virus Latent Membrane Protein 2 Antigen-specific Cytotoxic T Lymphocytes Using Human Leukocyte Antigen Tetramer-based Artificial Antigen-presenting Cells *Acta Biochimica et Biophysica Sinica* **38**: 157-163.

Lucas KG, Salzman D, Garcia A, and Sun Qi (2004) Adoptive immunotherapy with allogeneic Epstein-Barr virus (EBV)-specific cytotoxic T-lymphocytes for recurrent, EBV-positive Hodgkin disease *Cancer* **100**: 1892-1901.

Macallan,D.C., Wallace,D.L., Irvine,A.J., Asquith,B., Worth,A., Ghattas,H., Zhang,Y., Griffin,G.E., Tough,D.F., and Beverley,P.C. (2003) Rapid turnover of T cells in acute infectious mononucleosis *European journal of Immunology* **33**: 2655-2665.

Macsween,K.F., Crawford,D.H. (2003) Epstein-Barr virus--recent advances *The Lancet Infectious Diseases* **3**: 131-140.

Magrath,I. (1990) The pathogenesis of Burkitt's lymphoma *Adv Cancer Res* **55**: 270.

Malkki,M., Single,R., Carrington,M., Thomson,G., and Petersdorf,E. (2005) MHC microsatellite diversity and linkage disequilibrium among common HLA-A, HLA-B, DRB1 haplotypes: implications for unrelated donor hematopoietic transplantation and disease association studies *Tissue Antigens* **66**: 114-124.

Maran,A., Maitra,R.K., Kumar,A., Dong,B., Xiao,W., Li,G., Williams,B.R., Torrence,P.F., and Silverman,R.H. (1994) Blockage of NF-kappa B signaling by selective ablation of an mRNA target by 2-5A antisense chimeras *Science* **265**: 789-792.

Marie,I., Durbin,J.E., and Levy,D.E. (1998) Differential viral induction of distant interferon-α genes by positive feedback through interferon regulatory factor-7 *EMBO* **17**: 6660-6669.

Marshall,N.A., Vickers,M.A., and Barker,R.N. (2003) Regulatory T Cells Secreting IL-10 Dominate the Immune Response to EBV Latent Membrane Protein 1 *J Immunol* **170**: 6183-6189.

Maruo,S., Nanbo,A., and Takada,K. (2001) Replacement of the Epstein-Barr Virus Plasmid with the EBER Plasmid in Burkitt's Lymphoma Cells *J.Virol.* **75**: 9977-9982.

Mathur,A., Kamat,D., Filipovich,A., Steinbuch,M., and Shapiro,R. (1994) Immunoregulatory abnormalities in patients with Epstein-Barr virus-associated B cell lymphoproliferative disorders *Transplantation* **57**: 1042-1045.

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

McGeoch,D.J., Cook,S., Dolan,A., Jamieson,F.E., and Telford,E.A.R. (1995) Molecular Phylogeny and Evolutionary Timescale for the Family of Mammalian Herpesviruses *Journal of Molecular Biology* **247**: 443-458.

McGeoch,D.J., Rixon,F.J., and Davison,A.J. (2006) Topics in herpesvirus genomics and evolution *Virus Res* **117**: 90-104.

McKiernan,S.M., Hagan,R., Currie,M., McDonald,G.S.A., Kelly,A., Nolan,N., Walsh,A., Hegarty,J., Lawlor,E., and Kelleher,D. (2004) Distinct MHC Class I and II Alleles Are Associated With Hepatitis C Viral Clearance, Originating From a Single Source *Hepatology* **40**: 108-114.

McNally,J.M., Welsh,R.M. (2002) Bystander T cell activation and attrition *Current Topics in Microbiology and Immunology* **263**: 29-41.

McRae,B.L., Semnani,R.T., Hayes,M.P., and van Seventer,G.A. (1998) Type I IFNs Inhibit Human Dendritic Cell IL-12 Production and Th1 Cell Development *J Immunol* **160**: 4298-4304.

McShane,M.P., Longnecker,R. (2004) Cell-surface expression of a mutated Epstein-Barr virus glycoprotein B allows fusion independent of other viral proteins *PNAS* **101**: 17474-17479.

Medzhitov,R., Janeway,J. (1998) Innate immune recognition and control of adaptive immune responses *Seminars in Immunology* **10**: 351-353.

Meitinger,C., Strobl,L.J., Marschall,G., Bornkamm,G.W., and Zimber-Strobl,U. (1994) Crucial sequences within the Epstein-Barr virus TP1 promoter for EBNA2-mediated transactivation and interaction of EBNA2 with its responsive element *J.Virol.* **68**: 7497-7506.

Metes,D., Storkus,W., Zeevi,A., Patterson,K., Logar,A., Rowe,D., Nalesnik,M.A., Fung,J.J., and Rao,A.S. (2000) Ex vivo generation of effective Epstein-Barr virus (EBV)-specific CD8+ cytotoxic T lymphocytes from the peripheral blood of immunocompetent Epstein-Barr virus-seronegative individuals *Transplantation* **70**: 1507-1515.

Miller,C.L., Lee,J.H., Kieff,E., and Longnecker,R. (1994) An Integral Membrane Protein (LMP2) Blocks Reactivation of Epstein-Barr Virus from Latency Following Surface Immunoglobulin Crosslinking *PNAS* **91**: 772-776.

Miller,G., Robinson,J., Heston,L., and Lipmann,M. (1974) Difference between laboratory strains of Epstein-Barr virus based on immortilization, abortive infection, and interference *Proc.Nat.Acad.Sci.* **71**: 4006-4010.

Molesworth,S.J., Lake,C.M., Borza,C.M., Turk,S.M., and Hutt-Fletcher,L.M. (2000) Epstein-Barr Virus gH Is Essential for Penetration of B Cells but Also Plays a Role in Attachment of Virus to Epithelial Cells *J.Virol.* **74**: 6324-6332.

Moore,K.W., Waal Malefyt,R., Coffman,R.L., and O'Garra,A. (2001) Interleukin-10 and the in terleukin-10 receptor *Ann Rev Immunol* **19**: 683-765.

Moore,M., Cannon,MJ., Sewall,A., Finlayson,M., Okimoto,M., and Nemerow,GR. (1991) Inhibition of Epstein-Barr virus infection in vitro and in vivo by soluble CR2 (CD21) containing two short consenus repeats *J.Virol* **65**: 3559-3565.

Moormann,A.M., Chelimo,K., Sumba,O.P., Lutzke,M., Kazura,J., and Rochford,R. (2005) Exposure to holoendemic malaria results in elevated Epstein-Barr virus loads in children *Journal of Infectious Disease* **191**: 1233-1238.

Moretta,A., Bottino,C., Vitale,M., Pende,D., Cantoni,C., Mingari,M.C., Biassoni,R., and Moretta,L. (2001) Activating receptors and coreceptors involved in human natural killer cell-mediated cytoloysis *Ann Rev Immunol* **19**: 197-223.

Morrow,R.H.J. (1985) Epidemiological evidence for the role of falciparum malaria in the pathogenesis of Burkitt's lymphoma *IARC Sci Publ* **60**: 177-186.

Moss,D.J., Burrows,S.R., Silins,S.L., Misko,I.S., and Khanna,R. (2001) The immunology of Epstein-Barr virus infection *Philosophical Transactions of the Royal Society B: Biological Sciences* **356**: 475-488.

Moutschen,M., Leonard,P., Sokal,E.M., Smets,F., Haumont,M., Mazzu,P., Bollen,A., Denamur,F., Peeters,P., Dubin,G., and Denis,M. (2007) Phase I/II studies to evaluate safety and immunogenicity of a recombinant gp350 Epstein-Barr virus vaccine in healthy adults *Vaccine* **25**: 4697-4705.

Mueller,N., Evans,A., Harris,N., Comstock,G., Jellum,E., Magnus,K., Orentreich,N., Polk,B., and Vogelman,J. (1989) Hodgkin's disease and Epstein-Barr virus. Altered antibody pattern before diagnosis *New England Journal of Medicine* **320**: 689-695.

Mullen,M.M., Haan,K.M., Longnecker,R., and Jardetzky,T.S. (2002) Structure of the Epstein-Barr Virus gp42 Protein Bound to the MHC Class II Receptor HLA-DR1 *Molecular Cell* **9**: 375-385.

Munz,C. (2004) Epstein-Barr Virus Nuclear Antigen 1: from Immunologically Invisible to a Promising T Cell Target *J.Exp.Med.* **199**: 1301-1304.

Munz,C. (2005) Immune response and evasion in the host-EBV interaction. In *Epstein-Barr Virus*. Robertson,E.S. (ed). Caister Academic Press, pp. 197-232.

Munz,C., Bickham,K.L., Subklewe,M., Tsang,M.L., Chahroudi,A., Kurilla,M.G., Zhang,D., O'Donnell,M., and Steinman,R.M. (2000) Human CD4+ T Lymphocytes Consistently Respond to the Latent Epstein-Barr Virus Nuclear Antigen EBNA1 *J.Exp.Med.* **191**: 1649-1660.

Murray,P.G., Young,L.S. (2001) Epstein-Barr virus infection: basis of malignancy and potential for therapy *Expert Reviews in Molecular Medicine* **3**: 1-20.

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

Murray,R.J., Kurilla,M.G., Griffin,H.M., Brooks,J.M., Mackett,M., Arrand,J.R., Rowe,M., Burrows,S.R., Moss,D.J., Kieff,E., and Rickinson,A.B. (1990) Human Cytotoxic T-Cell Responses Against Epstein-Barr Virus Nuclear Antigens Demonstrated by Using Recombinant Vaccinia Viruses *PNAS* **87**: 2906-2910.

Nagaoka,H., Gonzalez-Aseguinolaza,G., Tsuji,M., and Nussenzweig,M.C. (2000) Immunization and infection change the number of recombination activating gene (RAG)-expressing B cells in the periphery by altering immature lymphocyte production *Journal of Experimental Medicine* **191**: 2113-2120.

Nalesnik,M., Zeevi,A., Randhawa,P., Faro,A., Spichty,K., Demetris,A., Fung,J., Whiteside,T., and Starzl,T. (1999) Cytokine mRNA profiles in Epsetin-Barr virus-associated post-transplant lymphoproliferative disorders *Clinical Transplantation* **13**: 39-40.

Nanbo,A., Inoue,K., Adachi-Takasawa,K., and Takada,K. (2002) Epstein-Barr virus RNA confers resistence to interferon-a-induced apoptosis in Burkitt's lymphoma *EMBO* **21**: 954-965.

Nanbo,A., Takada,K. (2002) The role of Epstein-Barr virus-encoded small RNAs (EBERs) in oncogenesis *Reviews in Medical virology* **12**: 321-326.

Nedwin,G.E., Naylor,S.L., Sakaguchi,A.Y., Smith,D., Jarrett-Nedwin,J., Pennica,D., Goeddel,D.V., and Gray,P.W. (1985) Human Lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization *Nucl.Acids Res.* **13**: 6361-6373.

Nemerow,G.R., Cooper,N.R. (1984) Early events in the infection of human B lymphocytes by Epstein-Barr virus: the internalization process *Virology* **132**: 186-198.

Nemerow,G.R., Mold,C., Schwend,V.K., Tollefson,V., and Cooper,N.R. (1987) Identification of gp350 as the viral glycoprotein mediating attachment of Epstein-Barr virus (EBV) to the EBV/C3d receptor of B cells: sequence homology of gp350 and C3 complement fragment C3d *J.Virol.* **61**: 1416-1420.

Nemerow,G.R., Wolfert,R., McNaughton,M.E., and Cooper,N.R. (1985) Identification and characterization of the Epstein-Barr virus receptor on human B lymphocytes and its relationship to the C3d complement receptor (CR2) *J.Virol.* **55**: 347-351.

Neri,A., Barriga,F., Inghirami,G., Knowles,D.M., Neequaye,J., Magrath,I.T., and la Favera,R. (1991) Epstein-Barr virus infection precedes clonal expansion in Burkitt's and acquired immunodeficiency syndrome-associated lymphoma [see comments] *Blood* **77**: 1092-1095.

Neuhierl,B., Feederle,R., Hammerschmidt,W., and Delecluse,H.J. (2002) Glycoprotein gp110 of Epstein-Barr virus determines viral tropism and efficiency of infection *PNAS* **99**: 15036-15041.

Newell,K.W. (1956) The reported incidence of glandular fever: an analysis of a report of the Public Health Laboratory Service *J Clin Pathol* **10**: 20-22.

Nguyen,K.B., Cousens,L.P., Doughty,L.A., Pien,G.C., Durbin,J.E., and Biron,C.A. (2000) Interferon [alpha]/[beta]-mediated inhibition and promotion of interferon [gamma]: STAT1 resolves a paradox *Nat Immunol* **1**: 70-76.

Niederman,J.C., McCollum,R.W., Henle,G., and Henle,W. (1968) Infectious mononucleosis. Clinical manifestations in relation to EB virus antibodies *JAMA* **203**: 205-209.

Niederman,J., Evans,A., Henle,W., and McCollum,R. (1970) Prevalence, incidence and persistance of EB virus antibody in young adults *N Engl J Med* **282**: 361-365.

Niens,M., Jarrett,R.F., Hepkema,B., Nolte,I.M., Diepstra,A., Platteel,M., Kouprie,N., Delury,C.P., Gallagher,A., Visser,L., Poppema,S., te Meerman,G.J., and van den Berg,A. (2007) HLA-A*02 is associated with a reduced risk and HLA-A*01 with an increased risk of developing EBV-positive Hodgkin lymphoma *Blood*: blood-2007.

Niens,M., van den Berg,A., Diepstra,A., Nolte,I.M., van der Steege,G., Gallagher,A., Taylor,G.M., Jarrett,R.F., Poppema,S., and te Meerman,G.J. (2006) The Human Leukocyte Antigen Class I Region Is Associated with EBV-Positive Hodgkin's Lymphoma: HLA-A and HLA Complex Group 9 Are Putative Candidate Genes *Cancer Epidemiol Biomarkers Prev* **15**: 2280-2284.

Nikiforow,S., Bottomly,K., and Miller,G. (2001) CD4+ T-Cell Effectors Inhibit Epstein-Barr Virus-Induced B-Cell Proliferation *J.Virol.* **75**: 3740-3752.

Niro,G.A., Fontana,R., Gioffreda,D., Valvano,M.R., Lacobellis,A., Facciorusso,D., and Andriulli,A. (2005) Tumor necrosis factor gene polymorphisms and clearance or progression of hepatitis B virus infection *Liver International* **25**: 1175-1181.

Nitsche,F., Bell,A., and Rickinson,A. (1997) Epstein-Barr virus leader protein enhances EBNA-2-mediated transactivation of latent membrane protein 1 expression: a role for the W1W2 repeat domain *J.Virol.* **71**: 6619-6628.

Nonkwelo,C., Ruf,I.K., and Sample,J. (1997) Interferon-independent and -induced regulation of Epstein-Barr virus EBNA-1 gene transcription in Burkitt lymphoma *J.Virol.* **71**: 6887-6897.

Oda,T., Imai,S., Chiba,S., and Takada,K. (2000) Epstein-Barr Virus Lacking Glycoprotein gp85 Cannot Infect B Cells and Epithelial Cells *Virology* **276**: 52-58.

Ollier,W.E.R. (2004) Cytokine genes and disease susceptibility *Cytokine* **28**: 174-178.

Opelz,G., Dohler,B. (2004) Lymphomas After Solid Organ Transplantation: A Collaborative Transplant Study Report *American Journal of Transplantation* **4**: 222-230.

Orange,J.S., Biron,C.A. (1996) Characterization of early IL-12, IFN-alphabeta, and TNF effects on antiviral state and NK cell responses during murine cytomegalovirus infection *J Immunol* **156**: 4746-4756.

Pallesen,G., Hamilton-Dutoit,S., Rowe,M., and Young,L. (1991) Expression of Epstein-Barr virus latent gene products in tumour cells of Hodgkin's disease *Lancet* **337**: 320-322.

Paludan,C., Munz,C. (2003) CD4+ T Cell Responses in the Immune Control Against Latent Infection by Epstein-Barr Virus *Current Molecular Medicine* **3**: 341-347.

Paludan,C., Bickham,K., Nikiforow,S., Tsang,M.L., Goodman,K., Hanekom,W.A., Fonteneau,J.F., Stevanovic,S., and Munz,C. (2002) Epstein-Barr Nuclear Antigen 1-Specific CD4+ Th1 Cells Kill Burkitt's Lymphoma Cells *J Immunol* **169**: 1593-1603.

Papadopoulos,E.B., Ladanyi,M., Emanuel,D., Mackinnon,S., Boulad,F., Carabasi,M.H., Castro-Malaspina,H., Childs,B.H., Gillio,A.P., Small,T.N., Young,J.W., Kernan,N.A., and O'Reilly,R.J. (1994) Infusions of Donor Leukocytes to Treat Epstein-Barr Virus-Associated Lymphoproliferative Disorders after Allogeneic Bone Marrow Transplantation *N Engl J Med* **330**: 1185-1191.

Parham,P., McQueen,K.L. (2003) Alloreactive killer cells: hindrance and help for haematopoietic transplants *Nat Rev Immunol* **3**: 108-122.

Parker,G., Crook,T., Bain,M., Sara,E., Farrell,P., and Allday,M. (1996) Epstein-Barr virus nuclear antigen (EBNA) 3C is an immortilising oncoprotein with similar properties to adenovirus E1A and papillomavirus E7 *Oncogene* **13**: 2541-2549.

Parker,G., Touitou,R., and Allday,M. (2000) Epstein-Barr virus EBNA 3C can disrupt multiple cell cycle checkpoints and induce nuclear division divorced from cytokinesis *Oncogene* **19**: 700-709.

Parolini,S., Bottino,C., Falco,M., Augugliaro,R., Giliani,S., Franceschini,R., Ochs,H.D., Wolf,H., Bonnefoy,J.Y., Biassoni,R., Moretta,L., Notarangelo,L.D., and Moretta,A. (2000) X-linked Lymphoproliferative Disease: 2B4 Molecules Displaying Inhibitory Rather Than Activating Function Are Responsible for the Inability of Natural Killer Cells to Kill Epstein-Barr Virus-infected Cells *J.Exp.Med.* **192**: 337-346.

Paya,C., Fung,J., Nalesnik,M., Kieff,E., Green,M., Gores,G., Habermann,T., Wiesner,R., Swinnen,L., Woodle,E., and Bromberg,J. (1999) Epstein-Barr virus-induced posttransplant lymphoproliferative disorders *Transplantation* **68**: 1517-1525.

Pegtel,D.M., Middeldorp,J., and Thorley-Lawson,D.A. (2004) Epstein-Barr Virus Infection in Ex Vivo Tonsil Epithelial Cell Cultures of Asymptomatic Carriers *J.Virol.* **78**: 12613-12624.

Pellet,P.G., Roizman,B. (2006) The family *Herpesviridae*: A brief introduction. In *Field's Virology*. Snipe,D.M., Howley,P.M. (eds). Lippencroft, Williams & Wilkins, pp. 2479-2499.

Pende,D., Rivera,P., Marcenaro,S., Chang,C.C., Biassoni,R., Conte,R., Kubin,M., Cosman,D., Ferrone,S., Moretta,L., and Moretta,A. (2002) Major Histocompatibility Complex Class I-related Chain A and UL16-Binding Protein Expression on Tumor Cell Lines of Different Histotypes: Analysis of Tumor Susceptibility to NKG2D-dependent Natural Killer Cell Cytotoxicity *Cancer Res* **62**: 6178-6186.

Peng,C.W., Xue,Y., Zhao,B., Johannsen,E., Kieff,E., and Harada,S. (2004) Direct interactions between Epstein-Barr virus leader protein LP and the EBNA2 acidic domain underlie coordinate transcriptional regulation *PNAS* **101**: 1033-1038.

Peng,C.W., Zhao,B., and Kieff,E. (2004) Four EBNA2 Domains Are Important for EBNALP Coactivation *J.Virol.* **78**: 11439-11442.

Peng,R., Tan,J., and Ling,P.D. (2000) Conserved Regions in the Epstein-Barr Virus Leader Protein Define Distinct Domains Required for Nuclear Localization and Transcriptional Cooperation with EBNA2 *J.Virol.* **74**: 9953-9963.

Pereira,M.S., Blake,J.M., and Macrae.A.D (1969) EB virus antibody at different ages *British Medical Jouranal* **4**: 526-527.

Perera,S., Thomas,J., Burke,M., and Crawford,D. (1998) Analysis of the T-cell micro-environment in Epstein-Barr virus-related post-transplantation B lymphoproliferative disease *Journal of Pathology* **184**: 177-184.

Perry,M., Whyte,A. (1998) Immunology of the tonsils *Immunology Today* **19**: 414-421.

Petti,L., Sample,C., and Kieff,E. (1990) Subnuclear localisation and phosphorylation of Epstein-Barr virus latent infection nuclear proteins *Virology* **176**: 563-574.

Piccioli,D., Sbrana,S., Melandri,E., and Valiante,N.M. (2002) Contact-dependent Stimulation and Inhibition of Dendritic Cells by Natural Killer Cells *J.Exp.Med.* **195**: 335-341.

Pinto,A., Gattel,V., Zagonel,V., Degan,M., De Iuliis,A., Rossi,F., Tassan Mazzocco,F., Godeas,C., Rupolo,M., Poletto,D., Gloghini,A., Carbone,A., and Gruss,H. (1998) Hodgkin's disease: a disorder of dysregulated cellular cross-talk *Biotherapy* **10**: 309-320.

Poppema,S., Potters,M., Visser,L., and van den Berg,A. (1998) Immune escape mechanisms in Hodgkin's disease *Ann Oncol* **9**: 521-524.

Pratesi,C., Bortolin,M., Bidoli,E., Tedeschi,R., Vaccher,E., Dolcetti,R., Guidoboni,M., Franchin,G., Barzan,L., Zanussi,S., Caruso,C., and De Paoli,P. (2006) Interleukin-10 and interleukin-18 promoter polymorphisms in an Italian cohort of patients with undifferentiated carcinoma of nasopharyngeal type *Cancer Immunology, Immunotherapy* **55**: 23-30.

Precopio,M.L., Sullivan,J.L., Willard,C., Somasundaran,M., and Luzuriaga,K. (2003) Differential Kinetics and Specificity of EBV-Specific CD4+ and CD8+ T Cells During Primary Infection *J Immunol* **170**: 2590-2598.

Pulliam,L., Moore,D., and West,D.C. (1995) Human cytomegalovirus induces IL-6 and TNF alpha from macrophages and microglial cells: possibe role in neurotoxicity *Neurovirology* **1**: 219-227.

Raab-Traub,N., Flynn,K. (1986) The structure of the termini of the Epstein-Barr virus as a marker of clonal cellular proliferation *Cell* **47**: 883-889.

Radkov,S.A., Bain,M., Farrell,P.J., West,M., Rowe,M., and Allday,M.J. (1997) Epstein-Barr virus EBNA3C represses Cp, the major promoter for EBNA expression, but has no effect on the promoter of the cell gene CD21 *J.Virol.* **71**: 8552-8562.

Radkov,S.A., Touitou,R., Brehm,A., Rowe,M., West,M., Kouzarides,T., and Allday,M.J. (1999) Epstein-Barr Virus Nuclear Antigen 3C Interacts with Histone Deacetylase To Repress Transcription *J.Virol.* **73**: 5688-5697.

Ragoczy,T., Miller,G. (1999) Role of the Epstein-Barr Virus Rta Protein in Activation of Distinct Classes of Viral Lytic Cycle Genes *J.Virol.* **73**: 9858-9866.

Rawlins,D.R., Milman,G., Hayward,S.D., and Hayward,G.S. (1985) Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen (EBNA-1) to clustered sites in the plasmid maintenance region *Cell* **42**: 859-868.

Reddehase,M.J., Jonjic,S., Weiland,F., Mutter,W., and Koszinowski,U.H. (1988) Adoptive immunotherapy of murine cytomegalovirus adrenalitis in the immunocompromised host: CD4-helper-independent antiviral function of CD8-positive memory T lymphocytes derived from latently infected donors *J.Virol.* **62**: 1061-1065.

Reddehase,M.J., Mutter,W., Munch,K., Buhring,H.J., and Koszinowski,U.H. (1987) CD8-positive T lymphocytes specific for murine cytomegalovirus immediate-early antigens mediate protective immunity *J.Virol.* **61**: 3102-3108.

Rentenaar,R.J., Gamadia,L.E., van derHoek,N., van Diepen,F.N.J., Boom,R., Weel,J.F.L., Wertheim-van Dillen,P.M.E., van Lier,R.A.W., and ten Berge,I.J.M. (2000) Development of virus-specific CD4+ T cells during primary cytomegalovirus infection *J.Clin.Invest.* **105**: 541-548.

Rickinson,A.B., Kieff,E.D. (2006) Epstein-Barr Virus. In *Field's Virology*. Snipe,D.M., Howley,P.M. (eds). Lippencroft, Williams & Wilkins, pp. 2657-2701.

Rickinson,A.B., Moss,D.J. (1997) Human cytotoxic T lymphocyte responses to Epstein-Barr virus infection *Ann Rev Immunol* **15**: 405-431.

Riddell,S., Wantanabe,K., Goodrich,J., Li,C., Agha,M., and Greenberg,P. (1992) Restoration of Viral Immunity in Immunodeficient Humans by the Adoptive Transfer of T Cell Clones *Science* **257**: 238-241.

Rivailler,P., Quink,C., and Wang,F. (1999) Strong Selective Pressure for Evolution of an Epstein-Barr Virus LMP2B Homologue in the Rhesus Lymphocryptovirus *J.Virol.* **73**: 8867-8872.

Rochford,R., Mosier,D.E. (1995) Differential Epstein-Barr virus gene expression in B-cell subsets recovered from lymphomas in SCID mice after transplantation of human peripheral blood lymphocytes *J.Virol.* **69**: 150-155.

Rooney,C., Howe,J.G., Speck,S.H., and Miller,G. (1989) Influence of Burkitt's lymphoma and primary B cells on latent gene expression by the nonimmortalizing P3J-HR-1 strain of Epstein-Barr virus *J.Virol.* **63**: 1531-1539.

Rooney,C., Smith CA, Ng CY, Loftin S, Li C, Krance,R., Brenner,M., and Heslop,H. (1995) Use of gene-modified virus-specific T lymphocytes to control Epstein-Barr-virus-related lymphoproliferation *Lancet* **345**: 9-13.

Rooney,C., Smith,C., Ng,C., Loftin,L., Sixbey,J., Gan,Y., Srivastava,D., Bowman,L., Krance,R., Brenner,M., and Heslop,H. (1998) Infusion of Cytotoxic T Cells for the Prevention and Treatment of Epstein-Barr Virus-Induced Lymphoma in Allogenic Transplant Recipients *Blood* **92**: 1549-1555.

Rosendorff,A., Illanes,D., David,G., Lin,J., Kieff,E., and Johannsen,E. (2004) EBNA3C Coactivation with EBNA2 Requires a SUMO Homology Domain *J.Virol.* **78**: 367-377.

Rowe,D., Rowe,M., Evan,G., Wallace,L., Farrell,P., and Rickinson,A. (1986) Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells *EMBO* **5**: 2599-2607.

Ruf,I.K., Rhyne,P.W., Yang,C., Cleveland,J.L., and Sample,J.T. (2000) Epstein-Barr Virus Small RNAs Potentiate Tumorigenicity of Burkitt Lymphoma Cells Independently of an Effect on Apoptosis *J.Virol.* **74**: 10223-10228.

Sadler,R.H., Raab-Traub,N. (1995) The Epstein-Barr virus 3.5-kilobase latent membrane protein 1 mRNA initiates from a TATA-Less promoter within the first terminal repeat *J.Virol.* **69**: 4577-4581.

Saiki,R.K., Scharf,S., Faloona,F., Mullis,K.B., Horn,G.T., Erlich,H.A., and Arnheim,N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia *Science* **230**: 1350-1354.

Salek-Ardakani,S., Arrand,J.R., and Mackett,M. (2002) Epstein-Barr Virus Encoded Interleukin-10 Inhibits HLA-Class I, ICAM-1, and B7 Expression on Human Monocytes: Implications for Immune Evasion by EBV *Virology* **304**: 342-351.

Sample,J., Young,L., Martin,B., Chatman,T., Kieff,E., Rickinson,A., and Kieff,E. (1990) Epstein-Barr virus types 1 and 2 differ in their EBNA-3A, EBNA-3B, and EBNA-3C genes *J.Virol.* **64**: 4084-4092.

Sauter,M., Mueller-Lantzsch,N. (1987) Characterisation of an Epstein-Barr virus nuclear antigen 2 variant (EBNA 2B) by specific sera *Virus Res* **8**: 141-152.

Savard,M., Gosselin,J. (2006) Epstein-Barr virus immunossuppression of innate immunity mediated by phagocytes *Virus Res* **119**: 134-145.

Savoldo,B., Cubbage,M.L., Durett,A.G., Goss,J., Huls,M.H., Liu,Z., Teresita,L., Gee,A.P., Ling,P.D., Brenner,M.K., Heslop,H.E., and Rooney,C.M. (2002) Generation of EBV-Specific CD4+ Cytotoxic T Cells from Virus Naive Individuals *J Immunol* **168**: 909-918.

Sawyer,R., Evans,A., Niederman,J., and McCollum,R. (1971) Prospective studies of a group of Yale university freshmen: ocurrence of IM *J Infect Dis* **123**: 263-270.

Sayos,J., Wu,C., Morra,M., Wang,N., Zhang,X., Allen,D., van Schaik,S., Notarangelo,L., Geha,R., Roncarolo,M.G., Oettgen,H., De Vries,J.E., Aversa,G., and Terhorst,C. (1998) The X-linked lymphoproliferative-disease gene product SAP regulates signals induced through the co-receptor SLAM *Nature* **395**: 462-469.

Schaefer,B.C., Strominger,J.L., and Speck,S.H. (1995) Redefining the Epstein-Barr Virus-Encoded Nuclear Antigen EBNA-1 Gene Promoter and Transcription Initiation Site in Group I Burkitt Lymphoma Cell Lines *PNAS* **92**: 10565-10569.

Schaft,N., Lankiewicz,B., Drexhage,J., Berrevoets,C., Moss,D.J., Levitsky,V., Bonneville,M., Lee,S.P., McMichael,A.J., Gratama,J.W., Bolhuis,R.L.H., Willemsen,R., and Debets,R. (2006) T cell re-targeting to EBV antigens following TCR gene transfer: CD28-containing receptors mediate enhanced antigen-specific IFN{gamma} production *Int.Immunol.* **18**: 591-601.

Schooley,R.T. (1995) Epstein-Barr virus (infectious monocucleosis). In *Mandell, Douglas and Bennett's principles and practice of infectious diseases*. Mandell,G.L., Bennett,J.E., and Dolin,R. (eds). New York: Churchhill Livingston, pp. 1599-1613.

Schwemmle,M., Clemens,M.J., Hilse,K., Pfeifer,K., Troster,H., Muller,W.E.G., and Bachmann,M. (1992) Localization of Epstein-Barr Virus-Encoded RNAs EBER-1 and EBER-2 in Interphase and Mitotic Burkitt Lymphoma Cells *PNAS* **89**: 10292-10296.

Shannon-Lowe,C.D., Neuhierl,B., Baldwin,G., Rickinson,A.B., and Delecluse,H.J. (2006) From the Cover: Resting B cells as a transfer vehicle for Epstein-Barr virus infection of epithelial cells *PNAS* **103**: 7065-7070.

Shapiro,R.S., McClain,K., Frizzera,G., Gajl-Peczalska,K.J., Kersey,J.H., Blazar,B.R., Arthur,D.C., Patton,D.F., Greenberg,J.S., and Burke,B. (1988) Epstein-Barr virus associated B cell lymphoproliferative disorders following bone marrow transplantation *Blood* **71**: 1234-1243.

Sharp,T.V., Schwemmle,M., Jeffrey,I., Laing,K., Mellor,H., Proud,C.G., Hilse,K., and Clemens,M.J. (1993) Comparative analysis of the regulation of the interferoninducible protein kinase PKR by Epstein - Barr virus RNAs EBER-1 and EBER-2 and adenovirus VA, RNA *Nucl.Acids Res.* **21**: 4483-4490.

Shi,Y., Lutz,C.T. (2002) Interferon-gamma Control of EBV-Transformed B Cells: A Role for CD8+ T Cells That Poorly Kill EBV-Infected Cells *Viral Immunology* **15**: 213-225.

Shibata,D., Weiss,L.M. (1992) Epstein-Barr virus-associated gastric adenocarcinoma *Am J Pathol* **140**: 769-774.

Shimizu,N., Takada,K. (1993) Analysis of the BZLF1 promoter of Epstein-Barr virus: identification of an anti-immunoglobulin response sequence *J.Virol.* **67**: 3240-3245.

Shrestha,S., Strathdee,S.A., Galai,N., Oleksyk,T., Fallin,M.D., Mehta,S., Schaid,D., Vlahov,D., O'Brien,S.J., and Smith,M.W. (2006) Behavioral Risk Exposure and Host Genetics of Susceptibility to HIV-1 Infection *The Journal of Infectious Diseases* **193**: 16-26.

Silins,S.L., Sculley,T.B. (1994) Modulation of Vimentin, the CD40 Activation Antigan and Burkitt's Lymphoma Antigen (CD77) by the Epstein-Barr Virus Nuclear Antigen EBNA-4 *Virology* **202**: 16-24.

Silins,S.L., Sherritt,M.A., Silleri,J.M., Cross,S.M., Elliott,S.L., Bharadwaj,M., Le,T.T.T., Morrison,L.E., Khanna,R., Moss,D.J., Suhrbier,A., and Misko,I.S. (2001) Asymptomatic primary Epstein-Barr virus infection occurs in the absence of blood T-cell repertoire perturbations despite high levels of systemic viral load *Blood* **98**: 3739-3744.

Sinclair,A., Palmero,I., Peters,G., and Farrell,P. (1994) EBNA-2 and EBNA-LP cooperate to cause G0 to G1 transition during immortilization of resting human B lymphocytes by Epstein-Barr virus *EMBO* **13**: 3321-3328.

Sixbey,J., Nedrud,J., Raab-Traub,N., Hanes,R., and Pagano,J. (1984) Epstein-Barr virus replication in oropharyngeal epithelial cells *N Engl J Med* **310**: 1225-1230.

Sixbey,J.W., Lemon,S.H., and Pagano,J.S. (1986) A second site for Epstein-Barr virus shedding: the uterine cervix *Lancet* **2**: 1122-1124.

Sixbey,J., Yao,Q. (1992) Immunoglobulin A-induced shift of Epstein-Barr virus tissue tropism *Science* **255**: 1578-1580.

Sjoblom,A., Yang,W., Palmqvist,L., Jansson,A., and Rymo,L. (1998) An ATF/CRE Element Mediates both EBNA2-Dependent and EBNA2-Independent Activation of the Epstein-Barr Virus LMP1 Gene Promoter *J.Virol.* **72**: 1365-1376.

Sokal,E.M., Antunes,H., Beguin,C., Bodeus,M., Wallemacq,P., de Ville de Goyet,J., Reding,R., Janssen,M., Buts,J., and Otte,J. (1997) Early signs and risk factors for the increased incidence of Epstein-Barr virus-related posttransplant lymphoproliferative diseases in paediatric liver transplant recipients with tacrolimus *Transplantation* **64**: 1438-1442.

Speck,S.H., Chatila,T., and Flemington,E. (1997) Reactivation of Epstein-Barr virus: regulation and function of the BZLF1 gene *Trends in Microbiology* **5**: 399-405.

Spink,C.F., Keen,L.J., Mensah,F.K., Law,G.R., Bidwell,J.L., and Morgan,G.J. (2006) Association between non-Hodgkin lymphoma and haplotypes in the TNF region *Br.J.Haematol* **133**: 293-300.

Stark,G.R., Kerr,I.M., Williams,B.R.G., Silverman,R.H., and Schreiber,R.D. (1998) How cells respond to interferons *Annual Review of Biochemistry* **67**: 227-264.

Stetson,D., Medzhitov,R. (2006) Type 1 interferons in host defense *Immunity* **25**: 373-381.

Steven,N.M., Annels,N.E., Kumar,A., Leese,A.M., Kurilla,M.G., and Rickinson,A.B. (1997) Immediate Early and Early Lytic Cycle Proteins Are Frequent Targets of the Epstein-Barr Virus-induced Cytotoxic T Cell Response *J.Exp.Med.* **185**: 1605-1618.

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

Stevens,S.J.C., Vervoort,M.B.H.J., van den Brule,A.J.C., Meenhorst,P.L., Meijer,C.J.L.M., and Middeldorp,J.M. (1999) Monitoring of Epstein-Barr Virus DNA Load in Peripheral Blood by Quantitative Competitive PCR *J.Clin.Microbiol.* **37**: 2852-2857.

Straathof,K.C.M., Bollard,C.M., Popat,U., Huls,M.H., Lopez,T., Morriss,M.C., Gresik,M.V., Gee,A.P., Russell,H.V., Brenner,M.K., Rooney,C.M., and Heslop,H.E. (2005) Treatment of nasopharyngeal carcinoma with Epstein-Barr virus-specific T lymphocytes *Blood* **105**: 1898-1904.

Subklewe,M., Marquis,R., Choquet,S., Leblond,V., Garnier,J., Hetzer,R., Swinnen,L., Oertel,S., Papp-Vary,M., Gonzalez-Barca,E., Hepkema,B., Schoenemann,C., May,J., Pezzutto,A., and Riess,H. (2006) Association of human leukocyte antigen haplotypes with posttransplant lymphoproliferative disease after solid organ transplantation *Transplantation* **82**: 1093-1100.

Subklewe,M., Paludan,C., Tsang,M.L., Mahnke,K., Steinman,R.M., and Munz,C. (2001) Dendritic Cells Cross-present Latency Gene Products from Epstein-Barr Virus-transformed B Cells and Expand Tumor-reactive CD8+ Killer T Cells *J.Exp.Med.* **193**: 405-412.

Sutkowski,N., Conrad,B., Thorley-Lawson,D.A., and Huber,B.T. (2001) Epstein-Barr Virus Transactivates the Human Endogenous Retrovirus HERV-K18 that Encodes a Superantigen *Immunity* **15**: 579-589.

Swerdlow,A.J. (2003) Epidemiology of Hodgkin's disease and non-Hodgkin's lymphoma *Eur Nucl Med Mol Imaging* **V30**: S3-S12.

Swerdlow,A., Higgins,CD., Hunt,B., Thomas,JA., Burke,M., Crawford,D., and Yacoub,M. (2000) Risk of lymphoid neoplasia after cardiothoracic transplanation *Transplantation* **69**: 897-904.

Swinnen,L.J., Mullen,G.M., Carr,T.J., Costanzo,M.R., and Fisher,R.I. (1995) Aggressive treatment for postcardiac transplant lymphoproliferation *Blood* **86**: 3333-3340.

Swinnen,L., Costanzo,M., Fisher,S., O'Sullivan,E., Johnston,M., Heroux,A., Dizikes,G., Pifarre,R., and Fisher,R. (1990) Increased incidence of lymphoproliferative disorder after immunosuppression with the monoclonal antibody OKT3 in cardiac transplant recipients *New England Journal of Medicine* **323**: 1723-1728.

Szekely,L., Selivanova,G., Magnusson,K.P., Klein,G., and Wiman,K.G. (1993) EBNA-5, an Epstein-Barr Virus-Encoded Nuclear Antigen, Binds to the Retinoblastoma and p53 Proteins *PNAS* **90**: 5455-5459.

Takada,K. (1984) Cross-linking of cell surface immunoglobulins induces Epstein-Barr virus in Burkitt lymphoma lines *Int J Cancer* **33**: 27-32.

Takada,K., Nanbo,A. (2001) The role of EBERs in oncogenesis *Seminars in Cancer Biology* **11**: 461-467.

Tamaru,J., Hummel,M., Zemlin,M., Kalvelage,B., and Stein,H. (1994) Hodgkin's disease with a B-cell phenotype often shows a VDJ rearrangement and somatic mutations in the VH genes *Blood* **84**: 708-715.

Tan,L.C., Gudgeon,N., Annels,N.E., Hansasuta,P., O'Callaghan,C.A., Rowland-Jones,S., McMichael,A.J., Rickinson,A.B., and Callan,M.F.C. (1999) A Re-Evaluation of the Frequency of CD8+ T Cells Specific for EBV in Healthy Virus Carriers *J Immunol* **162**: 1827-1835.

Tanner,J., Weis,J., Fearon,D., Whang,Y., and Kieff,E. (1987) Epstein-barr virus gp350/220 binding to the B lymphocyte C3d receptor mediates adsorption, capping, and endocytosis *Cell* **50**: 203-213.

Tanner,J., Whang,Y., Sample,J.T., Sears,A., and Kieff,E. (1988) Soluble gp350/220 and deletion mutant glycoproteins block Epstyein-Barr virus adsorption to lymphocytes *J.Virol* **62**: 4452-4464.

Tao,Q., Robertson,K.D., Manns,A., Hildesheim,A., and Ambinder,R.F. (1998) Epstein-Barr Virus (EBV) in Endemic Burkitt's Lymphoma: Molecular Analysis of Primary Tumor Tissue *Blood* **91**: 1373-1381.

Tarodi,B., Subramanian,T., and Chinnadurai,G. (1994) Epstein-Barr Virus BHRF1 Protein Protects against Cell Death Induced by DNA-Damaging Agents and Heterologous Viral Infection *Virology* **201**: 404-407.

Tattevin,P., Le Tulzo,Y., Minjolle,S., Person,A., Chapplain,J.M., Arvieux,C., Thomas,R., and Michelet,C. (2006) Increasing Incidence of Severe Epstein-Barr Virus-Related Infectious Mononucleosis: Surveillance Study *J.Clin.Microbiol.* **44**: 1873-1874.

Thomas,R., McAulay,K., Higgins,C., Wilkie,G., and Crawford,D. (2005) Interferon gamma polymorphisms in posttransplant proliferative disease *Blood* **106**: 1502-1503.

Thorley-Lawson,D.A. (2005) EBV persistence and latent infection *in vivo*. In *Epstein-Barr Virus*. Robertson,E.S. (ed). Caister Academic Press, pp. 309-358.

Thorley-Lawson,D.A., Geilinger,K. (1980) Monoclonal antibodies against the major glycoprotein (gp350/220) of Epstein-Barr virus can neutralize infectivity *PNAS* **77**: 5307-5311.

Timms,J.M., Bell,A., Flavell,J.R., Murray,P.G., Rickinson,A.B., Traverse-Glehen,A., Berger,F., and Delecluse,H.J. (2003) Target cells of Epstein-Barr-virus (EBV)-positive post-transplant lymphoproliferative disease: similarities to EBV-positive Hodgkin's lymphoma *The Lancet* **361**: 217-223.

Toczyski,D.P., Matera,A.G., Ward,D.C., and Steitz,J.A. (1994) The Epstein-Barr Virus (EBV) Small RNA EBER1 Binds and Relocalizes Ribosomal Protein L22 in EBV-Infected Human B Lymphocytes *PNAS* **91**: 3463-3467.

Toczyski,D.P., Steitz,J.A. (1991) EAP, a highly conserved cellular protein associated with Epstein-Barr virus small RNAs (EBERs) *EMBO* **10**: 459-466.

Tomkinson,B., Kieff,E. (1992) Second-site homologous recombination in Epstein-Barr virus: insertion of type 1 EBNA 3 genes in place of type 2 has no effect on in vitro infection *J.Virol.* **66**: 780-789.

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

Tosato,G., Jones,K., Brenig,M.K., McWilliams,H.P., and McKnight,J.L.C. (1993) Interleukin-6 production in posttransplant lymphoproliferative disease *J.Clin.Invest.* **91**: 2806-2814.

Tugizov,S., Herrera,R., Veluppillai,P., Greenspan,J., Greenspan,D., and Palefsky,J.M. (2007) Epstein-Barr Virus (EBV)-Infected Monocytes Facilitate Dissemination of EBV within the Oral Mucosal Epithelium *J.Virol.* **81**: 5484-5496.

Tugizov,S.M., Berline,J.W., and Palefsky,J.M. (2003) Epstein-Barr virus infection of polarized tongue and nasopharyngeal epithelial cells *Nat Med* **9**: 307-314.

Uccini,S., Monardo,F., Stppacciaro,A., Gradilone,A., Agliano,A., Faggioni,A., Manzari,V., Vago,L., Costanzi,G., and Ruco,L. (1990) High frequency of Epstein-Barr virus genome detection in Hodgkin's disease of HIV-positive patients *Int J Cancer* **46**: 581-585.

Udalova,I.A., Richardson,A., Denys,A., Smith,C., Ackerman,H., Foxwell,B., and Kwiatkowski,D. (2000) Functional Consequences of a Polymorphism Affecting NF-kappa B p50-p50 Binding to the TNF Promoter Region *Mol.Cell.Biol.* **20**: 9113-9119.

VanBuskirk,A., Malik,V., Xia,D., and Pelletier,R. (2001) A gene polymorphism associated with posttransplant lymphoproliferative disorder *Transplantation Proceedings* **33**: 1834.

Veronese,M., Veronesi,A., D'Andrea,E., Del Mistro,A., Indraccolo,S., Mazza,M., Mion,M., Zamarchi,R., Menin,C., and Panozzo,M. (1992) Lymphoproliferative disease in human peripheral blood mononuclear cell-injected SCID mice. I. T lymphocyte requirement for B cell tumour generation *Journal of Experimental Medicine* **176**: 1763-1767.

Voss,M.D., Hille,A., Barth,S., Spurk,A., Hennrich,F., Holzer,D., Mueller-Lantzsch,N., Kremmer,E., and Grasser,F.A. (2001) Functional Cooperation of Epstein-Barr Virus Nuclear Antigen 2 and the Survival Motor Neuron Protein in Transactivation of the Viral LMP1 Promoter *J.Virol.* **75**: 11781-11790.

Wade,M., Allday,M.J. (2000) Epstein-Barr Virus Suppresses a G2/M Checkpoint Activated by Genotoxins *Mol.Cell.Biol.* **20**: 1344-1360.

Waldmann,T.A., Tagaya,Y. (1999) The multifaceted regulation of interleukin-15 expression and the role of this cytokine in NK cell differentiation and host response to intracellular pathogens *Ann Rev Immunol* **17**: 19-49.

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

Wang,X., Kenyon,W.J., Li,Q., Mullberg,J., and Hutt-Fletcher,L.M. (1998) Epstein-Barr Virus Uses Different Complexes of Glycoproteins gH and gL To Infect B Lymphocytes and Epithelial Cells *J.Virol.* **72**: 5552-5558.

Weiss,L., Movahed,L., Warnke,R., and Sklar,J. (1989) Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease *New England Journal of Medicine* **320**: 502-506.

Welsh,R.M., McNally,J.M., Brehem,M.A., and Selin,L.K. (2000) Consequences of cross-reactive and bystander CTL responses during viral infections *Virology* **270**: 4-8.

Whitney,B.M., Chan,A.T.C., Rickinson,A.B., Lee,S.P., Lin,C.K., and Johnson,P.J. (2002) Frequency of Epstein-Barr virus-specific cytotoxic T lymphocytes in the blood of Southern Chinese blood donors and nasopharyngeal carcinoma patients *Journal of Medical Virology* **67**: 359-363.

Whittle,H., Brown,J., Marsh,K., Blackman,M., Jobe,O., and Shenton,F. (1990) The effects of Plasmodium falciparum malaria on immune control of B lymphocytes in Gambian children *Clinical & Experimental Immunology* **80**: 213-218.

Whittle,H., Brown,J., Marsh,K., Greenwood,B., Seidelin,P., Tighe,H., and Wedderburn,L. (1984) T-cell control of Epstein-Barr virus-infected B cells is lost during P.falciparum malaria *Nature* **312**: 449-450.

Wilkie,G.M., Taylor,C., Jones,M.M., Burns,D.M., Turner,M., Kilpatrick,D., Amlot,P.L., Crawford,D.H., and Haque,T. (2004) Establishment and Characterization of a Bank of Cytotoxic T Lymphocytes for Immunotherapy of Epstein-Barr Virus-Associated Diseases. *Journal of Immunotherapy* **27**: 309-316.

Williams,H., Macsween,K.F., McAulay,K., Higgins,C.D., Harrison,N., Swerdlow,A.J., Britton,K.M., and Crawford,D.H. (2004) Analysis of immune activation and clinical events in acute infectious mononucleosis. *J Infect Dis* **190**: 63-71.

Williams,H., Crawford,D.H. (2006) Epstein-Barr virus: the impact of scientific advances on clinical practice *Blood* **107**: 862-869.

Woisetschlaeger,M., Yandava,C.N., Furmanski,L.A., Strominger,J.L., and Speck,S.H. (1990) Promoter Switching in Epstein-Barr Virus During the Initial Stages of Infection of B Lymphocytes *PNAS* **87**: 1725-1729.

Woisetschlaeger,M., Strominger,J.L., and Speck,S.H. (1989) Mutually Exclusive Use of Viral Promoters in Epstein--Barr Virus Latently Infected Lymphocytes *PNAS* **86**: 6498-6502.

Wu,M.S., Huang,S.P., Chang,Y.T., Shun,C.T., Chang,M.C., Lin,M.T., Wang,H.S., and Lin,J.T. (2002) Tumour Necrosis Factor-á and Interleukin-10 promoter polymorphisms in Epstei-Barr virus associated gastric carcinoma. *J Infect Dis* **185**: 106-109.

Wu,T., Mann,R., Carache,P., Hayward,S., Staal,S., Lambe,B., and Ambinder,R. (1990) Detection of EBV gene expression in Reed-Sternberg cells of Hodgkin's disease *Int J Cancer* **46**: 801-804.

Xiao,J., Palefsky,J.M., Herrera,R., Berline,J., and Tugizov,S.M. (2008) The Epstein-Barr virus BMRF-2 protein facilitates virus attachment to oral epithelial cells *Virology* **370**: 430-442.

Xu,X.W., Lu,M.H., and Tan,D.M. (2005) Association between tumour necrosis factor gene polymorphisms and the clinical types of patients with chronic hepatitis B virus infection *Clinical Microbiology and Infection* **11**: 52-56.

Yalamanchili,R., Tong,X., Grossman,S., Johannsen,E., Mosialos,G., and Kieff,E. (1994) Genetic and Biochemical Evidence That EBNA 2 Interaction with a 63-kDa Cellular GTG-Binding Protein Is Essential for B Lymphocyte Growth Transformation by EBV *Virology* **204**: 634-641.

Yang,L., Aozasa,K., Oshimi,K., and Takada,K. (2004) Epstein-Barr Virus (EBV)-Encoded RNA Promotes Growth of EBV-Infected T Cells through Interleukin-9 Induction *Cancer Res* **64**: 5332-5337.

Yang,R.B., Mark,M.R., Gray,A., Huang,A., Xie,M.H., Zhang,M., Goddard,A., Wood,W.I., Gurney,A.L., and Godowski,P.J. (1998) Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling *Nature* **395**: 284-288.

Yao,Q., Rowe,M., Morgan,A.J., Sam,C.K., Prasad,U., Dang,H., Zeng,Y., and Rickinson,A.B. (1991) Salivary and serum IgA antibodies to the Epstein-Barr virus glycoprotein gp340: incidence and potential for virus neutralization *Int J Cancer* **48**: 45-50.

Yates,J.L., Warren,N., and Sugden,B. (1985) Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells *Nature* **313**: 812-815.

Yin,Y., Manoury,B., and Fahraeus,R. (2003) Self-Inhibition of Synthesis and Antigen Presentation by Epstein-Barr Virus-Encoded EBNA1 *Science* **301**: 1371-1374.

Young,L., Murray,PG. (2003) Epstein-Barr virus and oncogenesis: from latent genes to tumours *Oncogene* **22**: 5108-5121.

Zhao,B., Sample,C.E. (2000) Epstein-Barr Virus Nuclear Antigen 3C Activates the Latent Membrane Protein 1 Promoter in the Presence of Epstein-Barr Virus Nuclear Antigen 2 through Sequences Encompassing an Spi-1/Spi-B Binding Site *J.Virol.* **74**: 5151-5160.

Zheng,H., Matte-Martone,C., Li,H., Anderson,B.E., Venketesan,S., Tan,H.S., Jain,D., McNiff,J., and Shlomchik,W.D. (2007) Effector memory CD4+ T cells mediate graft-versus-leukemia without inducing graft-versus-host disease *Blood*: blood-2007.

Zimber-Strobl,U., Kremmer,E., Grasser,FA., Marschall,G., Laux,G., and Bornkamm,G.W. (1993) The Epstein-Barr virus nuclear antigen 2 interacts with EBNA2 responsive *cis*-element of the terminal protein 1 gene promoter *EMBO* **12**: 167-175.

Zimber-Strobl,U., Suentzenich,K.O., Laux,G., Eick,D., Cordier,M., Calender,A., Billaud,M., Lenoir,G.M., and Bornkamm,G.W. (1991) Epstein-Barr virus nuclear antigen 2 activates transcription of the terminal protein gene *J.Virol* **65**: 415-423.

Chapter 9:
Publications