
UNIVERSITY OF BIRMINGHAM

# CD4+ T cell recognition of Epstein-Barr Virus Nuclear Antigen (EBNA) – 1 in the Chinese population

by

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# Dedications

To family and friends

Many many many thanks…I just cannot stress in words how many more thanks I have to say, but without your support this would have never been possible

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# Abstract

The current treatment of nasopharyngeal carcinoma (NPC), a tumour common in southern Chinese people, is dependent on chemo/radiosensitivity. Patients with early stages of the disease are usually chemo and radiosensitive with 5 years survival rates of 90% whereas, patients with late-stages of the disease or relapse have between 20-70% survival rates. These statistics indicate a need for a treatment to improve the overall survival rates in NPC patients, especially for those with relapse. Immunotherapy is a prime candidate for such treatment due to its association with Epstein-Barr Virus (EBV) infection, expression of EBV antigens in NPC tumour cells and the presence of EBV-specific T cells at the tumour site. More importantly, studies in other EBV-associated diseases strongly indicated that CD8+ T cell based immunotherapy targeting EBV antigens could control EBV infection and also tumour regression in some patients, however, most patients show poor tumour response rate due to ineffective maintenance of EBV-specific CD8+ T cells. Studies have also shown that CD4+ T cells are important in the control of viral infection by maintaining an effective CD8+ T cell response and as effectors in its own right. CD4+ T cell responses to EBV antigens have only been studied in the Caucasian population and not in Chinese people where NPC incidence is high. This thesis work was prompted with these points in mind. In this thesis, the screening in 25 Caucasian donors (24 donors are EBV-seropositive and 1 donor was EBV-seronegative at the time of this study) for T cell responses (CD8+ and CD4+) against the predicted BART products and BARF1 protein. The BART and BARF1 transcripts are expressed in NPC tumour cells, but their expression as proteins have never been identified until Kienzle *et al* (1998) reported the finding of CD8+ T cell response to one of the products encoded by the BART transcript, BARF0. In this study, no CD8+ or CD4+ T cell responses were detected against the BART products or to BARF1 protein, and concluded that if responses do exist, they are probably rare responses and would have little or no immunotherapeutic value. The attention of this thesis work was then shifted to studying an EBV protein that is uniformly identified in NPC tumour cells, Epstein-Barr virus nuclear antigen (EBNA) 1. CD4+ T cell response against EBNA1 has been studied in the Caucasian population, however, little is known about CD4+ T cell

epitopes presented by HLA alleles in Chinese people. In this study, CD4+ T cell responses in 78 healthy Chinese EBV carriers were analyzed and found marked focusing of epitopes in the EBNA1 C-terminal region, including a DP5-restricted epitope recognized by almost half of the donors tested. More importantly, EBNA1-specific CD4+ T cell clones to this DP5-restricted epitope could recognize EBNA1-expressing, DP5-positive target cells. However, further studies are required to determine the role of these EBNA1-specific CD4+ T cells in T cell based therapy.

# Abbreviations

**AIDS-CNSL** – acquired immunodeficiency syndrome-related central nervous system lymphomas

**APC** - Antigen Presenting Cells

**BARTs** – *Bam* H1A rightward transcripts

**BL** – Burkett's Lymphoma

**bp** – base pair

**BSA** – Bovine Serum Albumin

**C** – Constant

**CD** – Clusters of Differentiation

**CLIP** – Class II-associated Invariant Chain Protein

**CSA** – Cyclosporin A

**D** – Diversity

**DC** - Dendritic Cells

**DRiPs** – Defective ribosomal products

**EBERs** – EBV-encoded small nonpolyadenylated RNAs

**EBNA** – EBV nuclear antigen

**EBV**- Epstein Barr Virus

**ELISA** – Enzyme-linked immunosorbant assay

**ELISpot** – Enzyme-linked immunospot

**ER** – Endoplasmic reticulum

**FACS** - Fluorescence Activated Cell Sorting

**FCS** – Foetal Calf Serum

**HCMV** – Human Cytomegalovirus

**hCSF** – human colony-stimulating factor

**HD** – Hodgkin's disease

**HIV** – Human Immunodeficiency Virus

**HK** – Hong Kong

**HLA** – Human Leukocyte Antigen

**HS** – Human Serum

**HSV** – Herpes Simplexvirus

**Ii** – Invariant chain

**IL** – Interleukin

**IM** – Infectious Mononucleosis

**ITAMs** – Immunoreceptor Tyrosine-based Activation Motifs

**IFN-γ** – Interferon-γ

**J** – joining

**KO** – Knockout

**LCL** – Lympoblastoid Cell Line

**LCV** – Lymphocrytoviruses

**LMP** – Latent Membrane Protein

**MAb** – Monoclonal Antibodies

**MCMV** – Murine CMV

**MHC** - Major Histocompatibility Complex

**MVA** – Modified vaccinia virus Ankara

**NK** - Natural Killer

**NPC** – Nasopharyngeal Carcinoma

**ORF** – Open Reading Frame

**PBMCs** – Peripheral Blood Mononuclear Cells

**PBS** – Phosphate Buffered Saline

**PCR** – Polymerase Chain Reaction

**PHA** - Phytohaemagglutinin

**PTLD** – post-transplant lymphoproliferative disease

**RER** – Rough endoplasmic reticulum

**RNA** – Ribonucleic acid

**SFC** – Spot Forming Cells

**STAT** – Signal Transducer and Activator of Transcription

**TAE** – Tris/Acetate Buffered EDTA

**TAP** – Transporter associated with antigen processing

**TCR** – T cells Receptor

**TGN** – Trans-Golgi Network

**TMB** – 3, 5', 5', 5' – tetramethyl benzidine

**TNF-α** – Tumour Necrosis Factor-α

**TR** – Terminal repeats

**UKB** – United Kingdom Blood Donors

**UKL** – United Kingdom Laboratory Donors

**V** – Variable

**VCA** – Viral Capsid Antigen

**WHO** – World Health Organisation

**ZAP-70** – 70 kDa Zeta Associated Protein

**$\beta_2$M** - $\beta_2$-Microglobulin

# Section 1

# Introduction

# Section 1.1
# Viruses linked to human cancers

## 1.1: Viruses linked to human cancers

In the last 40 years, considerable evidence has linked several infectious agents, especially viruses, to human cancers, (Parkin, 2002). Table 1.1 summarises the key associations between viruses and human cancer and the global incidence of virus-associated tumours, estimated to be >12% of total cancer burden worldwide. Summarised briefly, the overall picture is as follows: evidence for a causal association is strongest where viral genetic information is not only consistently retained in every cell of an individual tumour, but is also being expressed as viral proteins. There are four examples of this type.

Certain types of the human papilloma virus (HPV), especially HPV types 16 and 18, are associated with cervical cancer, a disease which accounts for around 5% of global cancer burden (Parkin, 2006). In every tumour specimen, HPV DNA fragments can be detected integrated into the host genome with the E6 and E7 genes still intact. Furthermore, expression of viral proteins E6 and E7 is consistently found in tumour cells (Pagano *et al*, 2004). The relevant HPV types are sexually transmitted and widespread in human populations. The incidence of cervical cancer differs between developing (7.7%) and developed countries (1.7%), this lower incidence in developed countries being largely due to the introduction of screening programmes.

As will be discussed later in detail, Epstein - Barr virus (EBV), a gamma herpesvirus, is associated with several human malignancies, together constituting around 1% of global cancer burden (Parkin, 2006). EBV infection is very common worldwide, with >95% of the world population are EBV-carriers. While the incidence of one EBV-associated tumour, Hodgkin Lymphoma (HL), a malignancy of Hodgkin/Reed Sternberg cells (HRS), is similar in all parts of the world, but this is not true of other tumours. For example, Burkitt lymphoma (BL), a type of B cell lymphoma, has a very high incidence rate in many parts of equatorial Africa and in New Guinea where malarial infection is holoendemic, compared with BL incidence worldwide. Therefore in some way malarial infection, combined with EBV, is thought to contribute to tumour development (Pagano *et al*, 2004). Another

| Infection | Cancer | %Virus-positive | No. of cases Worldwide/year |
|---|---|---|---|
| HPVs | Cervical cancer | 100% | 490,000 |
| HBV | Liver cancer | 50% | 340,000 |
| HCV | Liver cancer | 25% | 195,000 |
| EBV | Burkitt lymphoma | > 90% | 113,000 |
| | Hodgkin's Disease | > 50% | |
| | Post-transplant lymphoma | > 80% | |
| | Nasopharyngeal carcinoma | 100% | |
| KSHV | Kaposi Sarcoma | 100% | 66,000 |
| | Primary effusion lymphoma | 100% | |
| | Multi Castleman's | > 50% | |
| HTLV1 | Adult T cell leukaemia | 100% | 3,000 |

**Table 1.1: Viruses linked to human cancer**

This table was adapted from Cancer Research UK (2006) CancerStats – Infectious Agents and Cancer (info.cancerresearchuk.org/ cancerstats)

example, nasopharyngeal carcinoma (NPC), an epithelial cancer, is relatively rare on a world scale (0.7% of all cancers per year), but has moderately high incidence in certain North African countries and in Inuit people. More importantly, the tumour is common in South East Asia, and exceptionally common in Southern Chinese people. This observed geographical isolation of NPC incidences possibly indicates potential genetic susceptibility or environmental factors which may play a part in promoting tumour development. In all cases of EBV – positive malignancies, EBV episomal DNA and the expression of EBV latent proteins could be detected in the tumour cells, with different EBV - associated tumours expressing different patterns of viral latent cycle proteins.

The Kaposi Sarcoma Herpes Virus (KSHV) is associated with several human malignancies, together accounting for <1% of global cancer burden (Parkin, 2006). The best known association is with a tumour of endothelial cell origin, Kaposi Sarcoma (KS). There are four major forms of this tumour: (1) classic form of KS is mainly seen in elderly men of Mediterranean and Eastern European descent, possibly indicating genetic susceptibility but also reflecting the fact that KSHV incidence is higher in such populations than in Northern Europe. (2) Endemic KS, an aggressive from of KS, prevalent in certain parts of Africa before the HIV epidemic. (3) KS is relatively common among acquired immunodeficiency syndrome (AIDS) patients and is considered an AIDS-defining condition in human immunodeficiency virus (HIV)-positive carriers. A high incidence of KS is observed in Africa, where KSHV infection is common and where the epidemic of human immunodeficiency virus (HIV)/AIDS is severe. (4) KS occurs in rare organ transplant patients who are naturally KSHV-infected and receiving immunosuppressive therapy. KSHV viral DNA and KSHV encoded proteins could be detected in >95% of all KS lesions (Pagano *et al*, 2004).

Primary effusion lymphoma (PEL) is a very rare B cell tumour, typically seen in heavily immunocompromised HIV-infected individuals, particularly among homosexual cohorts in which KSHV has become endemic. This may reflect that sexual behaviour plays a part in the development of the disease. To date, all cases of PEL analysed are positive for KSHV

DNA. Multicentric Castleman's disease (MCD), another B cell proliferative condition, is observed in both AIDS and non-AIDS associated cases. In most, if not all, AIDS-associated MCD cases KSHV DNA could be detected in the tumour cells, while only approximately 50% of non-AIDS associated cases have detectable KSHV DNA.

The Human T Lymphotropic Virus type 1 (HTLV 1), the first human retrovirus to be discovered, is associated with Adult T-cell Leukaemia (ATL). ATL is a rare cancer, in global scales, accounting for 0.03% of the global cancer burden. However, ATL is seen at higher frequency in southern Japan where HTLV1 infection is relatively common and where in 3-5% of infected individuals would develop ATL over their life-time. Transmission of the virus from one individual to another is via HTLV1-infected T cells and not 'free' virus, thus HTLV1 has low levels of transmission to contacts but can be passed from mother to child in breast milk. In every case of ATL, proviral HTLV1 DNA can be detected integrated into the host genome. However, integration sites differ between ATL patients, and it is thought that the HTLV1-encoded Tax protein plays a role in lymphomagenesis.

In contrast to the above examples where viral gene expression in tumour cells is thought to actively contribute to the malignant process, the association between hepatitis viruses and hepatocellular carcinoma (HCC) is thought to be indirect. HCC is among the most common cancer in the world, with 42.5% of cases in developed countries and 92% in developing countries associated with hepatitis B or C virus (HBV or HCV) infection respectively. On a global scale, HBV infection is the major factor in the development of HCC, with the prevalence of HBV and incidence of HCC following the same geographical pattern of distribution. Prospective studies have shown that HBV carriers have 10-100 fold increased risk of HCC, while parallel studies with HCV are still on-going. In HBV-associated HCC, the HBV DNA is consistently integrated into the host genome. However the position of integration into the cellular genome shows no consistent pattern, no particular fragment of viral DNA is consistently retained and no particular viral antigens are consistently expressed. HBV, a DNA virus, and HCV, an RNA virus, naturally infects and replicate in

hepatocytes. This could activate virus-specific and non-specific cellular responses in the host which may contribute to liver injury and hence to chronic stimulation of liver regeneration. The continual cell turnover that results puts the regenerating population at risk of oncogenic change. In the case of HBV, integration of viral DNA adds a second contributory factor, since integration is potentially mutagenic and the resultant genomic instability could facilitate the oncogenic process.

# Section 1.2
# Immune strategies targeting virus-associated malignancies

## 1.2: Immune strategies targeting virus-associated malignancies

### 1.2.1: Prophylactic vaccines

Considering the impact of these virally-associated tumours on the global cancer burden, it is important to look at immune strategies to reduce that burden. Ideally, prophylactic vaccination, using a vaccine that protects the naïve healthy host against initial viral infection, would be the most direct way to achieve this. As an example, prophylactic vaccines have been developed to protect against primary HPV infection, a bivalent vaccine targeting HPV types 16 and 18 and a quadrivalent vaccine targeting HPV types 6, 11, 16, 18. They act by inducing a neutralising antibody response against the major capsid protein, L1, in the vaccinated individuals (Garcia and Saslow, 2007). Ongoing clinical trials demonstrate nearly 100% protection from persistent infection (Koutsky *et al*, 2002). Currently studies are underway to vaccinate large numbers of naïve healthy young girls against HPV infection. Possibly within 20 years time, a decline in HPV transmission would begin to be reflected in a falling incidence of new HPV-associated tumours. This would constitute formal proof of a causal association between viral infection and tumourgenesis.

As a second example, the HBV vaccine was developed around 20 years ago, using the viral surface antigen, HbS, to induce a neutralizing antibody response. For example, Galimska (2000) reports the effectiveness of the HBV vaccination programme in Poland. The vaccination programme started in 1993, where all newborns and people at high risk to HBV infection were vaccinated, these included, medical workers, medical students, people with HBV-infected partners, people with chronic liver disease and patients undergoing surgery. In total, around 3.7 million people or 10% of Poland's population where vaccinated between 1993 to1997. In the early 1990s, prior to HBV vaccination, HBV incidence in Poland was around 40 per 100,000 (or 13,296 cases of recorded HBV infected individuals a year), whereas in 1997, HBV incidence in Poland was around 12.7 per 100,000 (or 4896 cases/year). In a period of four years, a considerable decline in HBV incidence was noted. This shows that the HBV vaccine is effective at providing HBV naïve individuals protection against HBV infection (Galimska, 2000). In a separate study in Taiwan, Chien *et*

*al* (2006) reports a 10 year study of the HBV vaccine which started in July 1984. Between July 1984-June 1986, all newborns born to high risk HBV-positive mothers where vaccinated with the HBV vaccine and Hepatitis B immunoglobulin (HBIG). The programme was further extended in 1987 to include the vaccination of preschool children (who have not yet received the vaccination during birth) and to susceptible medical personnel. From 1988 to 1990, the vaccination programme was extended even further to include elementary school children. Prior to the vaccination programme, >90% of the general population <40 years of age were infected with HBV, with 15-20% of cases are chronic HBV infection. Several seroepidemiological studies were carried out as a 'follow-up' of the vaccination trial. Prior to the vaccine trial, the prevalence of Hepatitis B surface antigen (HBsAg) detected in children under the age of 5 in 1984 was 9.3% which dropped to 2% in 1989. This study demonstrated an effective protection (78%) in majority of HBV-negative children. Subsequent studies in 1992 and 1994 showed effective protection in HBV-negative young children reaching 85% and 87% respectively. In addition to HBV transmission, the incidence of HCC in children has also dropped significantly. The incidence of HCC in children between the ages of 6-14 was 0.7 per 100,000 between the years 1981-1986, and between 1986-1990 was 0.57 per 100,000 and 1990-1994 was 0.36 per 100,000. Thus the vaccination programme clearly demonstrates the potential efficacy of prophylactic vaccination as a strategy to reduce cancer incidence. It also served to directly link HBV infection in the causation of HCC.

Prophylactic vaccines for the other virally-associated cancers have yet not been developed despite extensive research. This reflects the fact that different agents have different biologies and generating truly sterile immunity is easier in some systems than in others.

### 1.2.2: Therapeutic vaccines

Facing with the current situation, with viruses for which no prophylactic vaccines is available, an alternative approach would be a therapeutic vaccine, given to patients with the tumour and aiming to boost the host cellular immune response against viral antigens that are expressed in the tumour. Therapeutic vaccines are still at an early stage of development

and aim to exploit the specificity and power of the CD8+ and CD4+ T cell responses that normally act to control infections against intracellular pathogens such as viruses. CD8+ T cells are important because they recognize endogenously expressed antigens in the context of Human Leukocyte Antigen (HLA) class I molecules (HLA I). Such T cells are known to have cytotoxic functions and so, if specific for viral antigens expressed in tumours, should in theory recognize and kill HLA class I-positive tumour cells. CD4+ T cells, on the other hand, usually recognize exogenously acquired antigens in the context of HLA II molecules. These T cells are known to produce cytokines which 'help' sustain CD8+ T cell responses. However recent studies suggest that, in some cases, CD4+ T cells can also recognize endogenously expressed antigens in HLA II-positive target cells, although the mechanism underlying this phenomenon is still under investigation.

The present thesis was undertaken with these general points in mind. The specific virus to be studied is Epstein-Barr virus, and the work was prompted by the possibility of developing a therapeutic vaccine against one particular EBV-associated tumour, nasopharyngeal carcinoma. Although the cellular response to EBV infection has been studied, information has largely come from work in Caucasian individuals and not on Chinese populations which are at highest risk of this tumour. The next section of the thesis reviews current understanding of how antigens are presented to T cells and the role of human leukocyte antigen (HLA) complex in that process. This is central to the theme of the thesis work since differences in HLA polymorphism between Caucasians and Chinese populations will influence T cell responsiveness.

# Section 1.3
# Antigen processing and presentation to T cells

### 1.3.0: Antigen processing and presentation to T cells

The adaptive immunity is elicited when antigen-specific cells are activated upon antigen recognition. Activation of T cells occur via the T cell receptor (TCR) recognition of antigenic peptide presented by HLA molecules that are displayed on the surface of infected cells or antigen presenting cells (APC). APC are specialised cells which processes antigens and display them through HLA class I or II molecules to T cells.

### 1.3.1: Antigen Presenting Cells (APC)

This section reviews the importance of profession APCs in the role of generating an effective adaptive immunity against pathogens. Much of the understanding in the role of APC came from study of dendritic cells (DCs). DCs play an important role in bridging the innate and adaptive immunity. The activation of adaptive immunity is initiated during innate response. This is achieved by the recognition of pathogen-associated molecular patterns (PAMPs) that signals through pattern recognition receptors, the toll-like receptors (TLR). TLR is a family of receptors that are expressed at the surface and within the cytoplasm and endosomal membranes of DCs (Hochrein and O'Keeffe, 2008). There are currently 13 mammalian TLRs identified and these receptors are capable of recognising distinct bacterial, fungal and viral patterns. For example, TLR3 (double stranded RNA), 7 (single stranded RNA), 8 (single stranded RNA) and 9 (CpG DNA) recognises genomic patterns (Diebold *et al*, 2004; Heil *et al*, 2004; Lund *et al*, 2004; Tabeta *et al*, 2004; Ahmad-Nejal *et al*, 2002). TLR2 (human cytomegalovirus glycoproteins gH and gB) and TLR4 (fusion protein of respiratory syncytial virus) recognises envelope proteins. In terms of innate immunity, TLR recognition of pathogen components leads to MyD88 – NFkB signalling and ultimately leading to inflammatory response (Doherty and Arditi, 2004). In addition, in the context of adaptive immunity, MyD88 signalling also upregulate the expression of CD80 and CD86 molecules which 'flags' HLA-pathogen complexes on the cell surface. T cells that interact with the HLA-pathogen complex, and express surface molecules CD28 or CTLA-4, would get the appropriate costimulatory signal for the activation of adaptive immunity.

In addition to recognition of pathogen components by the TLRs and the activation of adaptive immunity, APCs are also capable of processing antigens to be presented to CD8+ and CD4+ T cells of the adaptive immune system through HLA I and HLA II molecules respectively. These sections would be discussed separately.

**1.3.2: Presentation of endogenously expressed antigens to CD8+ T cells**

Every protein is subjected to continuous turnover and is degraded, at rates reflected by the half-life of the protein. In addition, recently synthesised proteins that are misfolded or abnormal (defective ribosomal products (DRiPs) are rapidly degraded. DRiPs account for 30% of all synthesised proteins in the cell (Princiotta *et al*, 2003). Proteins that are targeted for degradation are tagged with a small protein called ubiquitin (Ciechanover, 2006). Ubiquitinated proteins are then targeted by a multifunctional protease complex called proteasome which cleaves peptide bonds, in an ATP-dependent manner, generating peptide fragments. Two forms of proteosomes exist; (1) constitutive proteosomes are expressed in healthy cells, and (2) 'immunoproteosomes' are expressed by interferon gamma (INF-γ) or tumour necrosis factor alpha (TNF-α) stimulated cells and in primary and secondary lymphoid organs. In immunproteosomes, the active proteosome subunits are replaced by 'immuno' subunits (Lmp2, Lmp7 and MECL-1) to form immunoproteosomes (Duan *et al*, 2006). These immunoproteosomes are speculated to be more effective in generating HLA I antigenic peptides, however, certain HLA I antigenic peptides could only be generated by the constitutive proteasome (Duan *et al*, 2006).

In the endoplasmic reticulum (ER), newly synthesised HLA I α-chain is associated with a membrane bound chaperone protein, calnexin, which facilitates the correct folding of the molecule. ERp57, a thiol oxidoreductase was found to be associated with calnexin and calreticulin which was demonstrated to form disulphide bonds in protein folding (Zhang and Williams, 2006). $\beta_2$-microglobulin ($\beta_2$-m), calreticulin and tapasin then displaces calnexin forming a calreticulin-tapasin-HLA I complex. This complex, when the peptide-binding cleft is unoccupied, is unstable and is retained in the ER. Antigenic peptide is acquired through association of the protein complex and with transporters associated with

antigen processing (TAP). TAP is a heterodimer consisting of two proteins, TAP1 and TAP2, which has a high affinity to transport peptide fragments that are typically 8-10 amino acids in length into the ER (Romisch, 1994). Association of the protein complex with TAP would bring the HLA I molecule in close proximity to receiving transported antigenic peptides. Upon high affinity binding of antigenic peptide, the HLA class I molecule become stable and dissociates from the complex. The HLA I/antigenic peptide loaded complex is then transported to the cell surface membrane for presentation via the Golgi.

### 1.3.3: Presentation of exogenously acquired antigens to CD8+ T cells

Traditionally it was believed that antigens presented to CD8+ T cells are entirely derived from endogenously expressed proteins. However, while this remains essentially true for most cell types, it has been shown in DCs, macrophages, B lymphocytes, neutrophils and endothelial cells that they could acquire and present exogenously derived antigens to $CD8^+$ T cells (Basta and Alatery, 2007). This process is termed 'cross-priming'. Exactly how "cross-priming" is achieved remains hotly debated. It has been suggested that exogenously derived antigens are acquired by DCs, either by phagocytosis or macropinocytosis of apoptotic vesicles generated from dying infected or tumour cells (Rock and Shen, 2005; Winau *et al*., 2004; Chen *et al*., 2004), and then processed for HLA I molecule presentation. Donohue *et al* (2006) demonstrated that antigens of the cross-priming pathway are a separate pool of antigens from the direct-priming pathway. This is because antigens that are processed in the classical pathway are mainly derived from DRiP. DRiPs are 'short-lived' proteins that could only be visualised by inhibiting their rapid degradation shortly after their synthesis. Whereas, antigens that are processed by cross-priming do not require on-going synthesis to prime naïve CD8+ T cell responses. Possibly these proteins are more stable or they are stabilised by heat shock proteins (HSP), since inhibitors to HSP90 (geldanamycin and herbimycin A) would inhibit cross-priming *in vitro* (Basta *et al*, 2005). There are several proposed pathways, based on evidence using inhibitors of processing machineries, which may suggest the possible routes in which exogenously acquired proteins could be processed and presented to CD8+ T cells.

One such pathway is TAP and proteasome-independent (Storni and Bachmann, 2004; Kurotaki *et al*, 2007). This involves processing of internalised antigens through the endocytic pathway using the same mechanism as generating peptides for the HLA class II molecules and also endosomal proteases, cathepsin (Kurotaki *et al*, 2007). This is supported by the evidence that TAP-defective cell line, RMA-S, are capable of presenting HLA-I restricted epitopes to CD8+ T cells possibly through association with heat shock protein (Schirmbeck and Reimann, 1994). In another study, Schirmbeck *et al* (1995) demonstrated that lysosomal inhibitors (chloroquine, NH4Cl, primaquine, or leupeptin) would block TAP-defective T2 cell lines from presenting HLA I-restricted antigens to CD8+ T cells, whereas, proteosomal inhibitors (cycloheximide or brefeldin A) did not. Loading of peptides generated by lysosomal enzymes possibly takes place in the late endosomal compartments involving recycling HLA I molecules (Hochman *et al*., 1991; Gromme *et al*., 1999; Kurotaki *et al*, 2007). However, other studies have shown that newly synthesised MHC class I molecule may also be directly involved where the late endosomal compartment may have fused with the rough endoplasmic reticulum (RER), since RER derived membrane could be found in the endosomal membrane. In addition, RER derived enzymes could also be found in the endosomal compartment (De Bruijn *et al*., 1995; Gagnon *et al*, 2002).

Another suggested pathway, the TAP and proteosome-dependent pathway, was proposed that internalised antigens are transported into the cytoplasm where they are processed by the proteasome and then transported by TAP into the ER for HLA I presentation (Burgdorf and Kurts, 2008). The evidence came from study by Isenman and Dice (1993) where they demonstrated that radiolabelled peptide fragments internalised into the lysosome can make their way into the cytosol. In another experiment, Norbury *et al* (1995) showed that exogenous derived proteins may also possibly gain access into the cytosol where it is processed by the proteasome, since, proteosomal inhibitors (Brefeldin A and gelonin) would inhibit HLA-I presentation of internalised proteins. The mechanism involving the transfer of exogenously acquire proteins from the endocytic compartments to the cytoplasm

is not fully understood. Reis e Sousa and Germain (1995) argues that exogenous proteins possibly escape into the cytoplasm as a result of phagocytic overload leading to loss of phagosome membrane integrity, since increasing concentration of exogenous protein would lead to leaking of the protein into the cytosol. However, Norbury *et al* (1995) and Isenman and Dice (1993) showed that the lysosomal membrane integrity is still intact.

These experiments clearly show that there may be more than one possible route in which cross-priming can occur. Possibly some proteins are processed by the TAP-independent pathway, whereas, other proteins are processed by the TAP-dependent pathway. More importantly, these experiments strong support the presence of an alternative pathway in which antigens that escape the classical pathway are still processed and presented to CD8+ T cells.

### 1.3.4 MHC class II presentation of exogenous antigen

Peptides that are displayed through the HLA II molecule generally are derived from proteins acquired exogenously by APCs either through phagocytosis, endocytosis or micropinocytosis (Pillay *et al*, 2002). Each organelle of the endocytic pathway plays an important role in the processing of internalised substances. The first organelle, the early endosome, is the sorting station. This compartment has a slightly acidic environment, with a pH range of 6.3-6.5, which facilitates the dissociation of antigens that may be bound to receptors. The receptors are then recycled, whereas, the antigens are transported by endosome carrier vesicles to the late endosome (Killisch *et al*, 1992) or the early endosome may directly fuse with the late endosome (Jahraus *et al*, 1998). The late endosome is the main site of proteolysis (Tjelle *et al*, 1996). This organelle contains approximately 20% of the hydrolase pool which operate under acidic pH (pH 5.0-5.5). At this stage, most antigenic proteins are digested into peptide fragments which may have two possible fates. Antigen-derived peptides may be transported to the HLA II containing compartment (HIIC) where it is then loaded onto the HLA II molecule and exported to the plasma membrane for presentation to CD4+ T cells. Alternatively, the late endosome may fuse with the lysosomal compartment where the antigen is further digested into smaller fragments (Rocha and

Neefjes, 2008). Enzymes in the lysosomal compartment are activated in acidic pH between 4.5-5.0. The lysosomal compartment, which contains the majority of the hydrolase pool, only accounts for 20% of the total proteolysis that takes place inside a cell. It has been suggested that the lysosomal compartment may serve to be a storage compartment for hydrolases, that can be called upon when needed, such as during phagocytosis when large amounts of foreign substances are engulfed by the cell (Tjelle *et al*, 1996). Finally, antigen-derived peptide fragments are then transported to the HIIC for loading onto HLA II molecules for presentation.

Newly synthesised HLA class II αβ dimers are associated with a protein called invariant (Ii) chain in the ER (Brown *et al*, 1993). Ii has three major functions; (1) the folding of the α and β chains (Bikoff *et al*, 1993; Viville *et al*, 199), (2) the Class II-associated invariant chain peptide (CLIP) of Ii blocks the HLA class II peptide-binding cleft preventing inappropriate loading with peptides in the ER (Roche and Cresswell, 990), (3) the re-direction of the HLA class II molecules from the trans-golgi network (TGN) into the endosomal/lysosomal pathway for peptide loading, specifically to the HIIC (Bakke and Dobberstein, 1990; Roche *et al*, 1993).

The HIIC is the primary site where the HLA II molecule is loaded with antigenic peptide. The exact definition of an HIIC compartment is debatable, but several essential components are required for the loading of the HLA II molecule. The HIIC compartment is a late endosome-like compartment (Rocha and Neefjes, 2008) which has the feature of an acidic pH (Ziegler and Unanue, 1982) containing key enzymes cathepsin S and L, and a non-classical class II molecule HLA-DM (Honey and Rudensky, 2003). Within the HIIC compartment, Ii is broken down by cathepsin S and L leaving the CLIP section still intact and bound to the peptide-binding cleft of the HLA class II molecule. Inhibition of cathepsin S and L would subsequently prevent HLA II peptide loading (Riese and Chapman, 2000) and cell surface expression (Neefjes and Ploegh, 1992). Replacement of the CLIP by processed antigen-peptide requires a low acid pH and is regulated by a non-classical class II molecules, HLA-DM. HLA-DM is a dedicated chaperone (the only known function is its

interaction with HLA II molecules) which stabilises the HLA II molecule that is devoid of peptide, preventing aggregation and support peptide exchange until a high affinity peptide is bound (Sloan *et al*, 1995; Densin *et al*, 1996).

Peptide loading of HLA II molecules has also been observed in other compartments of the endocytic pathway, and including, the plasma membrane (Moss *et al*, 2007) which has a neutral pH, absence of cathepsin S and L, and absence of antigen preparation. This can occur when the HLA-DM along with the HLA II molecule is transported to the plasma membrane via the HIIC compartment (Wubbolts *et al*, 1996), then loading of the HLA II molecule can occur in the plasma membrane. More importantly, the HLA-DM molecule contains a classical tyrosine-based internalisation motif which signals the internalisation of the HLA-DM molecule into the endocytic pathway. Therefore, loading of HLA II molecules therefore could take place in every endocytic compartment.

### 1.3.5 MHC class II presentation of endogenously expressed antigen

As described above, HLA class II molecules predominantly present peptides derived from uptake of exogenous antigens. However, analysis of natural ligands eluted from the HLA class II molecule revealed a relatively large proportion of these ligands are of the cytoplasm, nucleus and mitochondrial origin (Rammensee *et al*., 1999; Zhou and Blum, 2004). This indicated that endogenously synthesised proteins are be processed and presented to CD4+ T cells. More importantly, the absence of these endogenously synthesised antigens within the endocytic pathway (Trombetta and Mellman, 2005) suggested the mechanism for the processing of endogenously expressed antigens do not involve the endocytic pathway, but possibly through some form of intracellular sorting, a phenomenon call autophagy.

Autophagy is a process where cellular components are transported to the lysosome for degradation. In most cells, autophagy occurs at a low baseline rate and this is increased in response to nutrient starvation (Strawbridge and Blum, 2007). There are three types of autophagy, microautophagy, macroautophagy and chaperone-mediated autophagy.

Research in microautophagy is still in its early stages. Microscopy studies in yeast system have shown that during cell starvation the cytosol and organelles are directly internalised by invagination of the lysosomal membrane (Roberts *et al*, 2003). More importantly, inhibition of Tor with rapamycin would enhance microautophagy, possibly indicating a similar pathway is used in the control of microautophagy to that of macroautophagy.

The understanding in the mechanism of macroautophagy is more defined. Macroautophagy is a process whereby the cytosol and organelles are engulfed by a membrane of unknown origin into a specialised compartment called the autophagosome (Yorimitsu and Klionsky, 2005). Autophagosome formation is enhanced during periods of starvation. Studies in yeast models indicated, when cells have excess nutrient, phosphatidylinositol 3-kinase (PI3K) is activated leading to downstream activation of Tor, and Tor phosphorylates Atg13 resulting in the dissociation from Atg1 and inhibition of macroautophagy. Inhibitors of PI3K, such as Wortmannin and 3-methyladenine (3-MA) would result in decreased HLA class II presentation of endogenously expressed bacterial cytoplasmic antigen (Nimmerjahn *et al*, 2003). Alternatively, inhibition of Tor with rapamycin or its analogue CCI-779 would enhance macroautophagy (Klionsky, 2005). These autophagosomes contain one or more intravesicular membranes which, when fused with the lysosomes and early endosomes (Berg *et al*., 1998), would render its contents susceptible to breakdown and processing in the MHC class II pathway. Paludan *et al* (2005) demonstrated that by inhibiting acidification of the lysosome would lead to build up of whole unprocessed endogenously expressed viral proteins in the lysosome, suggesting proteolysis of endogenously expressed antigens takes place in the lysosome and not in the cytoplasm before HLA class II molecule loading.

Chaperone-mediated autophagy (CMA) is a selective process of antigen transfer from the cytoplasm to the lysosome by specialised proteins, heat shock cognate protein (HSC) and HSP. It was noted by Dengjel *et al* (2005) that during the period of cell starvation there is an accumulation HSC-70, which is a key chaperone protein in CMA. HSC-70 transfers endogenously expressed proteins from the cytosol into the lysosome through interaction

with the lysosome-associated membrane transporter (LAMP)-2a, since overexpression of LAMP-2a or HSC-70 would lead to enhanced CMA (Zhou *et al*, 2005). Whereas, diminished expression of HSC-70 would result in reduced CMA induced CD4+ T cell response. In addition to HSC-70, Rajagopal *et al* (2006) demonstrated in mouse models that the inhibition of HSP-90 with geldanamycin would lead to a dose-dependent reduction in the presentation of endogenously expressed antigens by CMA.

The phenomenon of autophagy remains true for many endogenously expressed antigens, however, Taylor and Rickinson (2007) demonstrated that not all endogenously expressed antigens enter the HLA class II processing pathway through autophagy. In their experiments using lymphoblastoid cell lines (LCL), a B cell line immortalised by EBV expressing GFP-tagged endogenously expressed antigens, reported to be susceptible to autophagy, could be inhibited by 3-MA. However, 3-MA was unable to inhibit presentation of some endogenously expressed viral antigens in these LCLs, since these LCLs were able to induce antigen-specific CD4+ T cell responses (Taylor and Rickinson, 2007). More importantly, they demonstrated the transfer of antigens from a 'donor' cell to a 'recipient' cell through a process similar to acquiring exogenous antigen (endocytic pathway). In this particular experiment, the donor cells are described as cells expressing the relevant antigens but do not have the relevant HLA II molecules to present these antigens to antigen-specific CD4+ T cells. The recipient cells, in this case B cells, do not express the relevant antigens but do have the relevant HLA II molecules to present antigens to antigen-specific CD4+ T cells. When the donor cells were cultured with the recipient cells, the recipient cells induced antigen-specific CD4+ T cell responses. Antigen transfer does not require direct donor-recipient cell contact, since culturing recipient cells in filtered (cell free) donor cell media could also induce antigen-specific CD4+ T cell responses.

These experiments clearly show that endogenously expressed antigens could gain access to the HLA II processing pathway and be presented to CD4+ T cells. Some antigens gain access possibly through autophagy, while other endogenously expressed antigens are picked up and treated as exogenously acquired antigens. The central message from these

reviews is that both endogenously expressed and exogenously acquired antigens can gain access to the HLA I and II processing pathway and presented to CD8+ and CD4+ T cells.

# Section 1.4
# Epstein-Barr Virus

### 1.4.0 History of Epstein – Barr Virus

The study leading to the discovery of Epstein – Barr virus (EBV) was initiated in the 1950's where Denis Burkitt, an Irish surgeon working in Uganda, noted an unusual cancer of the jaw specifically affecting children (Burkitt, 1958). This cancer, now known as Burkitt's lymphoma (BL), was substantially found to occur throughout equatorial Africa and its distribution mirrored very clearly to that of holoendemic malaria (Burkitt, 1962). These observations, originally leading to speculations, that a vector-borne agent may be responsible for the disease led ultimately to the discovery by electron microscopy of a herpesvirus-like particle in a BL-derived cell line (Epstein *et al*, 1964). This virus proved to be antigenically different to the then known human herpesviruses and was subsequently named as Epstein-Barr virus after the discoverers.

Despite the geographic restriction over BL incidence, seroepidemiological studies, demonstrated that EBV infection is very common throughout all human populations with >90% adults seropositive (Henle *et al*, 1969). Clearly, therefore, its association with BL was complex since only very few infected individuals develop the tumour. Forty years on, although we understand many aspects of EBV biology, still the pathogenesis of EBV associated tumours, now including BL, Hodgkin's Disease (HD), post-transplant lymphoproliferative disease (PTLD), acquired immunodeficiency syndrome-related central nervous system lymphomas (AIDS-CNSL) and nasopharyngeal carcinoma (NPC), remains unresolved.

### 1.4.1 EBV infection *in vitro*: B cell entry

The understanding of EBV infection *in vitro* came from studies of EBV interaction with its natural host, B cells. One of the most abundant viral glycoprotein expressed on the surface of the viral envelope is gp350/220, which is responsible for high affinity binding of the virus to B cell surface protein, the complement receptor type2 (CR2). The natural ligand of CR2 is the C3dg component of complement binding (Fingeroth *et al*, 1984; Fingeroth *et al*, 1988). The gp350/220 CR2 binding site has a very similar amino acid sequence to that of C3dg CR2 binding site. Synthetic peptides correlating to the C3dg CR2 binding site would

inhibit gp350/220-CR2 binding (Nemerow *et al*, 1989). Studies using recombinant EBV virus lacking gp350/220 show reduced efficiency of these viruses to infect B cells, thus suggesting that gp350/220 is the dominant, but not the only route in which EBV could infect B cells (Janz *et al*, 2000). In addition, antibodies targeting gp350/220 and soluble forms of CR2 could neutralised virus infection of B cells (Moore *et al*, 1991; Tanner *et al*, 1988).

Complex formation of gp350/220 with CD21 would initiate receptor mediated endocytosis of the virus (Tanner *et al*, 1987) into a low pH compartment, but low pH is not required (Miller and Hutt-Fletcher, 1992). In addition, EBV requires glycoproteins gH, gL, gB and gp42 for efficient fusion and penetration (Backovic *et al*, 2007; McShane and Longnecker, 2004). The exact role of these glycoproteins in fusion is unclear, but studies show that they are essential component in fusion of the viral envelope with the host membrane leading to penetration of the virus into the host. The glycoprotein gL serves as a chaperone for gH, and these two glycoproteins exist as a noncovalently bound complex. In studies with recombinant gH-null viruses, gL is not expressed, but more importantly the virus retains the ability to bind B cells but is unable to mediate B cell fusion (Molesworth *et al*, 2000). Likewise, recombinant viruses with gB deleted would also prevent B cell infection (Lee and Longnecker, 1997).

Glycoprotein gp42 associates noncovalently with gHgL and has been shown to interact with HLA class II molecule which functions to mediate viral entry into B cellss. Monoclonal antibodies (MAbs) against gp42 or HLA II molecules would inhibit viral infection (Li *et al*, 1997).This finding was supported in a model using recombinant viruses with gp42 deleted are unable to infect B cells. Infection in B cells could be restored when a soluble gp42 is introduced that could re-associates with gHgL. However when a soluble truncated gp42 that could bind to HLA class II molecules but not re-associated with the gHgL complex was introduced, it demonstrated to still inhibit B lymphocytes infection. This finding suggests gp42 is vital to B lymphocytes infection and must associate with the gHgL complex (Wang *et al*, 1998).

### 1.4.2 EBV infection *in vitro*: Epithelial cell entry

EBV infection of epithelial cells is more complicated than in B cells, since CR2 is expressed at low levels on some epithelial cells in culture. However, Fingeroth *et al* (1999) demonstrated using culture epithelial cells expressing low level cell surface CR2, EBV infection can occur. However, it is still uncertain whether epithelial cells express CR2 *in vivo* since monoclonal antibodies used in these studies cross-reacted with unrelated epithelial cell proteins (Young *et al*, 1989).

In addition to the CR2 dependent mechanisms of infection by EBV, three CR2-independent mechanisms have been proposed. The first mechanism came from evidence that the EBV virus when coated with gp350/220-specific IgA antibody would bind to the IgA receptor (Sixbey and Yao, 1992). It is suggested that the route of infection might be related to the IgA receptor, since cells expressing IgA receptor are more susceptible to EBV infection induced by EBV-specific IgA. The second mechanism came from the observation that EBV derived from B cells expressing gHgL complex can bind to epithelial cells while gH-null recombinant viruses loose this ability (Molesworth *et al*, 2000). In addition, monoclonal antibodies against gH is able reduce EBV binding to epithelial cells. These observations suggest that epithelial cells express a receptor, which is yet to be identified and is termed the gHgL receptor, which is used by EBV to bind to epithelial cells. Finally, EBV express a membrane protein, encoded by the BMRF2 open reading frame, which has been demonstrated to bind integrin that are expressed on the surface of polarised epithelial cells (Tugizov *et al*, 2003).

Fusion of EBV in nonpolarised epithelial cells takes place at neutral pH and does not appear to require endocytosis (Miller and Hutt-Fletcher, 1992). Studies using gp42-null recombinant viruses and monoclonal antibodies specific for gp42 do not affect epithelial cell infection, which is expected since epithelial cells do not constitutively express HLA II molecules (Wang *et al*, 1998). However, association of gp42 with the gHgL complex would inhibit epithelial cell fusion. More importantly, Wu *et al* (2005) identified gH-specific

monoclonal antibodies that specifically inhibit epithelial cell fusion but do not inhibit B cell fusion. This indicated that the mechanism for gHgL complex mediated fusion in B cells is different from that of epithelial cell fusion. Stoichiometric analysis of virion glycoprotein composition confirms this findings showing the expression of two kinds of complexes, the gHgLgp42 and the gHgL complex, on the EBV envelope. In addition, Wu *et al* (2005) was able to show, by making different gH chimeras by joining EBV gH with gH homologue from rhesus lymphocrytovirus, that certain chimeras preferentially mediate fusion with B cells, others preferentially mediate fusion with epithelial cells, and then there are those that would mediate fusion with neither cell types. These results suggested that gH important fo fusion in both B cells and epithelial cells, but differs in the way which fusion is triggered. In addition to the gHgL complex, epithelial cell fusion also requires gB expressed at a higher protein level than seen with B cell infection (Oda *et al*, 2000). How the process of fusion takes place still remains to be determined.

### 1.4.3 The EBV genome

The EBV genome is a double-stranded linear DNA that is approximately 184 kb in length encoding approximately 100 genes. EBV was the first herpesvirus to have its genome completely sequenced (Baer *et al*, 1984). The EBV genome was sequenced from the B95.8 EBV strain that was broken into fragments of variable sizes using various endonuclease, and the *Bam* HI endonuclease notation will be used in this thesis to refer to the EBV genome. The different *Bam* HI fragments were sorted in descending order of fragment size, A to Z. Individual DNA fragments were than cloned into *Escherichia coli*, sequence and paste together. Open reading frames (ORFs), genes and sites of transcription or RNA processing are usually referred to *Bam* HI (B) fragments, fragment size (A-Z), direction of promoter or ORF (L, left; R, right), whether it is a promoter (P) or ORF (F), and follow by a number indicating which ORF is being referred to; e.g BARF1 would be ***B****am* HI - fragment **A** - **R**ightward - OR**F** – **1** (first ORF).

The B95.8 EBV genome is approximately 172kb and, in comparison to wild type strain, has an approximate 12 kb deletion in the *Bam* HI A region of the genome. This deleted region

was later sequenced from the Raji virus strain (Parker *et al*, 1990). At both ends of the DNA are terminal repeats (TR) that are approximately 0.5kb in length. In latently infected cells, the EBV genome is circularized, and the TR ends are joined together where the LMP2 exons are also encoded.

### 1.4.4 Latent Genes

During latency infection, latent genes are expressed; these include the EBERs, BART transcripts, EBNAs and LMPs. The functions of these genes are summarized to give a general idea of their role in EBV-associated diseases.

#### 1.4.4.1 EBERs

There has been considerable progress in understanding the function of Epstein-Barr virus (EBV)-encoded small nonpolyadenylated RNAs (EBERs) in EBV infection. EBER1 and EBER2 are the most abundant viral transcripts in latently EBV-infected cells (Lerner *et al*, 1981; Rymo, 1979). The EBERs have been demonstrated to associate with cellular proteins La (Lerner *et al*, 1981), ribosomal protein L22 (Toczyski *et al*, 1994) and double-stranded RNA activated protein kinase (Clemens *et al*, 1994). This association with cellular proteins may function to control expression or inhibition of cellular genes that may play a role in maintenance of EBV-infected cells (Kitagawa *et al*, 2000; Yang *et al*, 2004; Takada, 2000). Swaminathan *et al* (1991) demonstrated in their EBER negative recominbinant virus model that EBERs are not essential for immortalization of B lymphocytes or for the replication of the virus. However, Yajima *et al* (2005) demonstrated in their EBER negative recombinant virus models that EBERs do play a critical role in improving the efficiency of B-cell transformation.

#### 1.4.4.2 BART transcripts

The BARTs have the potential to encode for three polypeptides A73, RPMS1 and BARF0. To date, there is no substantial evidence showing that these polypeptides are ever expressed. However, many groups have tried to understand the biological functions of these polypeptides by transfection models. Inspection of the amino acid composition of RMPS1

ORF showed the presence of an amino acid sequence WWP, which is rarely found in protein sequences, but is also found in the EBV EBNA2 sequence (Smith *et al*, 2000). This WWP motif in EBNA2 is important in EBNA2 for the binding to cellular protein CBF1. RPMS1 was demonstrated to interact and regulate the functions of cellular proteins CBF-1, NotchIC and CIR (Smith *et al*, 2000) which may be important in the life-long persistent infection of the virus (Zhang *et al*, 2001). More importantly, interaction of RPMS1 with Notch seems to favour proliferation of epithelial cells. BARF0 was also demonstrated to bind to Notch and directs the protein for proteosomal degradation (Thornburg *et al*, 2004). These studies demonstrated, if RPMS1 or BARF0 really exist, the regulation of Notch is important in EBV biology since Notch appears to have tumor suppressor function in several cell types such as keratinocytes and squamous cell carcinomas (Weng and Aster, 2004).

A73 was demonstrated to bind to several cellular proteins; RACK1 (receptor for activated C kinase), KIAA0547 (protein of no known function), β5-integrin, and a protein which could not be identified in EBML, Genbank or the EST databse (Smith *et al*, 2000). It was shown that RACK1 interacts with Src and Lck and inhibiting their activity, resulting in reduced growth rate in NIH 3T3 cells (Chang *et al*, 1998). The exact role of A73 in EBV biology is yet to be determined, but possibly A73 functions to modulate the activity of these cellular proteins and contribute to the development of EBV tumours.

#### 1.4.4.3 EBNA 1

EBNA 1 is the only viral protein which is expressed in all different forms of latency and is absolutely required for episomal maintenance of the viral genome in proliferating cells by ensuring segregation at each cell division (Yates *et al*., 1985). The B95.8 EBV strain of EBNA 1 is a 641 amino acid nuclear protein. The protein has a high proline content and consists of four components: (1) 89 amino acid Arg-rich N-terminal, (2) gly-ala repeat domain between amino acids 90-327, (3) a second Arg-rich region between amino acids 328-386 which contains a nuclear localization sequence between amino acids 379-386, and (4) a C-terminal domain between amino acids 387-641 which can bind DNA and dimerise (Oddo *et al*, 2006).

EBNA1 binds specifically to two clusters of sites within the EBV plasmid origin of latent DNA replication (*OriP*). *OriP* is a 1.7 kbp *cis*-acting region of the EBV genome and contains two clusters of EBNA1 binding sites, the family of repeats (FR) and the dyad symmetry elements (DS). The FR and DS contain multiple copies of high affinity binding sites, the FR contain 20 tandem copies of a 30bp element and the DS contain 4 copies of the repeat, and these copies of repeats are separated by 980bp of spacer DNA (Ambinder *et al*, 1991; Chen *et al*, 1993). In addition, EBNA1 can also bind to three different promoters located in *Bam* HI- C, Q and W with different affinities, and the binding of EBNA1 to these promoters plays an important role in the maintenance of latent EBV infection.

#### 1.4.4.4 EBNA-2, -3s and -LP

EBNA2 is a key transactivator of gene expression of both viral (LMP1 and 2) and human genes (CD21 and CD23) through interaction with RBP-Jκ. EBNA2 is important in B lymphocyte transformation because mutant viruses that carry the EBNA2 deletion are transformation incompetent (Rabson *et al*, 1982; Sixbey *et al*, 1991).

Recombinant viruses with an EBNA3 gene deletion showed that EBNA3B is not essential in latent infection of primary B lymphocytes, LCL outgrowth, cell survival or lytic virus replication (Tomkinson and Kieff, 1992). While EBNA3A and EBNA3C are critical for the transformation of B lymphocytes and survival.

EBNA-LP appears to interact with EBNA2 in a cooperative manner to induce cellular or viral gene expression (Peng *et al*, 2004; Sinclair *et al*, 1994). However molecular genetic analysis showed that full length EBNA-LP is not an absolute requirement for B lymphocyte transformation because LCL could be generated from EBV with deleted EBNA-LP (P3HR1/633 EBV). However, EBNA-LP deficient LCL seems to grow poorly but growth could be restored by transfecting with a vector expressing wild type EBNA-LP.

#### 1.4.4.5 LMP-1 and -2

The LMPs are membrane bound proteins. LMP1 and LMP2 have very distinct biological functions. LMP1 is a potent transforming protein activating a range of cellular transcription factors, such as, NF-κB (Wu *et al*., 2005). These transcription factors complement the role of EBNA2 in the transformation of B cells. LMP2 expression was demonstrated to influence signaling pathways linked to cell adhesion and motility, a possible role in the migration of cancer cells (Allen *et al*., 2005; Pegtel *et al*., 2005).

The B95.8 EBV strain LMP1 is a 386 amino acid consisting of three domains: (1) a 20 amino acid arginine and proline rich N-terminus that is located in the cytoplasm, (2) six 20 amino acid α-helical transmembrane segments joined together by five 8-10 amino acid reverse turns, and (3) a 200 amino acid C-terminus rich in acidic residues also located within the cytoplasm (Eliopoulos and Young, 2001). LMP1 is a relatively unstable protein with a short half-life of less than 2 hours but is converted to a more stable cytoskeleton-associated form. LMP1 also aggregates either with itself or other cellular membrane proteins (i.e. vimentin) to form a large, non-covalently linked membrane complex. Much of the understanding of LMP1 function came from studies in the ability of EBV recombinants carrying specific mutated LMP1 to transform primary B lymphocytes into LCLs. Several important functional properties had been identified from such experiments: (1) the N-terminal tail of LMP1 contains sequences which hold the protein in the correct orientation for aggregation and oligomerization, (2) the cytoplasmic C-terminus of LMP1 is responsible for signal transduction leading to B cell transformation and phenotypic changes (Eliopoulos and Young, 2001). Biochemical studies of the mechanism that underlies the transformation ability of LMP1 showed that the protein could activate the NFκB pathway through interaction with the cellular protein tumor necrosis factor (TNF) receptor-associated factor (TRAF)-1, -2 and -3 and TNFR-associated death domain proteins, TRADD and RIP. This type of signaling is similar to that seen with human CD40 in the activation of NFκB.

The LMP2s are two integral membrane proteins, LMP-2A (497 amino acids) and -2B (378 amino acids). Both proteins are structurally similar with an N-terminus cytoplasmic domain (LMP2A not 2B) followed by 12 integral membrane segments joined together by short reverse turns and a 27 amino acid C-terminus (Miller *et al*, 1994). LMP2A N-terminal domain directly interacts with cellular proteins, the src family tyrosine kinases (syk, fyn and lyn) and inhibits B cell receptor signaling, preventing activation of EBV replication (Caldwell *et al*, 2000). Studies of EBV infected epithelial cell models suggest that LMP2A and 2B may have other roles in these cell types different from that observed in infected B cells (Allen *et al*., 2005). EBV infected epithelial cells were observed to have increased cell adhesion and motility. However, the mechanism is still yet to be elucidated but there is evidence which suggests that tyrosine kinases may play a role (Scholle *et al*., 2000).

#### 1.4.5.1 Lytic cycle antigens

Viral gene expression in lytic cycle follows a temporal and sequential order. The immediate early genes are expressed followed by the early genes and then the late genes. *In vitro* infection of B lymphocytes usually results in latency type infection and models of studying lytic infection usually requires inducing latently infected cells into lytic cycle (Amon *et al*, 2005). Viral immediate early gene expression is induced directly by signal transduction of the B cell receptor. Two immediate early mRNAs have been identified for EBV, encoding the proteins BZLF1 and BRLF1. BZLF1 appears to be the major immediate early gene product in EBV. BZLF1 plays a dominant role in the switch from latent cycle to productive infection (Flemington and Speck 1990; Lieberman and Berk 1990). BZLF1 is a 34 to 38kD DNA-binding protein related to the basic leucine-zipper family of transcription factors with some sequence homology to c-Fos. BZLF1 has been shown to bind to several early gene promoters and cellular gene promoters, as well as the lytic origin of replication. BZLF1 further activates its own expression by binding to its own promoter and also activates transcription of BRLF1 (Speck *et al*, 1997). BRLF1 is also a transcription factor, and together with BZLF1 would activate most of the early genes.

Lytic cycle replication of EBV DNA requires several early gene products; BALF5, BMRF1, BALF2, BBLF4, BSLF1 and BBLF2/3 (Fixman *et al*, 1995). BALF5 encodes a DNA polymerase catalytic subunit (Tsurumi *et al*, 1993), BMRF1 encodes the DNA polymerase accessory subunit (Tsurumi, 1993), BALF2 encodes a single-stranded DNA-binding protein (Tsurumi *et al*, 1998), and the proteins encoded by BBLF4, BSLF1 and BBLF2/3 encode a protein complex, predicted from sequence comparison with herpes simplex virus type 1 (HSV-1) genes, to act as helicase, primase and helicase-primase associated proteins (Fixman *et al*, 1995). These proteins are believed to work together to replicate the EBV genome. Finally, proteins identified as late lytic genes encode for viral structural proteins, such as that of the glycoproteins gp350/220, gH, gL, and gp42 that are expressed on the viral envelope, and also the viral capsid proteins.

#### 1.4.5.2 BARF1

BARF1 is a lytic cycle antigen, and it will be described in more detail in this section of the thesis because its expression can also be found in latency type infection. An x-ray crystallography study of BARF-1 showed that BARF-1 has a sequence and structural homology similar to human CD80 (Tarbouriech *et al*., 2006). Human CD80 (expressed on the surface of antigen presenting cells) interacts with CD152 or CD28 (expressed on the surface of T cells) either by emitting inhibitory or activating signals. Stockbine *et al* (1998) observed the binding of BARF-1 to human colony-stimulating factor 1 (hCSF1) thus inhibiting T cell differentiation. This may suggest that BARF-1 play an important role in regulating the host immune response to enhance EBV infection. More recently, Wange *et al* (2006) reported that cancer cells transfected with BARF-1 are more tolerant to chemotherapy drugs, such as taxol, suggesting a role for BARF-1 with anti-apoptotic function.

### 1.4.6 Early events in EBV infection

Once the virus has entered the host, the process leading to capsid dissolution and genome transport to the nucleus is not known. However, in almost all EBV-infected cells the EBV linear genome becomes a covalently closed circular EBV episome (Adams, 1987; Lindahl,

1976). There is evidence suggesting the transcription factor Sp1 (and maybe with the help of other proteins) may play an important role in circularisation of the EBV genome by binding and joining the TR (Sun *et al*, 1997). Inhibitors of DNA, RNA and protein synthesis added to cells at the time of viral infection will inhibit genome circularisation at least suggesting that active cell macromolecular synthesis is necessary to aid the circularisation process (Sinclair and Farrell, 1995).

The virus can undergo two types of infections, lytic and latent. Lytic infection usually takes place in epithelial cells *in vivo* resulting in active replication, a process where viruses are produced and are released from the infected cell (Sixbey *et al*, 1983). Lytic cycle infection normally takes place in the oropharynx where a high viral load can be found in saliva (Gerber *et al*, 1972; Yao *et al*, 1985). Latent infection of B lymphocytes *in vitro* will result in circularisation of the EBV genome into episomes, expression of viral latent genes and immortalising the B lymphocyte.

### 1.4.7 Latency gene expression

Latent gene expression varies between different types of human malignancies. Figure 1.4.1 shows three models of latent infections, Latency I, II and III, that roughly describes the transcription pattern of the different human malignancies. The latency III gene expression comes from observation of *in vitro* transformed B lymphocyte lines (LCL) (Henderson *et al*., 1977). The latency III transcription pattern includes the expression of (1) EBV early region (EBER) RNAs 1 and 2, (2) the *Bam* H1A RNAs (BARTs), a family of spliced polyadenylated transcripts with the potential of encoding 3 polypeptides (BARF0, A73 and RPMS1), (3) EBV nuclear proteins (EBNA) 1, 2, 3A, 3B, 3C and LP expression through the Wp/Cp promoter, and (4) three spliced *Bam* HI N RNAs encoding latent membrane proteins (LMP) 1, 2A and 2B. Latency I was first described in EBV-positive BL cells with the characteristics of expression of; EBERs; BARTs; silencing of the Wp/Cp promoter and activation of the FQp promoter. This shift in promoter usage only initiates EBNA1 expression and all other EBNAs are not expressed; silencing of the LMP promoters in the

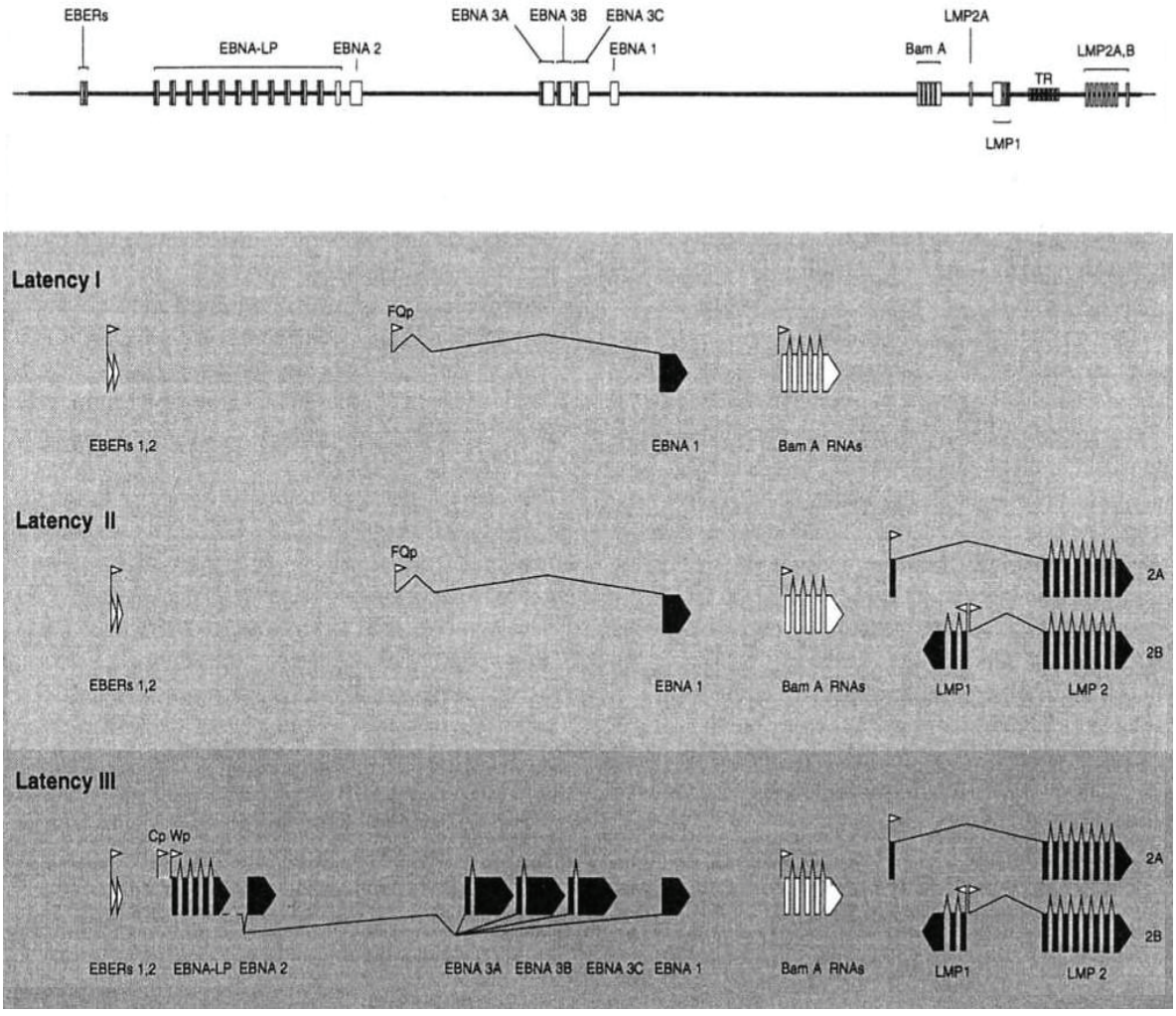

EBERs
EBNA-LP
EBNA 2
EBNA 3A
EBNA 3B
EBNA 3C
EBNA 1
LMP2A
Bam A
LMP2A,B
TR
LMP1
Latency I
FQp
EBERs 1,2
EBNA 1
Bam A RNAs
Latency II
FQp
EBERs 1,2
EBNA 1
Bam A RNAs
2A
2B
LMP1
LMP 2
Latency III
Cp Wp
EBERs 1,2
EBNA-LP
EBNA 2
EBNA 3A
EBNA 3B
EBNA 3C
EBNA 1
Bam A RNAs
2A
2B
LMP1
LMP 2

**Figure 1.4.1: The patterns of EBV latent gene transcripts in the three forms of latency.**

This Figure was taken from Kief and Rickinson (2001, p.2399). The genomic positioning of the exons is shown (*top*) on a linear map of the viral genome. It is important to note that the viral genome is circularized in latently infected cells through fusion of the terminal repeats (TR), and the LMP2 transcripts are spliced across this region. The transcripts are displayed in the correct orientation relative to one another with promoters (*pennants*), noncoding RNAs (*white*) and mRNAs (*black*).

*Bam* HI N region. Latency II was first recognised in EBV-positive Hodgkin Disease (HD) to express EBERs, BARTS, silencing of Wp/Cp promoter and activation of the FQp promoter for the expression of EBNA1, activation of one or more LMP promoter and BARF1 protein expression.

### 1.4.8 EBV infection *in vivo*

B cells are the primary target cells for EBV infection and establishment of latency. More importantly, EBV infection in B cells is usually non-productive, thus infection of B cells does not explain the successful spread of the virus in the human population. However, EBV can also infect epithelial cells, and studies in AIDS patient with oral hairy leukoplakia show that EBV undergo massive lytic replication and sheds virus from the epithelium of the lateral part of the tongue (Greenspan *et al*, 1985). It was believed that what was seen in HIV infected individual was an amplification of the situation in healthy non-HIV-infected EBV-infected individuals. But, this is not the case, since the EBV virus was not detectable from exfoliated epithelial samples collected from patients with infectious mononucleosis and from healthy individuals (Karajannis *et al*, 1997; Niedobitek *et al*, 1997). However, EBV lytic cycle antigen BZLF1 was detected in B cells and plasma cells, which suggested that the life cycle of EBV is restricted to B cells *in vivo* (Niedobitek *et al*, 1997). Laichalk and Thorley-Lawson (2005) showed that when memory B cells differentiate into plasma cells, EBV lytic cycle is activated through activation of BZLF1 promoter. However, the actual number of plasma cells that is producing EBV is minimal and does not correspond to the viral load found in saliva.

In a study by Shannon-Lowe *et al* (2005), the authors showed that when epithelial cell lines were directly cultured in cell-free recombinant EBV, expressing GFP, only <1 in $10^4$ epithelial cells were GFP-positive, this is termed direct infection. Whereas, when the same epithelial cell lines were cocultured with freshly prepared B cells coated with the same virus preparation, up to 25% of epithelial cells were GFP-positive. In addition, when the same experiment was repeated using a recombinant EBV with BZLF1-deleted but still expressing GFP, efficient epithelial cell infection was observed. This indicated that

infection of epithelial cells is not secondary to B cell infection, since the efficiency of infection by replication-deficient recombinant EBV was the same as that of wild type, but is a direct transfer from the B cell surface to the epithelial cells. This is termed transfer infection. Using different epithelial target cells (including cultures of normal nasopharyngeal epithelium) in the same experiments, Shannon-Lowe *et al* (2005) demonstrated efficient EBV infection in these epithelial cells, but was unable to infect normal fibroblasts, endothelial cells or primary T cells. They also demonstrated using different CR2-expressing donor cells (including EBV-negative Akata-BL, BJAB B cell lines, primary B cells and primary T cells) that primary B cells was the optimal donor cell type in transfer infection. Interestingly, glutaraldehyde fixation of B cells prior to exposure to EBV did not affect EBV binding but severely reduces transfer infection of their donor epithelial cells. This indicated that transfer infection requires active participation of the donor cell. Time course experiments, using monoclonal antibody staining for gp350 and fluorescence *insitu* hybridisation (FISH) using sensitive EBV DNA probe to detect for DNA containing EBV viral particle on the surface of B cells, showed that infected primary B cells is capable of transfer infection around 6 hours postinfection and remained competent to do so in up to 2 days. More importantly, EBV retained on the B cell surface was the source of transfer infection.

In studies using recombinant EBVs that lack gp350, gH, gp42 or BILF2 glycoprotein and recombinant EBV expressing low levels of gB, Shannon-Lowe *et al*,(2005)showed several important points: (1) gp350 is required for virus binding and transfer infection, and interestingly, gp350-null recombinant EBV consistently gave rate of direct infection equivalent to that seen in transfer infection, (2) as expected, gp42 is required for B cell infection, through interaction with HLA II molecule, but is not required in transfer infection, and (3) gH and gB are essential for EBV infection of B cells and epithelial cells.

Finally, Shannon-Lowe *et al* (2005) demonstrated, using confocal microscopy study and MAbs staining of CR2, gp350 and FISH staining of EBV DNA, that B cells at 24 hours post EBV infection would form conjugates with epithelial cells. More importantly, CR2

and gp350 accumulates at the junction where there was cell-cell contact. These data suggest that gp350 may possible inhibit direct infection of epithelial cells, and infection of epithelial cells is made possible when gp350 is bound to the B cell exposing the viral ligand to the epithelial receptor.

Persistent infection is achieved by infection of circulating naïve B cells. These EBV-infected naïve B-cells show a latency type III growth program. The EBV-infected naïve B-cells enter the germinal center where they proliferate and clonally expand, and therefore increase the EBV-infected B-cell pool. The germinal center infected cells show a latency type II EBV gene expression pattern (Thorley-Lawson, 2001). The EBV-infected germinal center cells differentiate into memory B-cells and functions as the long-term reservoir for EBV. Most of the EBV-infected memory B-cell population that circulates in the periphery do not show any EBV gene expression (termed latency type 0), but a small population of these EBV-infected memory B-cells would show a latency type I EBV gene expression pattern.

The next section of this thesis will review some of the current understanding of immune responses to EBV infection, specifically antibody responses and T cell responses to EBV antigens. This will set a theme for later sections of this thesis when reviewing the current immunotherapeutic strategies that are being studied in the treatment of EBV diseases.

# Section 1.5
# Immune responses to EBV infection

## 1.5 Immune responses to EBV infection

### 1.5.1 Antibody responses to EBV antigens

Henle *et al* (1968) first observed that infectious mononucleosis (IM) was associated with EBV seroconversion, and that IM patients would first develop IgM and IgG antibodies specific for viral capsid antigens (VCA), non-structural early antigens and membrane antigens. Evans *et al* (1983) showed in transmission studies in monkeys and in man that EBV infection would lead to production of heterophile antibodies and IM. This study provided a causal association between EBV infection and IM.

Interestingly, a sero-epidemiological study conducted over a period of 9 years in Uganda, where Denis Burkitt reported a high incidence of Burkitt's lymphoma (BL), showed that EBV-positive individuals who had high serum antibody titres against VCA compared to the mean control group are 30 times more likely to develop BL (de-Thé *et al*, 1978). This provided a causal link between EBV infection and development of BL. With the discovery of early antigens and the differentiation between early antigens that could be found in the cytoplasm and the nucleus ('diffuse' – D) and early antigens that could only be detected in the cytoplasm ('restricted' - R) components, Henle *et al* (1971) showed that different EBV diseases would have a different antibody profile. For example, most BL patients gave high antibody titres to EA-R. Sero-epidemiological study was also conducted on other suspected EBV diseases, such as in Hodgkin's disease (HD) patients have elevated antibody titres against VCA, EA-D and EA-R antigens (Mueller *et al*, 1989) and in nasopharyngeal carcinoma (NPC), high IgG and IgA antibody titres could be detected against VCA, EA (especially EA-D), EBNA and ZEBRA (Desgranges *et al*, 1977). Especially for NPC, antibodies to EA-D and EBV-specific IgA antibody titres is a characteristic of the disease (Henle and Henle, 1976)

### 1.5.2 $CD8^+$ T cell responses to EBV antigens

Studies in IM patients showed that these individuals have an expanded $CD8^+$ T cell numbers. Most of these $CD8^+$ T cells are lytic antigen specific (Callan *et al*., 1998) and accounts for approximately 25% of total $CD8^+$ T cell population. $CD8^+$ T cells directed at latent antigens can also be detected, but it is comparably much lower than responses against lytic antigens (Callan *et al*., 1998; Hislop *et al*., 2002). $CD8^+$ T cell responses to lytic antigens are poorly understood considering that there are ~70 lytic antigens and fewer than 20 of these antigens have been studied. However, of the antigens studied to date, there seems to be a clear immunodominance in $CD8^+$ T cell responses to the immediate early antigens (BZLF1 and BMRF1), and some of the early lytic antigens and the weakest responses to the late lytic antigens (Pudney *et al*, 2005). This may be due to impairment of the HLA II presentation pathway, as Keating *et al* (2002) demonstrated by FACS analysis. In their experiment, Keating *et al* (2002) showed, when latently infected cells (BL cell line Ag876 and B cell line (LCL)) were induced to go into lytic infection, that there was a decline in HLA I molecule staining by 5-fold and a 40-50% reduction in HLA II molecule staining relative to the latently infected cells. They also showed by monoclonal antibody staining for the presence of BZLF1 that these cells were in lytic infection. More importantly, downregulation of HLA I molecules happen early on during lytic infection, since the decrease in HLA molecules in late lytic cycle (by staining for VCA) and in early lytic cycle (by staining for presence of BZLF1) was approximately the same, 5-fold lower than seen in latently infected cells. These data suggests that $CD8^+$ T cell responses to the immediate early antigens are strong mainly because at this stage of infection the MHC class I presentation pathway is still intact, but as the virus infection progresses onto early and late gene expression, the MHC class I presentation pathway becomes impaired.

Phenotype studies of these $CD8^+$ T cell populations show that lytic antigen specific $CD8^+$ T cells are of the late $CD28^-/CD27^-$ differentiation phenotype (Hislop *et* al., 2001) whereas, latent antigen specific $CD8^+$ T cells are of the early $CD28^+/CD27^+$ differentiation phenotype also frequently expressing CCR7, a homing marker for secondary lymphoid organs (Appay *et al*., 2002; Hislop *et al*., 2001).

As for $CD8^+$ T cell responses to the latent antigens, a hierarchy of responses was also observed. The EBNA3s (EBNA3A, -3B and -3C) are the dominant $CD8^+$ T cell responses (Steven *et al*., 1996) followed by LMP2 responses (Khanna *et al*., 1992). Whereas, $CD8^+$ T cell responses to LMP1, measured by IFN-γ ELISpot assay (Meij *et al*., 2002), and EBNA1 (Lee *et al*., 2004) are less frequent. Approximately 10% (5/50) of healthy Caucasian donors have detectable HLA-A2 restricted responses against 2 LMP1 epitopes (LMP1 125-133 and LMP1 159-167) whereas responses to other LMP1 epitopes are less frequent (Meij *et al*., 2002). While, approximately 50% of the Caucasian population is HLA-A2 positive this clearly shows that only a minority of the Caucasian population responds to LMP1. On the other hand, responses to LMP2 are much more frequent, with more than 40% (12/28) of healthy Caucasian donors tested against LMP2 synthetic peptides had detectable $CD8^+$ T cell responses against two HLA-A2 restricted epitopes (LMP2 356-364 and LMP2 426-434). In addition, of the 12 LMP2 epitopes identified, 4 are also HLA-A2 restricted epitopes (Meij *et al*., 2002).

EBNA1 was first thought to be an immune evasive protein which prevents proteasome degradation through its gly-ala repeat domain (Levitskaya *et al*., 1995; Levitskaya *et al*., 1997). And the gly-ala repeat domain was found to downregulate its own protein expression and therefore reduces the chances of being detected by the immune system (Yin *et al*., 2003). More importantly, earlier work failed to detect EBNA1-specific $CD8^+$ T cell responses by cytotoxicity assay. These data led to the assumption that EBNA1-specific $CD8^+$ T cell responses are not present in the T cell repertoire. Even with these immune evasive strategies, EBNA1 does not fully escape detection by $CD8^+$ T cells in studies using LCL models. EBNA1-specific $CD8^+$ clones could be generated from EBV-immune donors with the capacity to recognize naturally infected target cells using IFN-γ as readout (Blake *et al*., 1997; Blake *et al*., 2000; Lee *et al*., 2004). More importantly, these IFN-γ producing EBNA1-specific $CD8^+$ T cells are capable of inhibiting outgrowth of LCLs. However, these clones are unable to recognize Burkitt's lymphoma cells or TAP-deficient cells (T2) which suggest that processing of EBNA1 for $CD8^+$ T cell recognition is through the conventional

proteosome/TAP-dependant pathway. This was confirmed using inhibitors of proteosomal function which would abolish LCL recognition by these clones (Lee *et al*., 2004; Tellam *et al*., 2004).

Immune responses to the *Bam*H1A rightward transcripts (BARTs) are poorly studied mainly because there is no substantial evidence to date which suggests that any of the predicted BART polypeptides are ever expressed at the protein level. But the main reason is because much of the work concerned with T cell responses has been focused on the well known EBV antigens (the LMPs and EBNAs).

However, $CD8^+$ T cell responses to BARF0 (one of the predicted BART polypeptides) have been studied (Kienzle *et al*., 1998) mainly initiated by the use of rabbit anti-serum which detected the expression of the product at the protein level (Fries *et al*., 1997). However, this same rabbit antiserum was later found to cross-react with cellular proteins (Kienzle *et al*., 1999). Therefore to date, it is still uncertain whether BARF0 is expressed at the protein level. However, in the study by Kienzle *et al* (1998), they showed that an HLA-A2 (LLWAARPRL) restricted BARF0-specific $CD8^+$ T cell clones could be generated from a single healthy virus carrier and, demonstrated that these clones have the capacity to kill an EBV-negative BL line overexpressing BARF0 using a vector but not naturally infected BL line. This suggested that possibly BARF0 is expressed naturally at very low levels thus evading detection by the immune system rather than immune evasion of the proteosome degradation pathway since, the epitope LLWAARPRL is processed for presentation when BARF0 is overexpressed in a EBV-negative BL line (DG75, HLA-A2 positive BL cell line). More importantly, Kienzle *et al* (1998) demonstrated, using the BARF0 peptide, that BARF0 peptide-specific CD8+ T cell response could be detected in HLA-A2 positive EBV seropositive individuals but not in HLA-A2 positive EBV seronegative individuals. In addition, BARF0-specific CD8+ T cells were capable of killing BARF0 peptide loaded target cells. These data strongly suggest that BARF0-specific CD8+ T cells do exist, since it could be detected only in EBV-seropositive but not in EBV-seronegative individuals.

### 1.5.3 $CD4^+$ T cell responses to EBV antigens

$CD4^+$ T cell responses to EBV have not been studied until quite recently mainly because EBV specific $CD4^+$ responses never reach the high frequency of EBV specific $CD8^+$ responses (Amyes *et al*., 2003; Precopio *et al*., 2003), and there was an assumption that they were not as important as $CD8^+$ T cells as effectors. But this was an assumption and not a fact.

Evidence supporting the importance of CD4+ T cells in the role for controlling persistent infection came from studies in MHC class II knockout (KO) mice models (Cardin *et al*., 1996). Respiratory challenge of the MHC class II KO mice with murine gammaherpesvirus 68 (MHV-68) leads to productive infection of the virus in both lungs and adrenal epithelial cells. But primary infection is effectively controlled by $CD8^+$ T cells and the virus develops persistent infection in lymph node and splenic B cells. However, over time, the control of persistent infection deteriorates due to progressive loss of $CD8^+$ T cells in the absence of $CD4^+$ T cell responses measured by increase of viral load in the lung, development of wasting and early death in MHC class II KO mice. This experiment clearly shows that $CD4^+$ T cell responses are important in the control of persistent infection, probably as 'helper' cells in maintaining an efficient $CD8^+$ T cell response, humoral response or as direct control.

In addition to 'helper' function of CD4+ T cells, *in vitro* studies of EBV-specific CD4+ T cells lines and clones suggest a more direct role for CD4+ T cells in the control of persistent infection (MacArther *et al*, 2007; Landais *et al*, 2004; Adhikary *et al*, 2006). MacArther *et al* (2007) demonstrated using EBV-specific CD4+ T cell lines, generated by reactivation of cord blood with irradiated autologous LCLs, in coculture assays would inhibit the growth of autologous LCLs. Furthermore, inhibition of LCL growth is dependent on the effector:target (E:T) ratio and inhibition of LCL growth is observed when E:T>2:1. Interestingly, E:T > 16:1 would lead to elimination of LCL growth. Transwell studies demonstrated that cell-cell contact is necessary for LCL growth inhibition, since

CD4+ T cell lines coculture with LCLs on one of the membrane does not inhibit LCL growth on the other side of the membrane.

MacArther *et al* (2007) also demonstrated in chromium release assays that their CD4+ T cell lines could kill autologous LCLs, however, the mechanism of cytotoxicity was slow and maximum kill was achieved only in a 24 hour assay compared to NK cell killing of LCLs which takes 5hours. FACS analysis of these CD4+ T cell lines showed a small proportion of CD4+ T cells stained for granzyme B and granulysin which suggests that only a small proportion of cells mediate killing and this is possibly mediated through granzyme B and granulysin.

Interestingly, EBV-specific CD4+ T cell lines were demonstrated to reduce the efficiency of virus transformation of B cells, but this is only achievable at a high EBV-specific CD4+ T cell line: B cell ratio of 10:1. Whereas, at low T cell:B cell ratio (1:1) the efficiency of virus transformation doubles. This indicated that possibly majority of the noncytotoxic T cells help with virus transformation, and reflected by increase in CD4+ T cell: B cell ratio, that the minority of cytotoxic T cells dominate the response and reduces the efficiency of virus transformation (MacArther *et al*, 2007). These studies demonstrated that CD4+ T cells play an important role, both as helper cells and effector cells, in the control of persistent and productive EBV infection.

Several groups have identified $CD4^+$ T cell responses to several lytic antigens (BZLF1, BMLF1 and BHRF1) (Precopio *et al*., 2003; Amyes *et al*., 2003; Landais *et al*., 2004), of which, the HLA-DR0401 restricted (BHRF1 122-133) $CD4^+$ T cell clones were demonstrated to have direct effector functions further suggesting the role of $CD4^+$ T cells in controlling EBV replication (Landais *et al*., 2004). Adhikary *et al* (2006) also demonstrated that BZLF1, BLLF1 and BALF4 –specific $CD4^+$ T cells were cytolytic and capable of inhibiting proliferation and outgrowth of LCL cells. Thus, in addition to control of persistent infection, these studies demonstrate a role for CD4+ T cells in the control of lytic infection *in vitro*.

*In vivo* studies of EBV-specific CD4+ T cell responses in AIDS patients with primary CNS lymphoma supports the importance of CD4+ T cells in the protective role against EBV-diseases (Gasser *et al*, 2007). In this report, Gasser *et al* (2007) studied PBMC samples from six patients that were obtained 0.5-4.7 years prior diagnosis of primary CNS lymphoma and 16 PBMC samples from HIV individuals who did not develop primary CNS lymphoma. They assessed the PBMC samples for EBV-specific CD4+ T cell responses by IFN-γ ELISpot assay using EBV peptides restricted through the HLA types of the samples or EBV-infected B cell lysates to stimulate EBV-specific CD4+ T cell IFN-γ response. Their results showed that 1/6 of the patients and 13/16 of the controls had detectable EBV-specific CD4+ T cell response. This suggested that, in the absence of CD4+ T cells would lead to primary CNS lymphoma. Whereas, the control group had EBV-specific CD4+ T cell responses and thus preventing these individuals from developing primary CNS lymphoma. However, more studies are needed to confirm this finding.

In another study by Sebelin-Wulf *et al* (2007) on post-transplant lymphoproliferative (PTLD) patients, they found by FACs analysis that PTLD patients had lower CD4+ T cell count when compared with patients with primary EBV infection or healthy EBV carriers. Whereas, CD8+ T cell count was similar to that of healthy EBV carriers. The author also measure the number of EBV-specific CD8+ T cells using HLA I tetramers for EBV-antigens and IFN-γ staining, and analysed by FACs, where double positively stained cells represent EBV-specific CD8+ T cells. They showed that in PTLD patients >30% of the EBV-specific CD8+ T cells were also IFN-γ stained, suggesting majority of the EBV-specific CD8+ T cells were not functional. Real time PCR assay also showed high EBV DNA load in the blood samples of PTLD patients, this was also true for patients with primary EBV infection. While EBV DNA load in healthy EBV-carriers were not detectable. Interestingly, the EBV DNA load in PTLD patients was inversely proportional to the total CD4+ T cell count (Sebelin-Wulf *et al*, 2007). When these patients were given a reduced immunosuppressive regimen, the frequency of EBV-specific CD8+ T cells remained constant during therapy and follow-up 6-12 months after therapy. Whereas, the

CD4+ T cell count remained stable during therapy and follow-up, however, EBNA1-specific CD4+ T cell count was undetected and this was proportional to the drop in EBV load. EBNA1-specific CD4+ T cells were detected by stimulation with recombinant EBNA1 protein followed by IFN-γ secretion assay. These data suggested that CD4+ T cells are required, possible, for the maintenance of EBV-specific CD8+ T cells. The author was unable to conclude why there was a decline in EBNA1-specific CD4+ T cells with decrease in viral load, since other studies suggested the importance of EBNA1-specific CD4+ T cells in the protection against EBV diseases in HIV patients (Piriou *et al*, 2005). However, Voo *et al* (2005) showed that EBNA1-specific CD4+ T cell clones selected on the basis of IFN-γ secretion in response to epitope peptide expressed phenotypic markers of T regulatory ($T_{reg}$) cells ($CD25^+GITR^+Foxp3^+$). More importantly, these $T_{reg}$ cells inhibited the proliferation of third party T cells. Thus the EBNA1-specific CD4+ T cells that were observed by Sebelin-Wulf *et al* (2007) could possibly be $T_{reg}$ cells and may possibly account for the inhibition of EBV-specific CD8+ T cell function. Therefore, in addition to having a protective role, CD4+ T cells could also promote EBV infection *in vitro*.

Based on work in Caucasians, $CD4^+$ T cell responses to the latent antigens show a completely different pattern of immunodominance seen with $CD8^+$ T cell responses. EBNA1 and EBNA3C specific $CD4^+$ T cell responses are readily detectable in healthy Caucasian EBV carriers by IFNγ ELISpot (Leen *et al*, 2001). Responses could be as frequent as 70% of healthy Caucasian donors tested would have EBNA1 or EBNA3C response. In this study, Leen *et al* (2001) describe finding 15 EBNA1 epitopes and 10 EBNA3C epitopes. However, LMP1 and LMP2 responses are poorly recognized (Leen *et al*., 2001) with frequency of responders to LMP1 and LMP2 between 10-15% of total donors tested. More importantly, only a few epitopes have been identified; 3 epitopes in LMP1 and 4 epitopes in LMP2.

Phenotype studies showed that majority of EBV specific $CD4^+$ T cells are biased towards secreting Th1 cytokines, especially IFNγ and are of the memory phenotype expressing surface markers $CD45Ro^+CD27^+CD28^+$ (Amyes *et al*., 2003). Bickham *et al* (2001) showed

that there are two populations of EBNA1 specific $CD4^+$ T cells, Th1 measuring IFNγ release and Th2 measuring IL-4 release, but the response is skewed towards Th1 population. This skewing of $CD4^+$ responses to Th1 has been demonstrated to be an effect of IL12 producing DCs (Cella *et al*., 1996; Koch *et al*., 1996) and is reflected *in vivo* with the skewing of IgG1 anti-body directed against EBNA1 (Bickham *et al*., 2001), since Th1 cytokines has been observed to bias IgG1 production (Hussain *et al*., 1999). In addition to the IFNγ anti-viral response, these Th1 polarised EBV specific $CD4^+$ T cells were demonstrated to inhibit the outgrowth of LCLs (Wilson *et al*., 2001) and this inhibition is not due to the effect of cytokine release but cell-cell interaction. Th2 polarised $CD4^+$ T cells, however, were unable to kill EBNA1 expressing target cells (Bickham *et al*., 2001).

These studies show that EBV-specific CD8+ and CD4+ T cell responses are frequently detected in healthy EBV carrier and patients with EBV diseases. More importantly, both CD8+ and CD4+ T cells are as important in the protection of EBV infection. These studies pointed to the importance in search for CD4+ T cell responses to EBV antigens.

# Section 1.6
# EBV tumours

### 1.6 EBV tumours

This section of the thesis will review some of the evidence which associate EBV to human tumours. More importantly, also look at the EBV gene expression pattern in these tumours. This will set a scene for the coming sections reviewing immunotherapeutic strategies that are studied.

#### 1.6.1 B-Cell tumours

B cells act as the reservoir for EBV to persist, therefore B cell tumours account for the majority of the EBV tumours. This section will review how EBV infection contributes to the development of Burkitt Lymphoma (BL), Hodgkin Disease (HD) and Post-Transplant Lymphoproliferative Disorder (PTLD).

##### 1.6.1.1 Burkitt Lymphoma

Burkitt lymphoma (BL) was first described in children in equatorial Africa by Denis Burkitt. BL is predominantly a disease of children and young adults, and rarely occurs after the age of 45 (Parkin, 2006). Evidence of the association of African BL with EBV came from sero-epidemiological studies conducted in Uganda (de-Thé *et al*, 1978). The authors showed that the level of antibodies to EBV, which could be detected months or years before the onset of disease, was considerably higher in children who subsequently developed BL than in normal control. In addition, EBV DNA could be detected in over 95% of BL tumours from sub-Saharan Africa (Parkin, 2006).

There are 3 variants of BL; endemic, sporadic and immunodeficiency related. BL described in equatorial Africa are endemic BL where EBV has been detected in almost all cases of endemic variant. Sporadic BL are found in North America and Europe, these cases of BL are much rarer than endemic BL and accounts for 20-30% of childhood non-Hodgkin Lymphoma (nHL) with a different clinical presentation. BL cases that occur in North Africa resembles that of sporadic BL clinical presentation, however, 85% of sporadic BL cases in North Africa have detectable EBV DNA (Parkin, 2006). Lymphomas arising in

immunodeficient individuals are relatively rare with the exception of AIDS cases. EBV DNA have been detected in 30-40% of immunodeficiency-related cases (Young and Rickinson, 2004).

A characteristic of BL is the translocation of chromosome 8 and either the immunoglobulin loci on chromosome 14 or 2 and 22 near the location of the *c-myc* oncogene resulting in altered regulation of *c-myc* expression (Pagano *et al*, 2004). This chromosomal translocation may be triggered during gene rearrangement or class switching, since BL cells display cell-surface markers that represent germinal centre B cells.

The endemic pattern of BL suggests that both genetic and environmental factors are likely to contribute to the development of the tumour. Malarial infection is a factor that has been linked to BL since in areas of endemic BL there is also a high incidence of malarial infection. More importantly, chronic malarial infection would lead to an expanded germinal centre and BL cells express markers of germinal centre B cells (Pagano *et al*, 2004).

Most EBV-positive endemic BL cases show a latency type I EBV gene expression pattern. However, in some cases, in addition to EBNA1 and EBERs, expression of EBNAs 3A, 3B, 3C and LP could also be detected but not EBNA2 and LMPs (Kelly *et al*, 2002).

#### 1.6.1.2 Hodgkin's Disease

Hodgkin's disease (HD) is a distinct disorder in which the characteristic malignant cells, known as the Hodgkin (large mono-nucleated) and Reed-Sternberg (multinucleated) cells (HRS), make up only 1-2% of the total tumour mass. HD has been categorised by the World Health Organisation (WHO) into two classifications; nodular lymphocyte-predominant, which represents 5% of all HD cases and is not typically EBV-associated, and classical HD (Gandhi *et al*, 2004). Classical HD is also further divided into four subtypes; nodular sclerosis which accounts for the majority of classical HD cases, mixed cellularity, lymphocyte-rich, and lymphocyte-depleted. Lymphocyte-rich and lymphocyte-depleted HD subtypes together account for less than 5% of classical HD cases, thus most studies of

EBV-associated HD came from the study of nodular sclerosis HD and mixed cellularity HD.

The association of EBV with HD appears to depend on age (Parkin, 2006), with the highest rate occurring in young adults in developed countries and in childhood in developing countries. EBV-positive HD seems to predominate in males. Mixed cellularity HD appears to affect older adult age group (45+ years of age). Whereas, nodular sclerosis HD is more frequent in young Caucasian adults (Gandhi *et al*, 2004; Parkin, 2006). Sero-epidemiological studies showed that IgA and IgG antibodies against EBNA1, VCA and EA-D are elevated in the serum of HD patients that were taken years prior diagnosis of the disease when compared to EBV-positive healthy controls (Mueller *et al*, 1989). In addition, elevated EBV DNA load could also be detected in the serum of HD patients years before the diagnosis of the disease when compared to EBV-negative HD patient or to healthy controls (Gandhi *et al*, 2004). Furthermore, EBV DNA could be detected in EBV-positive HD HRS cells.

Molecular analysis of Hodgkin/Reed-Sternberg cells showed that these cells are derived from preapoptotic postgerminal centre B cells (Kanzler *et al*, 1996). These cells were shown to have undergone crippling mutation, which under normal circumstances, would be eliminated by apoptosis. However, in the case of HD, these cells survive probably rescued by certain transforming events. The Hodgkin/Reed-Sternberg cells display a latency type II EBV gene expression program which indicates an important role for EBV in the rescue of these preapoptotic cells. This is because LMP1 and LMP2A may mimic and replace survival signals induced by activated CD40 and B-cell receptor.

#### 1.6.1.3 Post-transplant lymphoproliferative disease

Post-transplant lymphoproliferative disease (PTLD) is a major graft and life threatening complication of solid organ transplant. This condition is described as an uncontrolled proliferation of lymphocytes due to the effect of post-transplant immunosuppression. Sometimes this condition is reversible by reduction of immunosuppression (Dharnidharka

*et al*, 2007). The highest risk to developing PTLD is in cases where the recipient is EBV-seronegative receiving an allograft from and EBV-positive organ donor. Such patients acquire primary infection under the influence of immunosuppression during early post-transplant period and these patients do not have EBV-specific T cells (Mathur *et al*, 2004).

### 1.6.2 T/NK cell tumours

EBV can infect $CD4^+$, $CD8^+$ T cells as well as NK cells in a minority of IM patients and even fewer cases in transplant patients (PTLD). Thus T/NK cell lymphomas are rarely observed (Tsao *et al*, 2004). Most EBV-associated T-cell lymphomas show a cytotoxic phenotype which suggests that these tumours may have arisen as a result of proliferating cytotoxic T cells trying to kill the EBV-infected cells (Young *et al*, 2004). Most EBV-associated T-cell lymphoma arises after chronic active EBV infection. T and NK cell lymphomas typically show a latency type II EBV gene expression pattern (Yang *et al*, 2004; Tsao *et al*, 2004).

### 1.6.3 Carcinomas

Epithelial cells are relatively resistant to EBV infection, but they have been associated with many carcinomas with intense lymphoid infiltrate (lymphoepithelioma), such as carcinomas in organs (salivary gland, thymus and lung) (Takada, 2000). This part of the thesis will review two very important EBV associated carcinomas, gastric carcinoma and nasopharyngeal carcinoma.

#### 1.6.3.1 Gastric Carcinoma

The first evidence for EBV-association with gastric carcinoma came from the detection of the EBV genome in a case of undifferentiated lymphoepithelioma-like gastric carcinoma using polymerase chain reaction (Burke *et al*, 1990). Shortly after the first report of undifferentiated EBV presence in lymphoepithelioma-like gastric carcinoma, more cases of EBV genome was also found present in lymphoepithelioma-like gastric carcinoma (Min *et al*, 1991; Shibata *et al*, 1991; Pittaluga *et al*, 1992). The presence of the EBV genome in

lymphoepithelioma-like gastric carcinoma ranges between 75-100% of cases (Osato and Imai, 1996).

In addition to finding EBV DNA in these rare lymphoepithelioma-like gastric carcinoma cases, EBV DNA could also be detected in 2-16% of cases of the more common gastric adenocarcinoma (Osato and Imai, 1996). In a study of 970 cases of gastric carcinoma, Tokunaga *et al* (1993) detected using EBER1 in-situ hybridization, 67 cases (7%) were EBV-positive, of which, 8/9 (89%) cases were lymphoepithelioma-like carcinoma and 59/961 (6%) were poor or moderately differentiated adenocarcinomas. In another study by Imai *et al* (1994) analysis 1000 cases of gastric carcinoma using PCR and EBER1 in-situ hybridization showed that 70 (7%) of cases were EBV-positive. Of these 70 EBV-positive samples, 8/9 (90%) were EBV-positive undifferentiated lymphoepithelioma-like carcinoma, 27/476 (6%) were poorly differentiated adenocarinoma, and 35/515 (7%) were moderately to well-differentiated adenocarinomas. It became clear that EBV-association to gastric carcinoma is very similar to that of EBV-associated nasopharyngeal carcinoma, in that, EBV could almost always be detected in undifferentiated epithelial cells.

Epidemiological studies showed that EBV associated cases of gastric carcinoma in many countries is around 10%, with higher EBV-positive cases in the United Sates (16%), this may possibly be due to environmental and cultural factors (Qiu *et al*, 1997). EBV-association seems to correlate with age since EBV-association is higher in older patients (60+ years of age) compared to younger patients. EBV-association is also more common in male than in female by 3:1 (M:F) (Tokunaga *et al*, 1993).

In-situ hybridization showed that, in EBV-associated gastric carcinoma, EBERs, EBNA1, LMP2A, BARF0 and occasionally in small number of cells also the LMP1 transcripts are present (Osato and Imai, 1996). Western blotting confirms that the EBNA1 protein is present but failed to detect LMP1 or EBNA2. In a small number of cells in some tumors, the BZLF1 and BHLF1 proteins were also detected indicating that switching from latent to lytic infection occasionally occurs in gastric carcinoma.

### 1.6.3.2 Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is observed in all parts of the world but vary in incidence and histologic subtypes among population groups (Parkin, 2006). However, undifferentiated NPC is frequently observed in Southeastern Asian populations, especially among Southeastern Chinese people, with incidence rate up to $100/10^5$/year in some regions. The tumor is also frequently observed in Inuit populations and Mediterranean Africa. On the other hand, NPC incidence in Western populations is relatively low. Two other factors have been linked to NPC which may explain the high incidence in these areas. Firstly, it has been observed that individuals whose parents have NPC are at increased risk to developing NPC, therefore, there is genetic susceptibility among Southern Chinese, South East Asian and Inuit people. Secondly, the local diet, the consumption of chemical carcinogens in preserved food (preserved meat and salted-fish), influences the risk.

NPC is an epithelial tumor that has been categoried by the WHO into three categories; (1) WHO I tumors are highly differentiated with characteristics of epithelial growth patterns and keratin filaments; (2) WHO II tumors are poorly differentiated and non-keratinized but yet they have retained epithelial cell shape and growth patterns; and (3) WHO III tumors are undifferentiated and non-keratinized that lack distinctive epithelial cell growth patterns.

Association of EBV to NPC tumors first came from seroepidemiologic studies showing elevated antibody titers in the serum of NPC patients with WHO II and III tumours that are directed at EBV capsid antigens (VCA) and early lytic antigens (EA). More importantly, in addition to elevated IgG antibodies there were also elevated IgA antibodies directed against VCA and EA. It is still debatable as to whether WHO I NPC patients is associated with EBV since some studies suggests that these patients have serological profiles similar to the control population (Pearson *et al*., 1983) while other studies suggest that elevated IgA titres against VCA could be detected in these individuals (Sam *et al*., 1989).

*In situ* hybridization assays also confirms the detection of EBV DNA in WHO II and III tumors (Desgranges *et al*., 1975a; Desgranges *et al*., 1975b), however, the detection of EBV DNA in WHO I tumors is less consistent. The lack of clarity in the association with EBV and WHO I tumors is mainly because WHO I tumors are relatively uncommon and therefore very few samples could be studied. However, in WHO I tumor that had detected EBV DNA, LMP1 and EBER expression was relatively lower than that of undifferentiated tumors (Pathmanathan *et al*., 1995) which, may explain the inconsistency of EBV DNA detection in differentiated tumors.

EBV gene expression in undifferentiated NPC tumors is limited to the transcription of EBERs, EBNA1, LMP1, LMP2, *Bam* H1A transcripts (Busson *et al*., 1992; Gilligan *et al*., 1990; Gilligan *et al*., 1991) and BARF1 (Seto *et al*., 2005). At the protein level, EBNA1 is consistently expressed and LMP1 and LMP2 have been detected in approximately 50% of tumor samples studied (Heussinger., 2004; Young *et al*., 1988). Whereas there is still no substantial evidence which shows that any of the *Bam* H1A transcripts and BARF1 is expressed in the protein level. At least, in a immunotherapeutic point of view, EBNA1, LMP1, LMP2, the predicted *Bam* H1A polypeptides and BARF1 are potential antigens which could be targeted by T lymphocytes.

Elevated EBV VCA- and EA-specific IgA levels could be readily detected in NPC patients (Henle and Henle, 1976) and correlates with tumour burden and recurrence. More importantly, IgA antibodies to EBV could also be detected at an elevated level in NPC patients years before the diagnosis of the disease. Therefore, measuring IgA titres has been an important diagnostic tool to determine tumour burden.

# Section 1.7
# Immunotherapeutic strategies targeting EBV-positive tumours

### 1.7 Immunotherapeutic strategies against EBV-positive tumours

The study of immune-therapy as a treatment for EBV-associated disease started around the late 1970's, in terms of developing prophylactic vaccines and therapeutic vaccines. This section of the thesis will review the progress of some of these studies and more importantly, to address the importance of EBV-specific CD4+ T cells in immune-therapy.

#### 1.7.1 Prophylactic vaccination

The work with generating a vaccine as a prevention of EBV infection started around the late 1970s with the recognition of EBV associated cancers, endemic BL and undifferentiated NPC, aimed at reducing the tumor incidence amongst risk groups (Epstein, 1976). The glycoprotein gp340 was chosen as the candidate (Randle *et al*., 1985) for a prophylactive vaccine because of its ability to induce virus neutralizing antibodies in humans (Pearson *et al*., 1971). This vaccine was tested in an animal model, cottontop tamarins (*Saguinus Oedipus Oedipus*), because EBV infection of this species causes malignant lymphomas (Cleary *et al*., 1985). Several studies have shown that most gp340 vaccinated cottontop tamarins produce neutralizing antibody response (Randle *et al*., 1985; Morgan *et al*., 1988; Finerty *et al*., 1992). More importantly, when these immunized cottontop tamarins were challenged with B95.8 strain EBV, they did not develop lesions whereas, control animals did. A similar result was also observed using other animal models, marmoset (*Callithrix Jacchus*), where the authors observed an increase in gp340 specific antibodies and decrease in viral load in immunized animals when challenged with the M81 strain of EBV (Mackett *et al*., 1996; Cox *et al*., 1998). In the cottontop tamarin model, the authors demonstrated that gp340 vaccination could successfully prevent clinical disease. However, the vaccine was unable to prevent persistent infection of the viruses in both animal models discussed. Other studies using the gp340 vaccine showed that in addition to neutralizing antibody responses, there was also $CD4^+$ T cell responses directed against gp340 which could be detected from blood samples taken from healthy human EBV carriers (Ulaeto *et al*., 1988; Wallace *et al*., 1991). The gp340 vaccine can induce both humoral and cell-mediated responses. Furthermore, CD4+ T cell responses to gp340 may be important in the control of EBV infection and possibly a role in prevention.

A gp340-based vaccine has been tested in humans in China with promising results, where vaccinated children all developed antibodies against gp340 (and in most cases also neutralizing antibody response) with indication of protection against EBV infection (Gu *et al*, 1995). In a more recent report by Moutschen *et al* (2007) looking at phase I and II trials of a gp350-based vaccine in Belgium, they reported that the vaccine was immunogenic and can induce gp350-specific antibody response (including neutralizing antibody response) and cell-mediated response. Initial results with the gp340-based and gp350-based prophylactic vaccines show protection of naïve healthy host against EBV infection

### 1.7.2 Adoptive T cell therapy

Much of the work with adoptive T cell therapy was focused on treating transplant patients who are at a high risk of developing PTLD due to the use of immunosuppressive agents. The objective of adoptive T cell therapy in PTLD is to provide these patients with EBV-immunity. The first study of adoptive T cell transfer was conducted by Papadopoulos *et al* (1994) where 5 EBV-positive PTLD individuals were treated with unselected populations of donor-derived lymphocytes. In all cases regression of the tumour occured however, they also developed graft-versus-host disease. Rooney *et al* (1995 and 1998) further developed the method by enriching EBV-specific T cells *in vitro* by LCL stimulation. These T cells were then transferred into transplant recipients who were at high risk of developing PTLD. In all 39 cases, the recipients did not develop PTLD compared to 7/61 of the control group who developed PTLD. These data clearly suggest, at least for the treatment of PTLD, that adoptive transfer of EBV-specific T cells could prevent development of PTLD in high risk patients and possibly other EBV-associated diseases.

Adoptive T cell therapy has also been studied in the treatment of other EBV-associated diseases such as HD. Lucas *et al* (2004) described in their phase I clinical study the adoptive transfer of allogeneic EBV-specific T cells. The reason for using allogeneic T cells instead of autologous T cells is because other studies suggest that T cells from HD patients expand at a slower rate and have reduced expression of T cell receptor ζ chain

(Roskrow *et al*, 1998). This study suggests adoptive transfer of allogeneic EBV-specific T cells in the treatment of EBV disease is safe, since these T cells specifically recognise EBV antigens restricted through HLA I molecules and does not induce graft-versus-host disease (Lucas *et al*, 2004). More importantly, one patient out of six was disease free 22 months after infusion. However, 5/6 patients later showed progression of the disease. This study demonstrates adoptive transfer of EBV-specific T cells to treat EBV diseases is possible. However, further studies are required to improve the effectiveness of the treatment.

The study of CD8+ T cell responses to EBV antigens in Chinese healthy EBV carriers and in NPC patients suggest immunotherapy may be possible (Lee *et al*, 2000). In this study the authors detected, in the blood of NPC patients using cytotoxicity assay, CD8+ T cell responses against NPC-associated EBV protein LMP2 and EBNA1 but not to LMP1 or BARF0. Interestingly, the authors also detected EBNA1-specific CD8+ T cell responses from tumour infiltrating lymphocytes (TIL) but did not detect any responses against the other NPC-associated EBV proteins. More importantly, Lee *et al* (2000) demonstrated using an HLA-A11 north African-derived NPC cell line (C15) that the HLA I processing pathway is intact. In this experiment, the authors infected the C15 cell line with a vaccine vector expressing EBNA3B and were then presented to HLA-A11 restricted EBNA3B-specific CD8+ T cells. The EBNA3B vaccina infected C15 cells were recognised and killed by the HLA-A11 restricted EBNA3B-specific CD8+ T cells. These data show that NPC cells do have the capability to process and present antigens via the HLA I pathway and NPC patients have CTL responses against NPC-associated EBV proteins EBNA1 and LMP2. It was argued at the time of publication that the EBNA1 protein does not get processed and presented via the HLA I pathway, therefore EBNA1 would not have any therapeutic benefit. But recent research show strong evidence that EBNA1 is processed and presented by the HLA I pathway, thus EBNA1 and LMP2 are potential targets for immune therapy (Blake *et al,* 1997).

The adoptive transfer of LMP2-specific CD8+ T cells in NPC has been studied by Straathof *et al* (2005). In this study of 10 NPC patients, comprised of 4 patients who were in

remission but are at high risk of relapse and 6 patients were unresponsive to chemotherapy and radiation, autologous LMP2-specific CD8+ T cells administration was demonstrated to be safe with no immediate or long-term effects. Nine of ten patients had detectable EBV DNA in the PBMCs prior to T cell transfer and EBV load fell within 6 weeks after infusion in 6 patients (Straathof *et al*, 2005). The LMP2-specific T cell population was accessed in 8 patients after T cell infusion using IFN-γ ELISpot assays. In 4 of 8 patients, the number of LMP2-specific T cells increased by 2-fold, but this increase in LMP2-immunity was only transient and returned back to baseline level 6 weeks after infusion in 3 of the 4 patients. Clinical responses were evaluated from computed tomography and magnetic resonance imaging. Of the 6 patients, who were unresponsive to chemotherapy and radiation, 1 patient had stable disease for more than 14 months without additional therapy, 1 patient had partial response sustained for 12 months, 2 patients had no response and 2 patients had complete remission. All 4 patients who were in remission at the time of T cell therapy remained disease free after 19-27 months (Straathof *et al*, 2005). The outcome of this study show a promising future for T cell based therapy in the treatment of EBV diseases. However, further studies are needed to improve the tumour response rate, possibly by maintaining the number of EBV-specific T cells, since in 2 patients who had tumour response sustained LMP2-immunity for 3 months. This may be an implication that CD4+ T cells are needed in maintaining an effective CD8+ response.

The following conclusions could be made from adoptive T cell transfer studies; (1) EBV-specific T cells could be safely administered into patients with EBV diseases, (2) in most cases, tumour-specific responses are observed and in some cases would lead to remission, (3) adoptive T cell transfer studies in patients with increased risk of relapse or EBV-diseases show almost 100% prevention, and (4) as a direct treatment of EBV tumours, tumour response rate is poor because EBV-specific immunity could not be sustained. These points do strongly support that CD8+ T cells are important in the treatment of EBV diseases but also indicate CD4+ T cells are required.

### 1.7.3 Vaccine therapy

Studies on vaccine-based therapy have also showed promising results *in vitro* and *in vivo*, such as using polyepitope vaccine and EBV chimeric antigen MVA constructs. The objective of these vaccine therapies are to induce a broad EBV-specific response *in vivo*, possibly both CD8+ and CD4+ responses.

The polyepitope technology allows the expression of epitopes in a string-of-beads without the flanking sequence. This would limit or remove the oncogenic effect of certain proteins, such as LMP1. In a study by Smith *et al* (2006), the authors generated a polyepitope vaccine containing $CD8^+$-specific epitopes from EBNA1, LMP1 and LMP2 (E1-LMPpoly). In this study, PBMCs from 14 human volunteers (4 healthy EBV carriers and 10 HD patients) were infected with adenovirus expressing E1-LMPpoly (AdE1-LMPpoly). The authors report that their polyepitope vaccine was capable of inducing CD8+ responses against the EBV epitopes from EBNA1, LMP1 and LMP2 in healthy EBV carriers. But more importantly, they also showed that the polyepitope vaccine could restore EBV-specific $CD8^+$ T cell function in PBMC samples taken from HD patients, with a significant proportion of the cells expression CD62L which is required for entry into the lymph node and access the tumour site. However, in this study, Smith *et al* (2006) did not incorporate CD4+ T cell epitopes into their construct.

In another study, Taylor *et al* (2004) generated an EBNA1-LMP2 (EL) chimer MVA construct, by joining the C-terminal half of EBNA1 (EBNA1 363-641) with full length LMP2 (mutated at positions 74 and 112), and tested the ability of the construct to induce EBNA1 and LMP2 –specific $CD4^+$ and $CD8^+$ responses *in vitro*. In this study, the authors demonstrated that the EBNA1 and LMP2 in the construct did not retain biological function. More importantly, when the construct was infected into DC, these MVA-EL infected DCs were killed by EBNA1 and LMP2-specific $CD8^+$ T cell clones and induced $CD4^+$ T cells recognition.

# Section 1.8
# Aims

### 1.8 Project aims

The aim of this thesis was to determine potential NPC-associated EBV protein targets that could induce EBV-antigen-specific T cell responses which could be useful in the treatment of EBV-associated NPC.

The first objective of this thesis will explore the potential usefulness of BART products and/or BARF1 as T cell targets, both CD4+ and CD8+ responses. This section of the thesis was initiated from the study by Kienzle *et al* (1998) where they report the finding of CD8+ T cell response against an HLA-A2 restricted BARF0 epitope. The study by Kienzle *et al* (1998) is the first piece of evidence which indicated that these BART products and BARF1 protein may possibly be expressed and could be potential targets for T cell recognition. In the context of NPC, BART and BARF1 transcripts are expressed by NPC cells, and if T cell responses to these predicted BART products and BARF1 protein are detected, it would be a good indication that these polypeptides are expressed in NPC cells. More importantly, they are potential targets for immunotherapy.

The second objective of this thesis will focus on determining CD4+ T cell responses to the Chinese EBNA1 protein using PBMC samples from healthy S.E. Chinese EBV carriers (Hong Kong). CD4+ T cell responses to EBNA1 have been described in healthy Caucasian EBV carriers (Leen *et al*, 2001), however, HLA polymorphism between the two populations and EBNA1 polymorphism between the Chinese and Caucasian EBV strains may contribute to differences in antigen presentation to T cells. Therefore, it was important to revisit CD4+ T cell responses, using PBMCs from healthy Chinese EBV carriers, to Chinese EBNA1.

The third and final objective of this thesis was to generate Chinese EBNA1-specific CD4+ T cell lines and clone for use to accurately define epitopes identified within the Chinese EBNA1 protein. This section of work will provide vital information about the features of Chinese EBNA1-specific CD4+ T cells and their potential usefulness in T cell based immunotherapy for the treatment of NPC.

# Section 2

# Materials and Methods

## 2.0 Methods and Materials

### 2.1 Tissue culture media, supplements and reagents

#### 2.1.1 RPMI 1640

RPMI 1640 supplemented with L-glutamine (0.2M) purchased from GIBCO was used for culture of all lymphocyte cell lines

#### 2.1.2 Foetal calf serum (FCS)

FCS had been pre-membrane filtered, virus and mycoplasma screened, was purchased from GIBCO or Imperial Labs and stored at -20˚C in 20ml aliquoted until use.

#### 2.1.3 Human Serum (HS)

Sterile male HS of type AB purchased from HD supplies (UK) had been screened for virus and mycoplasma was stored in -20˚C in 25ml aliquots until use.

#### 2.1.4 Penicillin-Streptomycin

A combined solution of Penicillin ($10^4$ units/ml) and Streptomycin (10mg/ml) in 0.9% NaCl was purchased from SIGMA and stored at -20˚C in 5ml aliquots until use.

#### 2.1.5 MLA

The Gibbon cell line MLA-144 spontaneously release IL-2 into the supernatant which was used as a source of growth stimulus to supplement T cell media. These cells were sub-cultured for 2 weeks in RPMI 1640 with 10% FCS without further feeding, of which, supernatant was harvested by centrifugation at 1600rpm for 10 mins and filtered through a 146mm 0.2µm membrane (PALL). MLA supernatant was stored in 60ml aliquots at -20˚C until use at 30% of T cell media.

#### 2.1.6 IL-2

Lypophilised powder (Totem (Peprotech)) was reconstituted at $10^5$ IU/ml, filtered through a 0.2µm membrane and stored at -20˚C in 500µl aliquots until use.

### 2.1.7 Phytohaemagglutinin (PHA)

PHA was purchased from ICN.

### 2.1.8 Complete media

Complete media was prepared by combining 500ml of RPMI 1640, 50ml of FCS, and 1ml of Penicillin-Streptomycin. Lymphoblastoid cell lines were all cultured in complete media.

### 2.1.9 T cell line and clone media

T cell line and clone media was prepared by mixing 500ml RPMI 1640, 50ml FCS, 10ml HS, 1ml Penicillin-Streptomycin, 150ml MLA and IL2 (25IU/ml). T cell lines and clones were all cultured using this media.

### 2.1.10 Tissue culture plates

Tissue culture plates, 24-well plates and 96 U-bottom, V-bottom and flat-bottom plates were all purchased from IWAKI or NUNC. Falcon 50ml and 15ml test tubes were purchased from BioRad or Starstedt.

### 2.1.11 Blood donors

Blood was donated for the BamH1A and BARF1 work by healthy laboratory donors and buffy coats obtained from the Birmingham Blood Transfusion Service (Vincent Drive, Edgbaston, Birmingham). While for the EBNA1 work, peripheral blood mononuclear cells (PBMCS) samples came from Prince of Wales Hospital (Shatin, Hong Kong)

### 2.1.12 Preparation of PBMCs

PBMCs were obtained from peripheral blood collected by venepuncture into heparinised syringes or from buffy coats. Blood donations were diluted 1:1 in RPMI 1640 and layered onto 50ml Falcon test tubes containing 10ml lymphoprep (Axis-Shield diagnostics) and separated by density grade centrifugation at 1900rpm for 30mins. PBMCs were harvested from the interface of the lymphoprep and plasma and were washed several times at

decreasing rpm. PBMC samples were then either used fresh or kept in liquid nitrogen until needed.

### 2.1.13 EBV virus stock

EBV virus stock was generated from the B95.8 cell line cultured in RPMI 1640, 10% FCS and 2mM/ml L-glutamine. The cell line was maintained by feeding twice a week. The B95.8 virus stock was prepared by collecting the supernatant from B95.8 cell line and centrifuged at 1400rpm for 5 mins. The supernatant was then passed through a 0.45 μm filter and finally stored at 4ºC until use (but not stored for more than 3months).

### 2.1.14 Cyclosporin A (CSA)

CSA (Sanimmun, Sandoz) was reconstituted in RPMI 1640 to a stock concentration of 10μg/ml.

### 2.1.15 Generation of lymphoblastoid cell lines (LCL)

LCLs were generated by *in vitro* transformation of B cells from PBMC samples of HK Control donors for the EBNA1 work. $5x10^6$ PBMCs were pelleted by centrifuge at 1600rpm and all supernatant was removed. Cell pellet was resuspended in 500μl aliquot of B95.8 virus from the Marmoset B95.8 cell line and incubated for 1 hour in a 37˚C incubator with 5% $CO_2$ flicking every 15mins. After incubation, cells were washed and cultured in 24 well plates with RPMI 1640 with L-glutamine containing 10% FCS, penicillin/streptomycin and 0.1 μg/ml cyclosporin A for the first 2 weeks to suppress T cell activity. LCL cultures were subsequently split and re-fed with fresh medium twice weekly.

### 2.1.16 Treatment of PBMC with PHA

PBMCs ($10^6$ cells/ml ) were cultured for one hour with complete media containing 1μg/ml of PHA. After one hour, PBMCs were washed.

### 2.1.17 γ-irradition of PBMC and LCLs

PBMCs were irradiated at 4000rads and LCLs at 6000rads using a γ-irradition source.

### 2.1.18 Cryopreservation reagents

Dimethyl sulphoxide (DMSO- tissue culture grade) and Isopropanol (Propan-2-ol) were purchased from sigma. 1ml sterile plastic cryovials were from NUNC and Mr Frosty containers were from Nalgene.

### 2.1.19 Freezing media

100ml of freezing media was prepared by mixing 40ml RPMI 1640, 50ml FCS and 10ml DMSO.

### 2.1.20 Cryopreservation and recovery of cryopreserved cells

Cells to be cryopreserved were centrifuged at 1600rpm for 10mins and resuspended in freezing media and aliquoted in 1ml sterile plastic cryovials (Nunc). The aliquots were placed in -70˚C freezer overnight in a Mr Frosty (Nalgene) containing isopropanol and then transferred the following day in a freezer containing liquid nitrogen for long term storage.

Cells were recovered from liquid nitrogen by fast thawing in a 37˚C waterbath and transferred to a 15ml tube to which 5ml of RPMI 1640 containing 10% FCS was added drop-wise. Cells were allowed to rest for 1 min before pelleted by centrifuge at 1600rpm. The cells were further washed 2 times in RPMI 1640 containing 10% FCS before resuspended in their appropriate growth medium for culture.

### 2.1.21 Human IFNγ

Human IFNγ was purchased from R&D systems

### 2.1.22 Phosphate buffered saline (PBS)

10 tablets (OXOID) were dissolved per litre of sterile water

### 2.1.23 Peptides

Synthetic 20mer peptides were generated using 9-fluorenylmethoxycarbonyl chemistry (Alta Biosciences, University of Birmingham) and dissolved in dimethylsulfoxide (DMSO, Fischer Scientific). Synthetic peptides used for this study were a series of 20-mer peptides, overlapping by 15 amino acids, covering 126aa, 174aa and 103aa unique sequences of the B95.8 BamH1A polypeptide products A73, BARF0 and RPMS1 respectively and 220aa BARF1 and 641aa unique sequence of the CKL EBNA1. The peptide concentrations were determined by Biuret assay (Doumas, 1975)

### 2.1.24 Bovine serum albumin (BSA)

BSA (5mg) was purchased from Fermentas Life Sciences

### 2.1.25 Biuret reagent

Biuret reagent was prepared by dissolving 4.6g sodium tartrate and 2.5g potassium iodide in 250ml 0.6% copper sulphate solution. To this 50ml of sodium hydroxide, 300ml of distilled water was added.

### 2.1.26 Biuret assay

The Biuret assay was performed in a 96-well v-bottom plate.100µl of Biuret reagent was aliquoted to each well to which 20µl of the peptide solution was added followed by a further 100µl of Biuret reagent. The plate was left to incubate at room temperature for 10mins, to allow the colour to develop, and then centrifuged at 2000rpm for 3mins to remove precipitants. 150µl of supernatant from each well was transferred into a 96-well flat-bottomed plate and the absorbance read using an automated plate-reader (Bio-Tek) at 540nm. The peptide concentration was calculated from a standard curve (2.5mg-40mg/ml) of bovine serum albumin (BSA) prepared during the same assay.

### 2.1.27 Mycoplasma test

Cell cultures were tested weekly for mycoplasma infection using Immuno-Mark immunofluorescence mycoplasma testing kit (ICN) in accordance to the manufacture instructions.

### 2.1.28 Antibodies

Antibodies used in the depletion of CD8+ T cell population (magnetic bead coated Anti-CD8 monoclonal antibody) was purchased from Dynal UK Ltd.

Rabbit anti-human IgM CD16 antibodies used in the depletion of CD16+ NK cells were purchased from Serotec.

Antibodies used in flow cytometry were purchased from BD Biosciences (FITC-conjugated anti-CD8, FITC-conjugated anti-CD4, FITC-conjugated anti-CD16.

Antibodies used in the identification of epitope HLA-restriction were purchased from Serotec (anti-HLA-DQ, SPVL3), established in this laboratory (anti-HLA-DR, produced by the L243 ATCC clone), and provided by Dr. A.M de Jong from the University of Leiden, the Netherlands (anti-HLA-DP B7.21).

Antibody used in the detection of Epstein-Barr Virus capsid antigens (VCA staining), the FITC-conjugated goat anti-human IgG, was purchased from BD Biosciences.

Antibodies used in the ELISpot assay were purchased from Mabtech (Stockholm, Sweden).

Antibodies used in the ELISA assay were purchased from Pierce Endogen.

### 2.1.29 Rabbit complement

Rabbit complement was purchased from serotec.

### 2.1.30 $CD16^+$ NK cell depletion of PBMC

For ELISpots with high background, PBMC samples were depleted of $CD16^+$ NK cell population using CD16 IgM-induced complement killing. PBMCs were incubated in the dark with rabbit antihuman CD16 IgM antibodies for 30 minutes. During this time, the rabbit complement (Serotec) was reconstituted in ice cold distilled water and kept on ice until use. After the incubation, the rabbit complement was added to the CD16 IgM labeled PBMCs, incubate for 1 hour at 37˚C and then washed 3 times with RPMI 1640 containing 10% FCS.

### 2.1.31 $CD8^+$ T cell depletion of PBMCs

For the EBNA1 work, PBMC samples were depleted of $CD8^+$ T cell population using Dynabead depletion (Dynal, UK Ltd). PBMCs were incubated with magnetic beads pre-conjugated to anti-$CD8^+$ antibodies in a 4˚C cold room for 30 mins on a cell roller. 4 beads were used per target cell, assuming that the $CD8^+$ populations make up one third of the total PBMC count. After incubation, a magnet was used to retain the $CD8^+$ T cells and the enriched $CD4^+$ T cell population in the supernatant was removed.

## 2.2 Generation of T cell lines and clones

### 2.2.1 ELISpot assay plate

ELISpot assay plates, 96 well Millipore plates, were purchased from Bedford MA

### 2.2.2 Identification of EBNA1-specific CD4+ T cell responses by ELISpot assay

96 well Millipore plates with a 0.45μm hydrophobic high protein-binding immobilon-P polyvinylidene difluoride membrane were coated with 15μg/ml of anti-IFN-γ mAbs, 1-DIK (Mabtech, Stockholm, Sweden) overnight at 4˚C. The wells were washed 6 times in the following morning with RPMI 1640 and then blocked for 1 hour with RPMI 1640 with

10% FCS. $5x10^5$ CD8-depleted or whole PBMCs were added to single wells to which individual peptides were added to make a final concentration of each peptide of 2μM. The plates were incubated overnight at 37˚ and 5% $CO_2$. In the following day, cells were removed from the plate by washing 6 times with PBS containing 0.05% Tween (wash buffer), and 1μg/ml solution of biotinylated anti-IFN-γ mAb, 7-B6-1 (Mabtech), was added to each well and left to incubate for 2-4 hours at room temperature. The wells were washed again 6 times with wash buffer and PBS streptavidin-conjugated alkaline phosphatase (Mabtech) was added for 2 hours. The wells were given a final wash and an alkaline phosphatase-conjugated substrate kit containing 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad, Richmond, CA) was added to the wells for 15-20mins. The plate was developed by running under cold water to which spots representing individual IFN-γ producing cells could be visualized. Spots in each well were counted under a dissection microscope by eye 3 times and an average taken as the result.

### 2.2.3 Isolation of EBNA1-specific $CD4^+$ T cell lines

HK control donor PBMCs were thawed from liquid nitrogen and depleted of $CD8^+$ T cell population. $5x10^6$ CD8-depleted PBMCs per reactivation were pelleted by centrifugation, resuspended in 1ml of RPMI 1640 containing 5μg/ml peptide and incubated for 1 hour at 37˚C with flicking every 15mins. After 1 hour incubation, each reactivation was washed 3 times with RPMI 1640 and resuspended in 2ml of RPMI 1640 with L-glutamine and penicillin/streptomycin in a 24 well plate (IWAKI) and in cubated for 7 days at 37˚C. On day 7, the culture were tested for peptide specificity by IFN-γ ELISpot.

### 2.2.4 Preparation of peptide-loaded LCLs

LCLs ($10^6$ cells/ml) were incubated for 1 hour with 5μg/ml of relevant peptides at 37˚C. After 1 hour, the LCLs were washed 3 times in complete media.

### 2.2.5 Cloning media

Cloning media was prepared by mixing 500ml RPMI 1640 with 10ml HS, 1ml penicillin-streptomycin, $10^6$ cells/ml of γ-irradiated PHA-treated PBMC and $10^4$ cells/ml of γ-irradiated peptide-loaded LCLs.

### 2.2.6 Generation of EBNA1-specific $CD4^+$ T cell clones

Epitope-specific $CD4^+$ T cell clones were isolated by limiting dilution cloning from polyclonal PBMC cultures. T cells from the reactivations were seeded, in 100µl RPMI 1640 with L-glutamine and 10% human serum, at between 0.3 and 30 cells per well in 96-well U-bottom plates (Nunc or IWAKI) with $10^4$ cells per well of γ-irradiated autologous LCLs (6000 rads) preloaded with 5µM of the relevant peptide and $10^5$ cells per well of allogeneic γ-irradiated phytohaemagglutinin (PHA)-treated PBMCs (4000 rads) as feeders. Feeder cells were separated from a pool of 3 fresh buffy coats obtained from the Birmingham National Blood Transfusion Service, and activated with 10µg/ml PHA for 1 hour before washing with RPMI 1640 and γ-irradiated. On day 3, the wells were fed with 100µl RPMI 1640 with L-glutamine, 10% human serum, 60% MLA and 20 IU/ml IL-2.

### 2.2.7 Identification of epitope-specific CD4+ T cell clones

After 4-6 weeks depending on the growth of the microcultures, proliferating wells were tested for peptide specificity by IFNγ Enzyme linked immunosorbant assay (ELISA). Cells were prepared by taking 50µl of each growing clone into a 96-well v-bottom plate, centrifuged, washed 3 times in RPMI 1640 and resuspended in 250µl medium. 100µl of each clone was incubated overnight with LCL alone or LCL pre-exposed to 5µM epitope peptide and, on the following day, the supernatants were tested by IFNγ ELISA to identify the epitope-specific clone, as those giving increased IFNγ release in response to LCL pre-exposed to epitope peptide than LCL alone.

### 2.2.8 IFN-γ ELISA assay

A known number of T cells (200-5000) were incubated in U- or V-bottom microtest plate wells with standard numbers of autologous, HLA-matched or HLA-mismatched LCLs that were either unmanipulated, pre-loaded for 1 hour with 5μM peptide (or an equivalent concentration of DMSO solvent as a control) and then washed, or pre-infected with MVA expressing gly-ala deleted EBNA1 (E1ΔGA) or LAMP-targeted E1ΔGA (or MVA-pSC11) at an moi. of 10 and then washed. In blocking assays, LCLs were pre-incubated (either as LCLs alone or pre-loaded with epitope peptide) with MAbs specific for HLA-DR (L243, ATCC clone HB-55), HLA-DQ (SPV-L3, Serotec) and HLA-DP (B7.21 provided by DR A.M. de Jong, Leiden University, The Netherlands) for 1 hour before T cells were added to the assay. The supernatant was harvested 18 hours later and was assayed for IFNγ by ELISA (Endogen) following the manufacturer's protocol. 96-well Maxisorp plates (Nunc) were coated with 50μl of a 0.75μg/ml solution of a mouse $IgG_{1K}$ anti-human IFNγ MAb (Pierce, Endogen) overnight at 4°C and, on the following morning, the antibody was flicked out of the wells and then blocked at room temperature for 1 hour with 1% BSA, 0.05% Tween 20 in PBS. The plates were then washed 4 times in wash buffer (0.05% Tween 20 in PBS) and 50μl of neat or diluted supernatant from the T cell assay was added to each well and left at room temperature for 2-4 hours. After incubation, the supernatant was discarded, washed 6 times in wash buffer and biotinylated mouse $IgG_{1K}$ anti-human IFNγ MAb (Pierce, Endogen) was added at 0.375μg/ml and left at room temperature for 1-2 hours, followed by further washing and addition of Extravidin peroxidase conjugate (SIGMA) for a further 30 minutes. After a final 8 washes of the plates in wash buffer, 100μl of developing solution TMB (3,5',5',5'-tetramethyl benzidine, Tebu-Bio laboratories) was added and left at room temperature for 20 minutes to allow the colour to develop. 100μl of 1M HCL was added to each well to stop the reaction and the absorbencies were measured at 450nm using an automated spectrophotometer (Wallace Victor$^2$ 1420 multilabel Counter). All results presented were the mean of triplicate wells.

### 2.2.9 Flow Cytometry

Flow cytometry was performed to confirm the percentage of $CD8^+$, $CD4^+$ and/or $CD16^+$ cells within whole PBMC, $CD8^+$ depleted PBMC, $CD16^+$ depleted PBMC and T cell clones. $1x10^6$ cells were pelleted by centrifugation at 13,000 rpm for 1 minute in a 15 ml Falcon tube pre-coated with FCS to prevent cells adhering to the tube. The cells were washed in PBS and resuspended in 500µl 1% BSA in PBS and aliquoted into four 10ml Falcon tubes, to which, either 2µl of a FITC-conjugated anti-$CD8^+$ MAb (BD Biosciences), 2µl of a FITC-conjugated anti-$CD4^+$ MAb (BD Biosciences), 2µl FITC-conjugated anti-CD16 MAb (BD Biosciences) or 10µl of an IgG control MAb were added. Cells were incubated for 30 minutes at room temperature before washing in 1% BSA in PBS three times to remove excess antibody and resuspended in 500µl 1% paraformaldehyde in PBS. The samples were kept at 4˚C until analysis on a Becton Dickinson 440 flow cytometer.

### 2.2.10 MVA

The different MVA constructs were prepared and provided by Dr. Graham Taylor at the University of Birmingham

### 2.2.11 $^{51}$ Chromium

$^{51}$ Chromium was purchased from Amersham Biosciences.

### 2.2.12 Cytotoxic T lymphocyte (CTL) assay

$CD4^+$ T cell clones were tested for cytotoxicity against target cells by chromium release assays. $1x10^6$ target LCL cells were pelleted by centrifugation at 1600 rpm, supernatant media was removed by pipetteing and the cells were resuspended in 15µl (75µCi) of $^{51}$Cr-sodium chromate (Amersham Biosciences). Target cells were then incubated at 37°C, 5% $CO_2$ for 90 minutes to allow uptake of the radioisotope. To appropriate targets, 5µM epitope peptides (or an equivalent volume of DMSO solvent as a control) were added for the final hour of incubation. During this time, the T cell clones were counted and diluted in RPMI 1640 containing 10% FCS and aliquoted in 100µl into 96-well V-bottom plates providing the required number of cells for various effector:target ratios. Following

incubation with the radioisotope, the target cells were washed twice with RPMI 1640 containing 10% FCS, counted and diluted to allow 2500 target cells to be added to the T cells per 100µl aliquot. For each target, the radioactivity spontaneously released from the labeled targets when incubated with 100µl of the medium alone and the maximum radioactivity released when incubated with 100µl 1% SDS solution served as controls. The plates were centrifuged at 1200rpm for 3 minutes and incubated for 5 hours or 18 hours at 37°C, 5% $CO_2$. After this time, 100µl was harvested from each well into LP2 tubes (Luckman) and the radioactivities were counted on a gamma-counter (Cobra Auto-Gamma counting system, Hewlett Packard). Results for the CTL assays are expressed as a percentage specific lysis where;

$$\% \text{ specific lysis} = \frac{(\text{release from target with T cells} - \text{spontaneous release})}{\text{Maximum release}} \times 100$$

And all results are expressed as the means from triplicate wells.

**2.2.13 VCA staining**

The EBV status of the blood donors participating in this study was tested by immunofluorescent staining for antibodies against the viral capsid antigen (VCA). Cells from a B95.8 LCL and a control EBV-negative cell line, BJAB, were washed in PBS and resuspended at a concentration of $10^7$ cells/ml. The cell suspension were transferred onto the holes of SM-011 slides (Hendley-Essex) in 10µl aliquots, air dried and fixed in acetone for 5 minutes at -20°C. Once fixed, the slides were air dried at room temperature and either used immediately for the detection of VCA antibodies or stored at -20°C for later use. Plasma collected from blood donors, during the preparation of PBMCs, was diluted 1:10 – 1:5120. 20µl of donor plasma, at each dilution, was added onto a hole of B95.8 LCL-fixed slide and a BJAB LCL-fixed slide. The slides were incubated for 1 hour at 37°C in a moist chamber to prevent drying out. Following washing in PBS twice for 10 minutes, the areas of the slide around the spots of cells were dried with a cotton bud to prevent transfer of

antibodies between the different cells. 10µl of 1:50 dilution of a FITC-conjugated anti-human IgG MAb was added to each spot of cells and incubated for 1 hour at 37°C in the humidified box. The washing and drying procedures were repeated and DABCO was dotted on the slides and covered with coverslips (22x70x1, Surgipath). The slides were examined under a UV microscope and donors who were positive for anti-VCA antibodies detected against B95.8 LCL but not BJAB LCL were designated EBV-positive. And donors without detectable fluorescence to either cell lines were designated EBV-negative.

### 2.2.14 Human Leukocyte Antigen (HLA) typing

47 donors from the EBNA1 work were HLA typed at the class II A, B and C loci and at the class II DR and DQ loci at the Birmingham Blood Transfusion Service and class II DP typed at the National Blood Authority (Tissue Typing Laboratory, Churchill Hospital, Oxford, United Kingdom) by PCR-based DNA typing.

### 2.2.15 DNA extraction for HLA typing and the sequencing of BARF1 and EBNA1

DNA was extracted from spontaneous LCL samples generated from Healthy Chinese donors. $5x10^5$ cells was washed once with PBS, transferred to a 1.5ml Eppendorf tube and kept cold on ice for 10 minutes. The cells were then centrifuged at 13,000 rpm for 10 minutes with a desk-top microcentrifuge to get a pellet of cells and the excess PBS was removed with a pipette, to which, 400µl of 50mM Tris at pH8 and 500µl of warm phenol was added, vortex for 30 seconds and centrifuged at 13,000 rpm for 10 minutes. The aqueous layer was extracted with a pipette into a fresh 1.5ml Eppendorf tube, 500µl of warm phenol was added, vortex for 30 seconds and centrifuged for 2 minutes at 13,000 rpm. The aqueous layer was extracted to a fresh 1.5ml Eppendorf tube and 500µl of chloroform was added, vortex for 30 seconds and centrifuged for 2 minutes at 13,000 rpm, and this procedure was repeated once. The aqueous layer was extracted to a fresh 1.5ml Eppendorf tube, 100µl of 3M sodium acetate at pH 5.2 and 200µl of 100% ethanol was added and vortex for 30 seconds. The mixture was stored overnight in a -70˚C.

## 2.3 Sequencing of BARF1 and EBNA1

### 2.3.1 Sodium Acetate

3M sodium acetate was prepared by dissolving 24.6g of sodium acetate (Sigma) into 100ml of distilled water

### 2.3.2 Tris/acetate buffered EDTA (TAE)

500mls of a concentrated stock of TAE (50x) was prepared by addition of 121g Tris base, 28.6ml glacial acetic acid and 5ml 0.5M EDTA to sterile water.

### 2.3.3 Ethidium Bromide

(10mg/ml) Ethidium Bromide was purchased from Sigma (UK)

### 2.3.4 PCR and Sequencing primers

The sequencing primers were generated by Sigma-Genosys Ltd (UK). The primers used in the PCR reaction and sequencing for BARF1 and EBNA1 are listed in table 2.1

### 2.3.5 PCR reagents

Taq polymerase was purchased from Roche

### 2.3.6 Polymerase Chain Reaction (PCR)

DNA extracts generated from Healthy Chinese donor spontaneous LCL samples were used in the amplification of the BARF1 and EBNA1 gene. A master mix for one reaction was prepared in a 50µl PCR thermal eppendorf tube by mixing 2.5µl of 10x PCR in $Mg^{2+}$ buffer, 0.5µl 10mM dNTP, 1.25µl 10mM primer 1, 1.25µl primer 2, 17.5µl de-ionised water and 0.2µl Taq polymerase. To this, 2µl of DNA preparations (or B95.8 DNA extract was used as positive control and de-ionised water was used as negative control) was added to the master mix. The solution was overlaid with 50µl of mineral oil and subjected to 40 cycles at 94˚C for 30 seconds and 72˚C for 30 seconds.

| | Coordinate of the primers in relation to the EBV genome | Primer Sequence |
|---|---|---|
| **BARF1** | | |
| **PCR primer 1** | 165383-165403 | 5'-TGTAGACTTGGCTGGCCTCAT-3' |
| **PCR primer 2** | 166324-166305 | 5'-AGCAGCTGTGACAATCTTCA-3' |
| **Seq-primer 1*** | 166118-166099 | 5'-ATTTTTCCCAACGCAGGTCA-3' |
| **Seq-primer 2** | 165890-165873 | 5'-GACAGACAGCGTTAGAGG-3' |
| **Seq-primer 3** | 165618-165635 | 5'-AGCCTCGGTCCAGACATT-3' |
| **EBNA1** | | |
| **PCR primer 1** | 107659-107704 | 5'-AATTTGAGGCCTGGCTTGAG-3' |
| **PCR primer 2** | 110149-110130 | 5'-TAAGGGGGCCAGGCTAAAGC-3' |
| **Seq-primer 1** | 107830-107849 | 5'-ACAGCGACCATGAAGGGGAT-3' |
| **Seq-primer 2** | 107967-107947 | 5'-TGGCCCCTCGTCAGACATGAT-3' |
| **Seq-primer 3** | 109083-109102 | 5'-AAAGAGGCCCAGGAGTCCCA-3' |
| **Seq-primer 4** | 109115-109095 | 5'-GATGACTGACTACTGGGACTC-3' |
| **Seq-primer 5** | 109323-109342 | 5'-CGCAAAAAAGGAGGGTGGTT-3' |
| **Seq-primer 6** | 109869-109850 | 5'-CTGCCCTTCCTCACCCTCAT-3' |
| **Seq-primer 7** | 109904-109885 | 5'-TAAGGGGGCCAGGCTAAAGC-3' |

**Table 2.1: Primers used in the sequencing of the BARF1 and EBNA1 gene**

*** Sequencing primer**

### 2.3.7 Agarose gel powder for the separation of PCR products

Agarose gel powder was purchased from Sigma.

### 2.3.8 Preparation of electrophoresis

PCR products were purified using gel electrophoresis. 1.5g of agarose gel powder was dissolved in 100ml of distilled water and 2ml of 50x TAE and 5µl of (10mg/ml) ethidium bromide was added to the mixture. The gel was allowed to set in a rectangular container with a comb placed at one end of the gel. The set gel was loaded onto an electrophoresis tank containing running buffer (1x TAE and ethidium bromide). 10µl of PCR product with 2µ of dye contain 5x TAE was loaded into the wells of the gel and 4µ of DNA ladder was loaded at the end wells. The gel was allowed to run at 180V for 45 minutes. The gel was taken into the dark room and observed under a UV light.

### 2.3.9 Extraction of PCR products from the agarose gel

DNA extraction from the agarose gel was conducted using QIAquick kit (Qiagen) following the instruction manual provided.

### 2.3.10 Sequencing reaction reagents

Reagents used in the sequencing reaction were purchased from ADI Prism

### 2.3.11 Sequencing reaction using BIGDYE terminator (v2)

20µl of sequencing mix was prepared by mixing 4µ of BIGDYE reagent (ADI Prism), 4µl of 2.5x buffer (ADI Prism), 5-20ng of PCR product, distilled water and 1µl of 3mM sequence primers in a thermal eppendorf. The sequencing mix was subjected to 10 seconds at 96˚C and 25 cycles; 96˚C for 10 seconds, 50˚C for 5 seconds and 60˚C for 4 minutes.

### 2.3.12 Ethanol Precipitation of sequencing products

Sequencing products were purified by ethanol precipitation by mixing 20µl of sequencing products with 2µl 3M sodium acetate and 50µl 100% ethanol and vortex thoroughly in a 1.5 ml microfuge tube. The mixture was left on ice for 10-15 minutes, and centrifuged for 10-

20 minutes in a desktop micro-centrifuge. The supernatant was carefully removed without disturbing the DNA pellet using a pipette. The pellet was rinsed with 250µl of 70% ethanol, centrifuged for 5 minutes and supernatant discarded. The DNA pellet was dried at 90-100˚C for 5-10 minutes, 10µl of Hi-Di solution (Roche) was added to the pellet and left at 90-100˚C for 1 minute. The sequencing samples were then analysed.

# Section 3

# Results

# Section 3.1

# Screening Caucasian donors for T-cell memory to BART products and to BARF1

### 3.1.0 Screening Caucasian donors for T-cell memory to BART products and to BARF1

### 3.1.1 Introduction

NPC biopsies and an NPC-derived cell line (C15) have been demonstrated to express a set of *Bam* HIA rightward transcripts (BARTs) polyadenylated RNAs and BARF1 RNA. However, the expression of BARTs at the protein level is still unclear, but they have the potential to encode at least three predicted polypeptide products A73, RPMS1 and BARF0. Figure 3.1.1 shows the location of the BARTs and BARF1 on a map of the EBV genome and, in addition, the 13kbp region that is deleted from the B95.8 strain. The BARTs are heavily spliced: the introns are represented by a line; boxes represent the exons; and the arrow shows the direction of transcription. The BARF1 gene is approximately 5kbp downstream from the BART genes (Figure 3.1.1).

This phase of the thesis work was initiated because the postulated BART-encoded polypeptides and BARF1 protein represent potential sources of T cell epitopes that might be exploited as therapeutic targets in the context of NPC. This section of work was setup to find other T-cells responses ($CD4^+$ or $CD8^+$) against the three predicted BART-encoded polypeptides or against the BARF1 protein. The long term aim of such work is to find the full spectrum of immunogenic viral epitopes potentially presented by NPC cells.

At the time this work began, we still did not have access to blood samples from healthy Chinese donors and so the screening work in this Chapter had to be carried out using healthy Caucasian donors. The results of these initial screening experiments are presented in full at this point of the thesis. However, the long-term direction of the work switched back towards what appears to be a potentially more useful target, EBNA1.

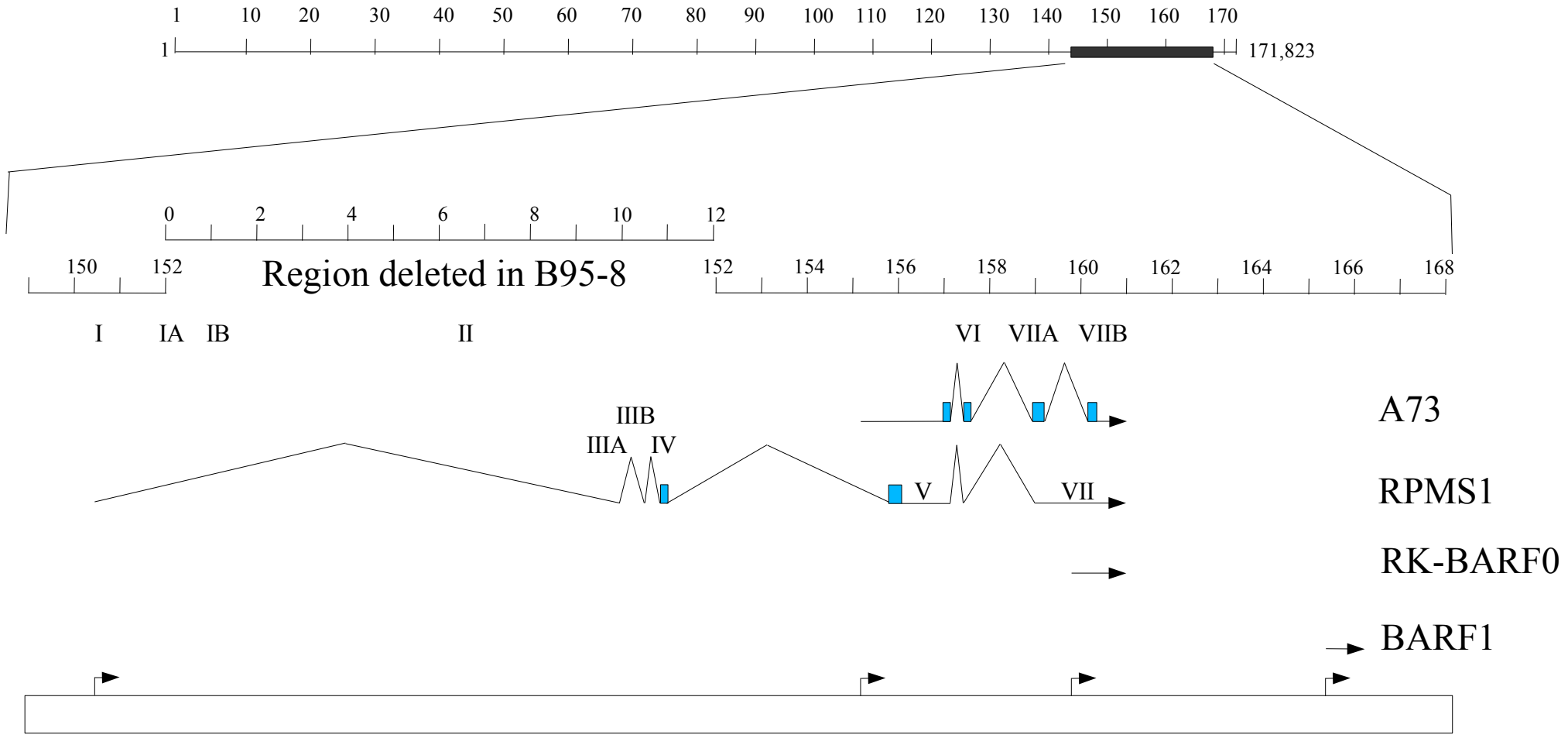

1 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170
1
171,823
0 2 4 6 8 10 12
150 152
Region deleted in B95-8
152 154 156 158 160 162 164 166 168
I IA IB II VI VIIA VIIB
IIIB
IIIA IV
V VII
A73
RPMS1
RK-BARF0
BARF1

**Figure 3.1.1: Location of the BART and BARF1 gene within the EBV genome**
This Figure was adapted from Smith *et* al (2000) showing the location of the BARTs and BARF1 on a map of the linear EBV genome and, in addition, the 13kbp region that is deleted from the B95.8 strain. The BARTs are heavily spliced: the introns are represented by a line; the exons are shown with the numbers directly above it; and the arrow shows the direction of transcription. The BARF1 gene is approximately 5kbp downstream from the BART genes.

### 3.1.2 Predicted BamH1A and BARF1 peptide panels.

Since this study was carried out on healthy Caucasian donors, the Caucasian EBV strain B95.8 sequence was used to infer the primary sequence of the predicted BARF0 (Baer *et al*, 1984), RMPS1 (Smith *et al*., 2000) and A73 (Smith *et al*., 2000) polypeptides and the BARF1 protein (de Turenne-Tessier *et al*., 1999). Figure 3.1.2 shows these primary sequences. RPMS1, BARF0 and A73 are predicted to be 103, 174 and 126 amino acids in length respectively and BARF1 is a 220 amino acid protein. Panels of 20-mer peptides (overlapping by 15 amino acids) were constructed for each of the sequences by Altabiosciences (The University of Birmingham). These four peptide panels are shown in Tables A3.1.1 - A.3.1.4 of the Appendix: there were 22 overlapping peptides covering A73 (Table A3.1.1); 32 overlapping peptides covering BARF0 plus the previously published 9-mer epitope LLWAARPRL (Kienzle *et al*, 1998) (Table A3.1.2); 18 overlapping peptides covering RPMS1 (Table A3.1.3); and 42 overlapping peptides covering BARF1 (Table A3.1.4).

### 3.1.3 Preliminary assays

Since in the long-term, our aim was to examine Chinese donor PBMC samples cryopreserved in Hong Kong, preliminary experiments were conducted to test if the ELISpot assay could detect IFN-γ release from samples that had been cryopreserved over a period of 6 months. Prior to conducting BART and BARF1 work, a mini-project was conducted to look for $CD4^+$ T cell responses against a panel of 58 EBNA3A peptide pools. Firstly, this has never been done before and, secondly, to optimize the ELISpot protocol. In total, 15 donors were screened of which one donor, UK-SL, tested positive against one of the EBNA3A peptide pools, pool 41. From this experiment, the following optimization is described. A freshly prepared PBMC sample from a laboratory donor in Birmingham, UK-SL, was divided into two samples. One was cryopreserved immediately (frozen PBMC), while the other was rested overnight in complete culture media at 37˚C before being tested the next day. The frozen PBMC sample was thawed after 6 months in cryopreservation, and again rested overnight in complete media at 37˚C before testing. This resting stage was found to give lower background than cells tested immediately after thawing. Figure 3.1.3

RPMS1

[1]MAGARRRARC[10] [11]PASAGCAYSA[20] [21]RPPPLSTRGR[30] [31]RISAGSGQPR[40]

[41]WWPWGSPPPP[50] [51]DTRYRRPGPG[60] [61]RRARSCLHAG[70] [71]PRGRPPHSRT[80]

[81]RARRTSPGAG[90] [91]GGGWRGGSCT[100] [101]SQR[103]

RK-BARFO

[1]MAATLPLPRC[10] [11]TDSMAARVPI[20] [21]EELRELRHLR[30] [31]GHCREDVVGV[40]

[41]QRSGRPLCLR[50] [51]PPRARDRALL[60] [61]WAARPRLLLS[70] [71]LQQVPEPRLA[80]

[81]DFILKQSRDR[90] [91]LRIHRHRQVV[100] [101]TGDVGPLCRG[110] [111]RVAVVGQNHQ[120]

[121]LAHTAPAGHR[130] [131]GDVEARVWDG[140] [141]TYAPKAAQQI[150] [161]QGPFQALQPH[170]

[171]GVRHAIKHAI[180] [181]DSLH[184]

A73

[1]MSMPPKGFLK[10] [11]KEMKPETRLL[20] [21]NKPPTVLTRP[30] [31]AMFCAWKLYS[40]

[41]RKMPSRSKTL[50] [51]EARCSSRPPC[60] [61]DSPACQTRDT[70] [71]GCPRRSGTGR[80]

[81]RGWRARRLGK[90] [91]ESWFADAWRM[100] [101]ARYWGCAVKA[110] [111]AAQSAFSAST[120]

[121]ASPEEL[126]

BARF1

[1]MARFIAQLLL[10] [11]LASCVAAGQA[20] [21]VTAFLGEERV[30] [31]TLTSYWRRVS[40]

[41]LGPEIVSWFK[50] [51]LGPGEEQVLI[60] [61]GRMHHDVIFI[70] [71]EWPFRGFFDI[80]

[81]HRSANTFFLV[90] [91]VTAANISHDG[100] [101]NYLCRMKLGE[110] [111]TEVTKQEHLS[120]

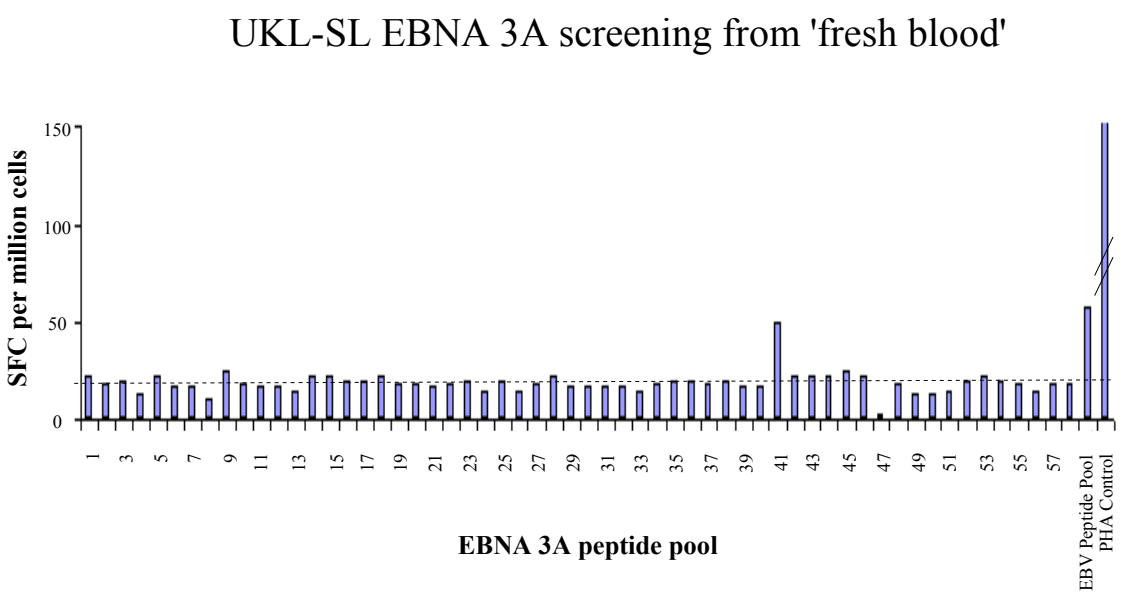
[121]VVKPLTLSVH[130] [131]SERSQFPDFS[140] [141]VLTVTCTVNA[150] [151]FPHPHVQWLM[160]

[161]PEGVEPAPTA[170] [171]ANGGVMKEKD[180] [181]GSLSVAVDLS[190] [191]LPKPWHLPVT[200]

[201]CVGKNDKEEA[210] [211]HGVYVSGYLS[220] [221]Q

**Figure 3.1.2: The amino acid sequence of the predicted BART products and BARF1**
This Figure shows the amino acid sequences of the three predicted polypeptide products (RPMS1, RK-BARF0 and A73) and BARF1. The superscripted numbers shows the coordinate of the amino acids relative to the polypeptide. The amino acid sequences were taken from Smith *et al* (2000) and the RK-BARF0 and BARF1 sequences were taken from the nucleotide database at http://www.ncbi.nlm.nih.gov/.

**A**


UKL-SL EBNA 3A screening from 'fresh blood'
SFC per million cells
150
100
50
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57
EBV Peptide Pool
PHA Control
EBNA 3A peptide pool

**B**

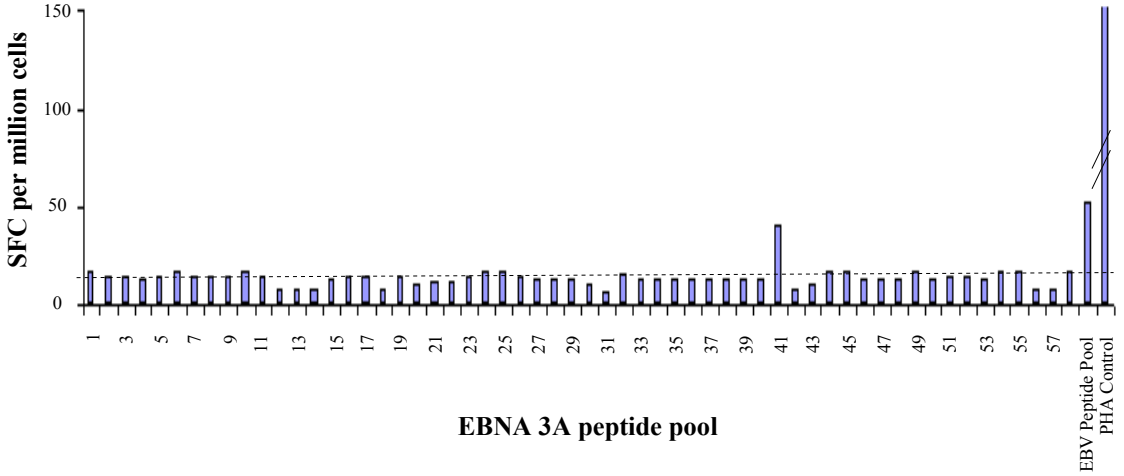

UKL-SL EBNA 3A screening from 'frozen PBMC'
SFC per million cells
150
100
50
0
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37 39 41 43 45 47 49 51 53 55 57
EBV Peptide Pool
PHA Control
EBNA 3A peptide pool

**Figure 3.1.3: IFN-γ ELISPOT assay of 'fresh' and 'frozen' PBMC samples**

Results of IFN-γ ELISPOT assays using (A) 'fresh' and (B) 'frozen' CD8-depleted PBMCs from UKL-SL exposed to a panel of 58 B95.8 strain EBNA3A peptide pools or assayed in the presence of known EBV antigen pool or PHA. Results are shown as numbers of spot-forming cells per $10^6$ CD8-depleted PBMCs tested. The dotted line represents the average background reading of the assay. Note that responses occasionally mapped to a single peptide pool, pool 41.

shows the ELISpot data of UK-SL fresh (Figure 3.1.3A) and frozen (Figure 3.1.3B) samples screening against 58 EBNA3A pools; each peptide pool contains five adjacent overlapping peptides (20-mer peptides overlapping by 15 amino aicds). For positive controls we included in Table A3.1.5 a list of peptides pulled together as a peptide pool control. The peptide pool contains eight known $CD4^+$ and four known $CD8^+$ EBV epitope peptides. This selection of known $CD4^+$ EBV epitope peptide pool is to detect response in the majority, if not all, EBV positive carriers that are screened in these experiments. And, (2) a polyclonal stimulus, PHA. As a negative control, we included wells without peptide but with an equivalent concentration of DMSO solvent alone (not shown in the figure). Both fresh and cryopreserved samples responded by IFN-γ detection against EBNA3A peptide pool 41 and against the known EBV epitope peptide pool with similar intensity (50 SFC per million cells). This assay, and similar experiments on other laboratory donor samples, demonstrated that cryopreservation of PBMC samples does not affect the ability of T-cells to respond (by IFN-γ release) against EBV-specific peptides.

Although not applicable in work with donor UK-SL, we found that high backgrounds is one of the biggest problems in an ELISpot assay as such background responses will tend to mask weak or borderline responses. Because NK cells are potential sources of non-specific IFN-γ production from PBMC samples, experiments were conducted to compare whole PBMC and NK-depleted PBMC preparations as responders in ELISpot assay. NK cells were removed by exposure to anti-CD16 IgM antibody followed by rabbit complement to lyse CD16-positive cells. The quality of the depletion was assessed by staining with FITC-labelled IgG MAbs to CD16, CD4 and CD8 or with an irrelevant FITC-labelled isotype-matched (IgG1) MAb. As can be seen from the FACS profiles in Figure 3.1.4, after CD16 depletion, $CD4^+$ and $CD8^+$ populations are still present whereas the $CD16^+$ population is completely removed. An ELISpot was conducted on CD16-depleted and undepleted PBMCs from a buffy coat sample which in earlier assays had given high background responses. Figure 3.1.5 shows a representation of such a result where the background response is significantly reduced while the positive control EBV peptide pool and PHA control response was unaffected. This protocol was therefore used for re-testing samples

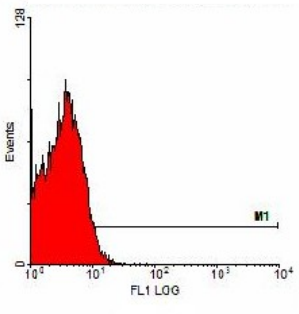
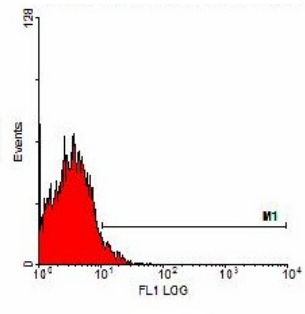
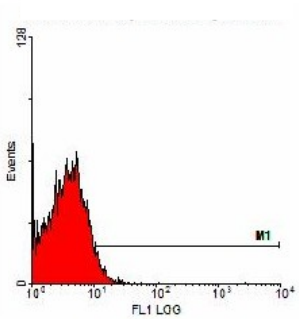
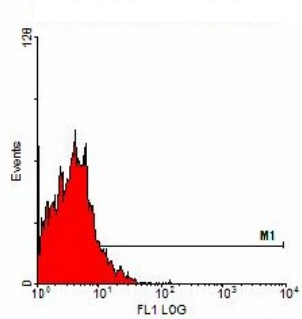
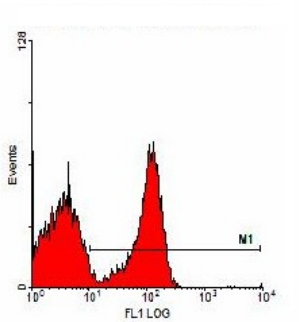
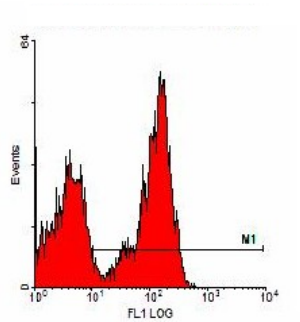
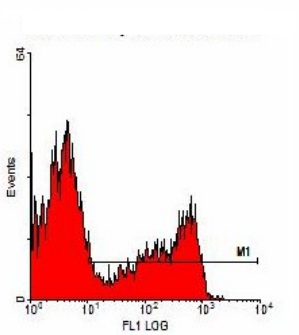
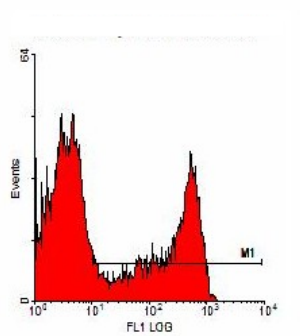
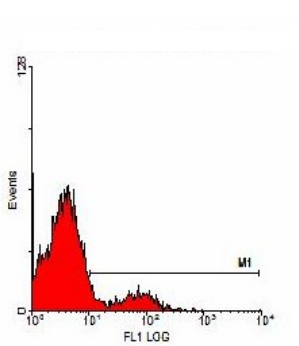
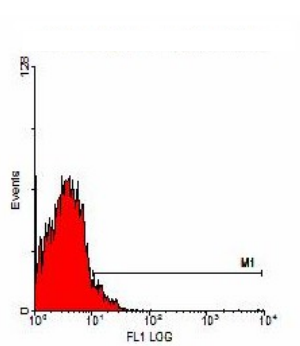
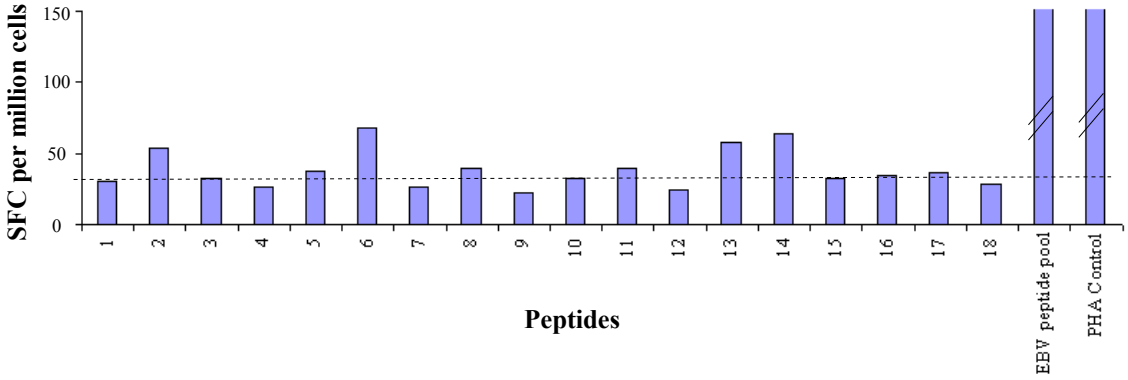
| | Before CD16 depletion | After CD16 depletion |
|---|---|---|
| **No Antibodies** |  |  |
| **IgG1 FITC** |  |  |
| **CD4 FITC** |  |  |
| **CD8 FITC** |  |  |
| **CD16 FITC** |  |  |

**Figure 3.1.4: CD16-depletion of whole PBMC**

The result shows the FACs analysis of whole PBMCs from UKL-SL before and after CD16-depletion. Before CD16-depletion (Left column, reading from top to bottom) shows the individual PBMC subpopulation staining using FITC conjugated antibodies for the respective cellular markers for $CD4^+$, $CD8^+$ and $CD16^+$ cells. After CD16-depletion (Right column), the $CD4^+$ and $CD8^+$ subpopulations of PBMC are unaffected and the $CD16^+$ subpopulation of cells were removed from the PBMC.

**A**

ELISpot with high background before NK cell depletion




**B**

ELISpot background after NK cell depletion

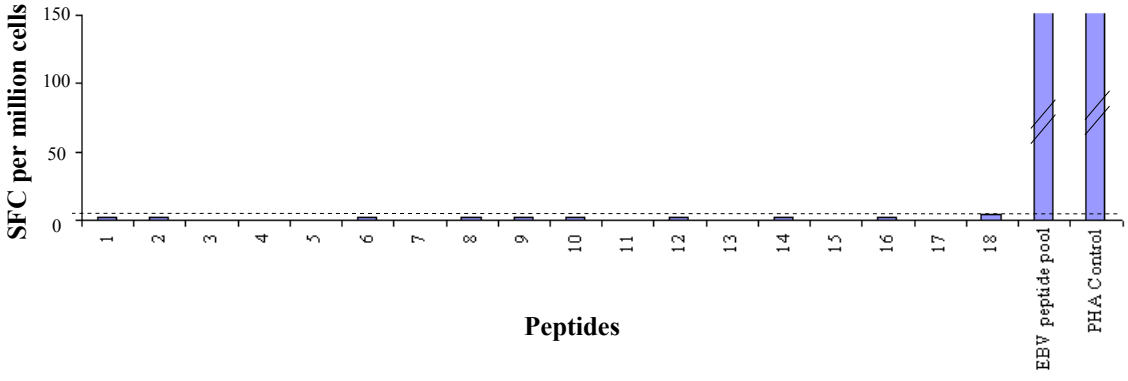


**Figure 3.1.5: INF-γ ELISpot assay before and after CD16-depletion**

Results of IFN-γ ELISpot assays before CD16-depletion (A) and after CD16-depletion (B) of PBMCs from UKL-SL exposed to a panel of the first 18 of 58 B95.8 EBV strain peptide pools. Results are shown as numbers of spot-forming cells (SFC) per $10^6$ CD8-depleted PBMCs tested. Note the average background reading (dotted line) before CD16-depletion (A) is approximately 40 SFC per $10^6$ CD8-depleted cells, and after CD16-depletion (B), the background reading is lowered to approximately 0-5 SFC per $10^6$ CD8-depleted cells.

that gave high backgrounds in the initial screening assays. Majority of the samples that were screened using BART and BARF1 peptides gave high background responses and therefore, they were NK cell depleted, unless otherwise indicated.

### 3.1.4 Screening assays

The EBV serological status of 25 healthy Caucasian donor serum samples, whom were used in the ELISpot screening for BARTs and BARF1 responses, was determined using anti-IgG monoclonal antibodies (MAbs). The results are summarised in Table 3.1.1, where 24 of 25 donor serum samples were tested positive and UKL25 serum sample was negative for IgG antibodies.

ELISpot screening of BART and BARF1 peptides was conducted using PBMC samples taken from these same 25 healthy Caucasian donors either using 60-100ml bleeds of laboratory donors (denoted UKL) or from buffy coat donations (denoted UKB). PBMC preparations from laboratory donors were usually tested immediately on the day of bleed while PBMC preparations from buffy coats were usually cryopreserved until testing at a later date. Buffy coats gave high enough PBMC yields for multiple freezings (6-8 vials of $50x10^6$ PBMC/vial of freezing), ideal for a rescreen if initial screening gave positive results. In every assay, whole PBMC preparations (containing both $CD4^+$ and $CD8^+$ T-cell populations) were tested against the peptide panels and therefore could in theory detect either $CD4^+$ or $CD8^+$ memory against the BART encoded polypeptides or BARF1 protein.

The 20-mer peptides were used individually rather than in pools of 3 adjacent peptides as used in EBNA1 screening work described by Leen *et al* (2001). This was possible because each peptide panel contained fewer peptides (42 peptides at most) compared to the EBNA1 work (80+ peptides) done earlier by Leen *et al* (2001). The same controls were used as described earlier in the preliminary assays.

The ELISpot plates were all counted by eye under a dissection microscope because at this stage of work an ELISpot reader for automated reading was unavailable. Note that all 25

| UK donor | VCA test |
|---|---|
| *UKL1 | †+ |
| UKL2 | + |
| UKL3 | + |
| UKL4 | + |
| UKL5 | + |
| UKL6 | + |
| UKL7 | + |
| UKL8 | + |
| ††UKB9 | + |
| UKB10 | + |
| UKB11 | + |
| UKB12 | + |
| UKB13 | + |
| UKB14 | + |
| UKB15 | + |
| UKB16 | + |
| UKB17 | + |
| UKB18 | + |
| UKB19 | + |
| UKB20 | + |
| UKL21 | + |
| UKL22 | + |
| UKB23 | + |
| UKB24 | + |
| | |
| UKL25 | ‡— |

*UK Laboratory donors

†† UK Blood transfusion donors

†Positive staining for IgG antibodies against EBV

‡Negative staining for IgG antibodies against EBV

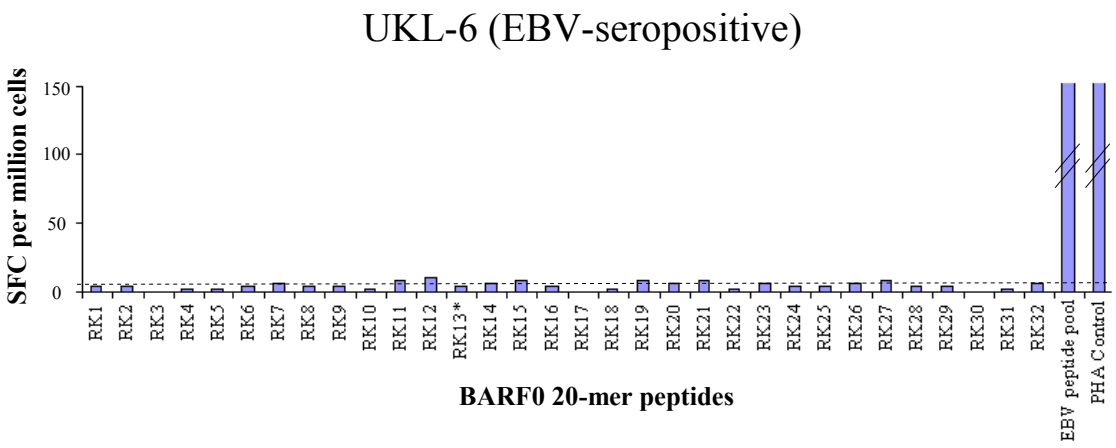
**Table 3.1.1: VCA staining of 25 UK donors**

donors gave strong positive response against the PHA control wells reflected by the wells, turning completely dark purple (this is represented as >1000 SFC per million cells in the assay Figures but the exact value is not known). A value 500 SFC per million cells is given to greater than half the donors tested (17/27), where the known EBV peptide pools induced very strong T cell responses such that the spots on the ELISpot plate were difficult to count. The DMSO control wells were used to set the background base line.

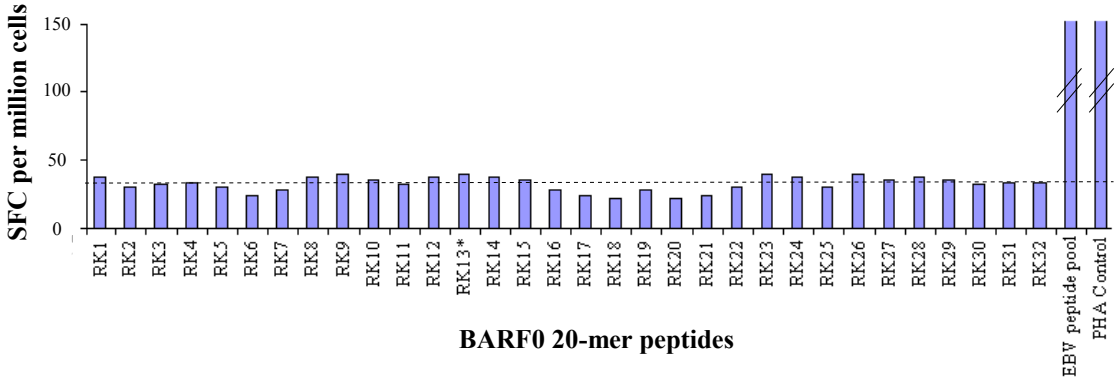
### 3.1.5 Screening from predicted BART-encoded polypeptides (RPMS1, A73 and BARF0)

Figure 3.1.6 A, B and C show three representative results from the screening of the BARF0 peptide panel. Responses to individual peptides are shown as histograms and are expressed as the number of spots forming cells (SFC) per $10^6$ PBMC. Clear positive responses above background values (mean SFC count ±3 standard deviation from negative control wells; see dotted line) were observed in the positive control wells containing the pool of known EBV peptides. Scanning the graph immediately shows that none of the donors responded against any of the BARF0 peptides above background (dotted line). Seropositive donors UK6 (Figure 3.1.6A) and UKB18 (Figure 3.1.6B) showed responses to the EBV epitope peptide pool control wells and also the PHA control whereas seronegative donor UKL 25 (Figure 3.1.6C) did not show any responses towards the EBV epitope peptide pool control wells but did respond against the PHA control. Of the 24 healthy seropositive Caucasian donors screened in this manner, the majority of the donors gave strong responses against the EBV peptide pool control. Data from the BARF0 peptide panel assays are summarised in Table 3.1.2. Most donors did not show any responses against any of the BARF0 peptides. The only responders are UKL 8 (to peptides RK3 and RK5) and UKB 17 (to peptide RK17). However, both donors were re-tested on two subsequent occasions against these and the adjacent peptides and, on each occasions, no responses was seen. Note that the background response in the re-test is significantly reduced in donor UKL8 because of NK cell depletion from whole PBMC, yet even in the optimal circumstances there was no hint of response to peptides RK3 and RK5. Note that peptide RK13 is the reported HLA-A*0201 restricted epitope LLWAARPRL (Kienzle *et al*, 1998); no donors responded against this peptide.

**A**


UKL-6 (EBV-seropositive)
SFC per million cells
150
100
50
0
RK1 RK2 RK3 RK4 RK5 RK6 RK7 RK8 RK9 RK10 RK11 RK12 RK13* RK14 RK15 RK16 RK17 RK18 RK19 RK20 RK21 RK22 RK23 RK24 RK25 RK26 RK27 RK28 RK29 RK30 RK31 RK32 EBV peptide pool PHA Control
BARF0 20-mer peptides

**B**


UKL-8 (EBV-seropositive)
SFC per million cells
150
100
50
0
RK1 RK2 RK3 RK4 RK5 RK6 RK7 RK8 RK9 RK10 RK11 RK12 RK13* RK14 RK15 RK16 RK17 RK18 RK19 RK20 RK21 RK22 RK23 RK24 RK25 RK26 RK27 RK28 RK29 RK30 RK31 RK32 EBV peptide pool PHA Control
BARF0 20-mer peptides

**C**

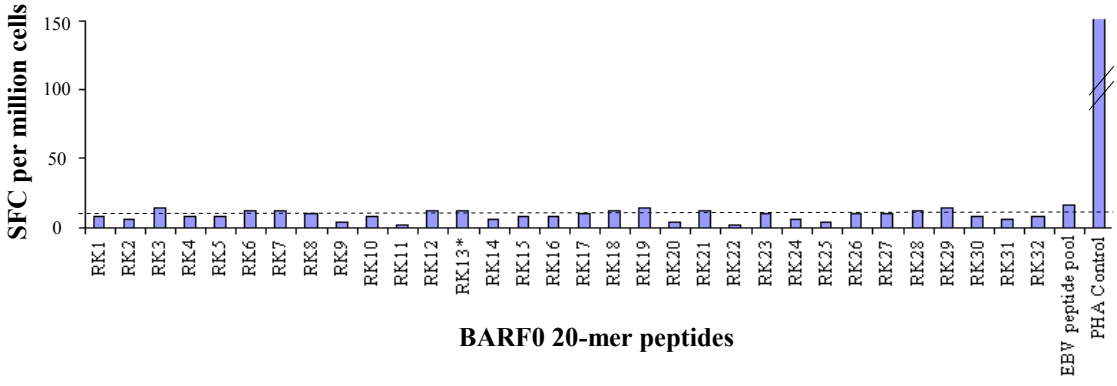

UKL-25 (EBV-seronegative)
SFC per million cells
150
100
50
0
RK1 RK2 RK3 RK4 RK5 RK6 RK7 RK8 RK9 RK10 RK11 RK12 RK13* RK14 RK15 RK16 RK17 RK18 RK19 RK20 RK21 RK22 RK23 RK24 RK25 RK26 RK27 RK28 RK29 RK30 RK31 RK32 EBV peptide pool PHA Control
BARF0 20-mer peptides

**Figure 3.1.6: Screening for T cell responses to BARF0 20-mer peptides**

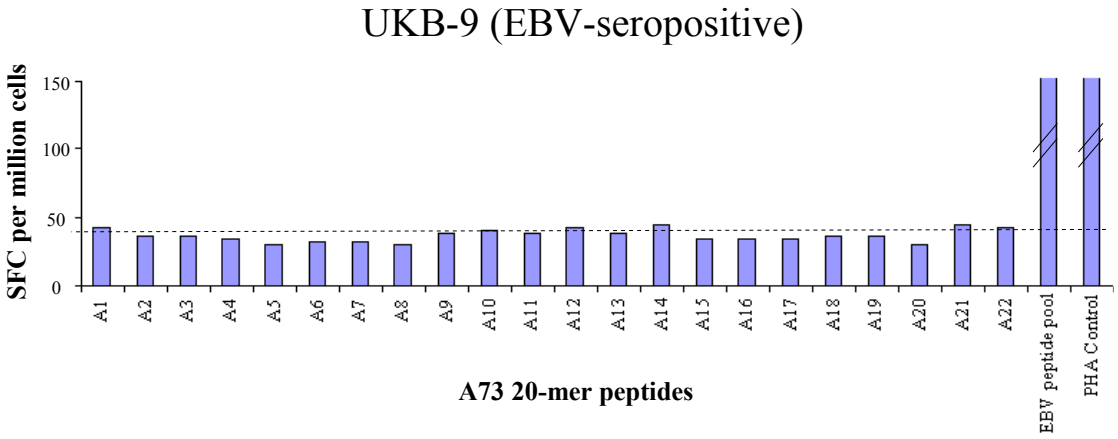
Result show three typical IFN-γ ELISpot assays using whole PBMCs from donors UKL-6 (A), UKL-8 (B) and UKL-25 (C) exposed to a panel of 32 B95.8 EBV strain predicted BARF0 20-mer peptides. Results are shown as SFC per $10^6$ cells. Peptide RK13 (*) is the reported HLA-A*0201 restricted epitope LLWAARPRL (Kienzle *et al*, 1998). Both EBV-seropositive donors, UKL-6 and UKL-8, responded to the EBV peptide pool control, whereas, EBV-seronegative donor (UKL-25) did not. All three donors responded against PHA. Note that there are no significant T cell responses to any of the BARF0 peptides above the background (dotted line) level from any of the three donors tested. Also note, no response was detected against the reported HLA-A*0201 restricted epitope.

| | | BARF0 Peptide panel | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Donor** | **Background** | **RK1** | **RK2** | **RK3** | **RK4** | **RK5** | **RK6** | **RK7** | **RK8** | **RK9** | **RK10** | **RK11** | **RK12** | **RK13*** | **RK14** | **RK15** | **RK16** | **RK17** | **RK18** | **RK19** | **RK20** | **RK21** | **RK22** | **RK23** | **RK24** | **RK25** | **RK26** | **RK27** | **RK28** | **RK29** | **RK30** | **RK31** | **RK32** | **EBV peptide pool** | **PHA Control** |
| **UKL 1** | 0-6 | 2 | 0 | 2 | 0 | 0 | 0 | 2 | 6 | 4 | 2 | 4 | 0 | 4 | 0 | 0 | 2 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 2 | 6 | 2 | 6 | 4 | 2 | 2 | 2 | 2 | 500(+) | 1000(-) |
| **UKL 2** | 22-40 | 22 | 38 | 22 | 30 | 32 | 22 | 38 | 24 | 40 | 40 | 32 | 34 | 38 | 22 | 26 | 32 | 32 | 38 | 28 | 38 | 34 | 36 | 34 | 26 | 28 | 22 | 34 | 34 | 36 | 32 | 38 | 40 | 174 | 1000 |
| **UKL 3** | 4-16 | 4 | 4 | 14 | 6 | 6 | 18 | 16 | 12 | 16 | 12 | 6 | 18 | 8 | 12 | 8 | 10 | 8 | 16 | 2 | 4 | 6 | 8 | 16 | 12 | 4 | 8 | 4 | 10 | 8 | 12 | 16 | 10 | 500 | 1000 |
| **UKL 4** | 14-38 | 14 | 20 | 38 | 20 | 30 | 34 | 34 | 28 | 30 | 34 | 18 | 30 | 16 | 28 | 22 | 38 | 38 | 38 | 32 | 34 | 14 | 18 | 22 | 26 | 24 | 14 | 34 | 16 | 26 | 34 | 30 | 24 | 500 | 1000 |
| **UKL 5** | 4-16 | 8 | 4 | 10 | 12 | 6 | 2 | 10 | 14 | 6 | 10 | 8 | 4 | 10 | 6 | 16 | 4 | 8 | 6 | 10 | 8 | 2 | 0 | 6 | 8 | 6 | 6 | 10 | 10 | 4 | 8 | 6 | 6 | 126 | 1000 |
| **UKL 6** | 0-10 | 4 | 4 | 0 | 2 | 2 | 4 | 6 | 4 | 4 | 2 | 8 | 10 | 4 | 6 | 8 | 4 | 0 | 2 | 8 | 6 | 8 | 2 | 6 | 4 | 4 | 6 | 8 | 4 | 4 | 0 | 2 | 6 | 500 | 1000 |
| **UKL 7** | 2-14 | 8 | 6 | 6 | 6 | 12 | 12 | 2 | 12 | 12 | 6 | 2 | 12 | 4 | 4 | 4 | 4 | 12 | 10 | 12 | 8 | 8 | 8 | 10 | 2 | 6 | 2 | 4 | 10 | 12 | 14 | 8 | 8 | 340 | 1000 |
| **UKL 8** | 48-76 | 48 | 46 | ****112** | 66 | **114** | 64 | 56 | 30 | 64 | 22 | 60 | 38 | 62 | 50 | 60 | 66 | 50 | 52 | 68 | 62 | 70 | 76 | 50 | 40 | 56 | 58 | 48 | 64 | 56 | 48 | 38 | 60 | 500 | 1000 |
| **UKL 8 retest 1#** | 2-6 | | 0 | 4 | 0 | 2 | 0 | | | | | | | | | | | | | | | | | | | | | | | | | | | 250 | 1000 |
| **UKL 8 retest 2** | 2-6 | | 2 | 6 | 0 | 0 | 0 | | | | | | | | | | | | | | | | | | | | | | | | | | | 250 | 1000 |
| **UKB 9** | 6-24 | 14 | 6 | 20 | 10 | 24 | 22 | 18 | 10 | 12 | 16 | 24 | 18 | 22 | 22 | 24 | 24 | 24 | 20 | 16 | 12 | 22 | 16 | 10 | 14 | 12 | 10 | 18 | 16 | 8 | 24 | 14 | 24 | 500 | 1000 |
| **UKB 10** | 2-12 | 2 | 10 | 2 | 6 | 6 | 0 | 12 | 0 | 2 | 2 | 2 | 2 | 0 | 6 | 4 | 4 | 2 | 6 | 2 | 6 | 12 | 6 | 8 | 10 | 0 | 6 | 0 | 4 | 8 | 10 | 0 | 2 | 310 | 1000 |
| **UKB 11** | 16-36 | 26 | 26 | 18 | 16 | 26 | 30 | 6 | 16 | 22 | 30 | 34 | 20 | 30 | 18 | 34 | 22 | 22 | 36 | 36 | 32 | 30 | 18 | 16 | 26 | 22 | 24 | 30 | 18 | 12 | 16 | 30 | 32 | 500 | 1000 |
| **UKB 12** | 24-36 | 24 | 36 | 36 | 30 | 32 | 30 | 24 | 30 | 34 | 30 | 36 | 34 | 34 | 32 | 30 | 26 | 30 | 24 | 34 | 32 | 30 | 34 | 32 | 26 | 28 | 30 | 28 | 30 | 36 | 34 | 32 | 30 | 500 | 1000 |
| **UKB 13** | 24-38 | 28 | 26 | 24 | 24 | 34 | 38 | 30 | 34 | 36 | 32 | 34 | 34 | 30 | 34 | 38 | 38 | 36 | 24 | 28 | 28 | 36 | 34 | 36 | 34 | 30 | 34 | 26 | 30 | 32 | 24 | 26 | 24 | 500 | 1000 |
| **UKL 14** | 4-18 | 6 | 8 | 16 | 6 | 18 | 10 | 18 | 10 | 6 | 4 | 8 | 18 | 8 | 6 | 6 | 4 | 18 | 4 | 6 | 0 | 6 | 18 | 8 | 10 | 6 | 4 | 14 | 8 | 14 | 12 | 18 | 10 | 400 | 1000 |
| **UKL 15** | 0-14 | 10 | 4 | 12 | 6 | 8 | 8 | 8 | 4 | 8 | 8 | 10 | 10 | 12 | 10 | 10 | 12 | 6 | 12 | 6 | 12 | 16 | 22 | 14 | 6 | 6 | 8 | 8 | 4 | 0 | 6 | 14 | 12 | 246 | 1000 |
| **UKB 16** | 30-52 | 36 | 30 | 34 | 48 | 30 | 30 | 38 | 32 | 40 | 44 | 40 | 48 | 40 | 36 | 40 | 42 | 48 | 38 | 36 | 42 | 46 | 42 | 36 | 40 | 48 | 44 | 40 | 40 | 36 | 46 | 42 | 42 | 500 | 1000 |
| **UKB 17** | 0-14 | 8 | 2 | 0 | 2 | 4 | 10 | 4 | 12 | 6 | 8 | 10 | 2 | 6 | 8 | 4 | 14 | **68** | 0 | 4 | 2 | 6 | 8 | 2 | 0 | 4 | 4 | 2 | 4 | 4 | 2 | 0 | 4 | 350 | 1000 |
| **UKB 17 retest 1** | 2-12 | | | | | | | | | | | | | | | | 4 | 10 | 2 | | | | | | | | | | | | | | | 300 | 1000 |
| **UKB 17 retest 2** | 2-12 | | | | | | | | | | | | | | | | 8 | 12 | 6 | | | | | | | | | | | | | | | 332 | 1000 |
| **UKB 18** | 22-40 | 38 | 30 | 32 | 34 | 30 | 24 | 28 | 38 | 40 | 36 | 32 | 38 | 40 | 38 | 36 | 28 | 24 | 22 | 28 | 22 | 24 | 30 | 40 | 38 | 30 | 40 | 36 | 38 | 36 | 32 | 34 | 34 | 500 | 1000 |
| **UKB 19** | 34-56 | 34 | 36 | 50 | 44 | 50 | 56 | 36 | 50 | 50 | 52 | 42 | 46 | 48 | 52 | 56 | 48 | 52 | 54 | 54 | 42 | 52 | 52 | 46 | 56 | 38 | 56 | 52 | 44 | 48 | 56 | 52 | 48 | 500 | 1000 |
| **UKB 20** | 6-20 | 14 | 8 | 12 | 18 | 16 | 18 | 16 | 18 | 16 | 18 | 12 | 20 | 12 | 18 | 10 | 16 | 16 | 10 | 20 | 14 | 16 | 18 | 12 | 12 | 18 | 20 | 20 | 10 | 20 | 18 | 16 | 6 | 500 | 1000 |
| **UKL 21** | 2-14 | 2 | 10 | 10 | 10 | 8 | 6 | 10 | 4 | 12 | 6 | 4 | 2 | 10 | 8 | 8 | 2 | 6 | 6 | 6 | 14 | 12 | 0 | 6 | 8 | 8 | 12 | 6 | 6 | 4 | 2 | 12 | 14 | 500 | 1000 |
| **UKL 22** | 0-12 | 8 | 0 | 4 | 2 | 10 | 0 | 2 | 6 | 12 | 6 | 6 | 2 | 6 | 0 | 4 | 8 | 2 | 4 | 6 | 2 | 8 | 12 | 0 | 6 | 2 | 6 | 4 | 6 | 6 | 4 | 4 | 8 | 500 | 1000 |
| **UKB 23** | 0-14 | 2 | 6 | 4 | 4 | 6 | 14 | 12 | 2 | 2 | 4 | 6 | 6 | 2 | 4 | 10 | 10 | 0 | 0 | 0 | 2 | 0 | 8 | 0 | 0 | 8 | 6 | 6 | 8 | 4 | 6 | 0 | 2 | 500 | 1000 |
| **UKB 24** | 0-10 | 0 | 0 | 10 | 0 | 8 | 2 | 8 | 8 | 6 | 6 | 8 | 4 | 4 | 4 | 10 | 10 | 4 | 8 | 0 | 2 | 0 | 0 | 8 | 0 | 8 | 2 | 0 | 2 | 2 | 2 | 6 | 4 | 500 | 1000 |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **UKL 25** | 2-14 | 8 | 6 | 14 | 8 | 8 | 12 | 12 | 10 | 4 | 8 | 2 | 12 | 12 | 6 | 8 | 8 | 10 | 12 | 14 | 4 | 12 | 2 | 10 | 6 | 4 | 10 | 10 | 12 | 14 | 8 | 6 | 8 | 16 | 1000 |

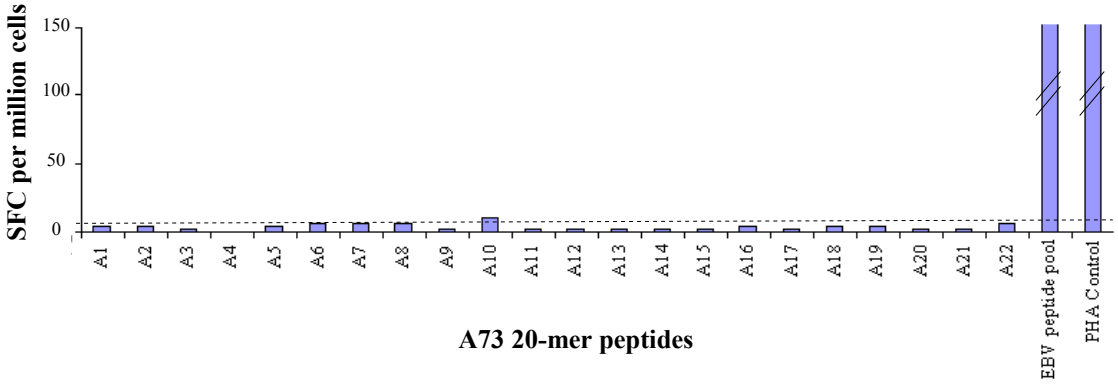
**Table 3.1.2: SUMMARY OF BARF0 PEPTIDE SCREENING**

UKL - Laboratory Donors
UKB - Buffy Coat Samples
**Positive ELISpot results are bolded
#All positive results are retested twice
* Peptide LLWAARPRL as tested by Kienzle *et al (*1998)
(+) Wells that exceed countable by eye are given a value of 500 SFC per million cells
(-) ELISpot wells containing PHA show up as a dark purple well and are given the value of 1000 SFC per million cells

**A**


UKB-9 (EBV-seropositive)
SFC per million cells
150
100
50
0
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
A13
A14
A15
A16
A17
A18
A19
A20
A21
A22
EBV peptide pool
PHA Control
A73 20-mer peptides

**B**


UKL-22 (EBV-seropositive)
SFC per million cells
150
100
50
0
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
A13
A14
A15
A16
A17
A18
A19
A20
A21
A22
EBV peptide pool
PHA Control
A73 20-mer peptides

**C**

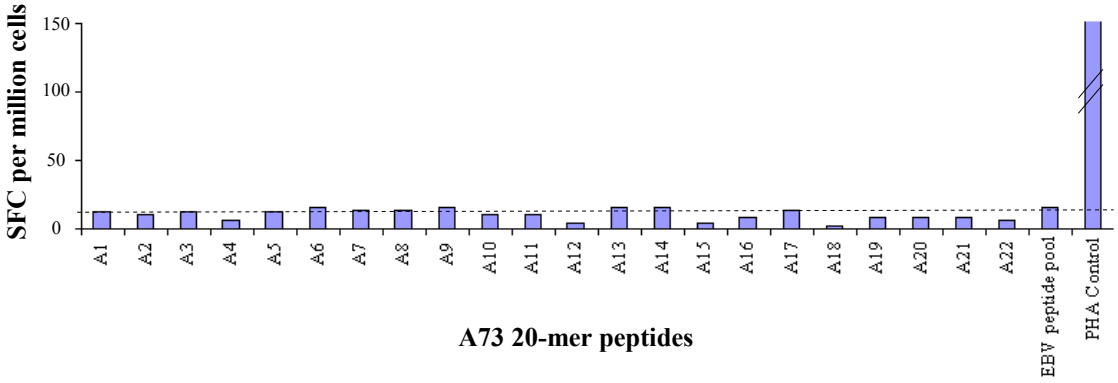

UKL-25 (EBV-seronegative)
SFC per million cells
150
100
50
0
A1
A2
A3
A4
A5
A6
A7
A8
A9
A10
A11
A12
A13
A14
A15
A16
A17
A18
A19
A20
A21
A22
EBV peptide pool
PHA Control
A73 20-mer peptides

**Figure 3.1.7: Screening for T cell responses to A73 20-mer peptides**

Result show three typical IFN-γ ELISpot assays using whole PBMCs from donors UKB-9 (A), UKL-22 (B) and UKL-25 (C) exposed to a panel of 22 B95.8 EBV strain predicted A73 20-mer peptides. Results are shown as SFC per $10^6$ cells. Both EBV-seropositive donors, UKB-9 and UKL-22, responded to the EBV peptide pool control, whereas, EBV-seronegative donor (UKL-25) did not. All three donors responded against PHA. Note that there are no significant T cell responses to any of the A73 peptides above the background (dotted line) level from any of the three donors tested.

Figures 3.1.7 A, B and C show data from two representative donors UKL19 and UKB22 and seronegative donor UKL25 screened against the panel of 22 A73 20-mer peptides; no responses were observed. Table 3.1.3 summarises the overall results. Again, there were two potential responses from the initial screening; UKL8 (peptides A4 and A5) and UKB17 (peptides A6, A10 and A1, but none of these were observed in the two subsequent repeat assays.

Likewise Figure 3.1.8 A, B and C show negative data from two seropositive donors UKB13 and UKB23 and the seronegative donor UKL25 screened against 18 RPMS1 peptides. Table 3.1.4 summarises the overall results and again 2 donors gave possible responses in the initial assays; UKL4 (peptides R6, R13 and R14) and UKL6 (R3, R5, R6 and R8), but these responses were not repeatable in the two retest assays that could be performed.

### 3.1.6 Screening of BARF1 peptide panel

Figure 3.1.9 A, B and C show the corresponding data from two seropositive donors UKL6 and UKB19 and the seronegative donor UKL25 screened against the 42 BARF1 peptide panel. The Figures show that none of the donors responded against any of the peptides. Again, both UKL6 and UKB19 responded to the known EBV epitope peptide pool and to PHA whereas, UKL25 only responded to PHA. The 25 donors screened against the BARF1 peptide panel are summarised in Table 3.1.5. Scanning the Table show that one donor responded against several BARF1 peptides, UKL5 (peptides B10, B11, B15, B16, B17 and B20), however re-test assays again show that these responses did not repeat.

### 3.1.7 Discussion

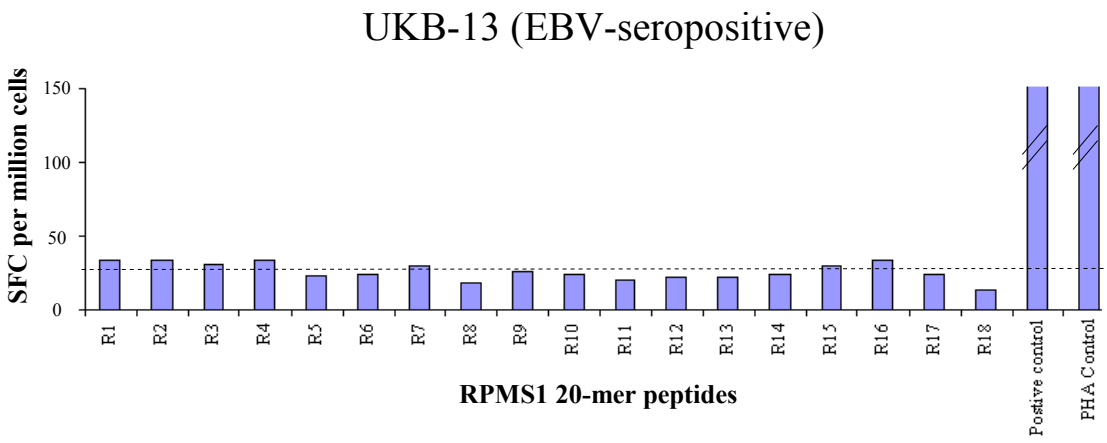
Looking overall at the results of these experiments, there was never a reproducible response to any of the peptides tested in the 24 EBV-immune donors examined. How might such negative results be interpreted? To date, there is still no unequivocal evidence showing that the postulated BART-encoded polypeptides are expressed. Evidence which may suggest the expression of the postulated BART-encoded products came from studies showing the potential functions of these predicted proteins (Smith *et al,* 2000; Thornburg *et al,* 2004).

| | | A73 peptide panel | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Donor** | **Background** | **A1** | **A2** | **A3** | **A4** | **A5** | **A6** | **A7** | **A8** | **A9** | **A10** | **A11** | **A12** | **A13** | **A14** | **A15** | **A16** | **A17** | **A18** | **A19** | **A20** | **A21** | **A22** | **EBV peptide pool** | **PHA Control** |
| **UKL 1** | 0-8 | 8 | 2 | 2 | 0 | 8 | 0 | 0 | 0 | 2 | 0 | 2 | 0 | 4 | 2 | 4 | 0 | 2 | 0 | 2 | 4 | 2 | 0 | 500(+) | 1000(-) |
| **UKL 2** | 0-20 | 20 | 8 | 18 | 14 | 12 | 20 | 16 | 20 | 16 | 2 | 12 | 0 | 4 | 14 | 18 | 12 | 12 | 8 | 8 | 12 | 8 | 4 | 174 | 1000 |
| **UKL 3** | 0-18 | 18 | 8 | 8 | 6 | 2 | 10 | 0 | 6 | 16 | 12 | 2 | 6 | 8 | 8 | 14 | 6 | 8 | 18 | 4 | 12 | 0 | 8 | 500 | 1000 |
| **UKL 4** | 12-42 | 32 | 30 | 34 | 24 | 20 | 12 | 14 | 12 | 22 | 24 | 20 | 12 | 60 | 14 | 22 | 38 | 42 | 8 | 30 | 42 | 22 | 12 | 500 | 1000 |
| **UKL 5** | 4-14 | 8 | 4 | 10 | 8 | 6 | 8 | 6 | 8 | 12 | 8 | 6 | 4 | 4 | 14 | 10 | 6 | 4 | 4 | 6 | 8 | 8 | 6 | 126 | 1000 |
| **UKL 6** | 0-12 | 6 | 4 | 12 | 4 | 0 | 0 | 6 | 0 | 6 | 2 | 2 | 2 | 4 | 4 | 6 | 6 | 6 | 8 | 6 | 4 | 2 | 6 | 500 | 1000 |
| **UKL 7** | 2-14 | 8 | 2 | 12 | 10 | 8 | 6 | 10 | 4 | 6 | 2 | 6 | 6 | 4 | 6 | 8 | 8 | 14 | 10 | 8 | 8 | 8 | 8 | 340 | 1000 |
| **UKL 8** | 78-90 | 90 | 62 | 78 | ****172** | **138** | 48 | 52 | 46 | 38 | 72 | 30 | 60 | 80 | 66 | 64 | 54 | 52 | 38 | 36 | 38 | 42 | 60 | 500 | 1000 |
| **UKL 8 retest 1#** | 2-4 | | | 0 | 2 | 0 | 0 | | | | | | | | | | | | | | | | | 250 | 1000 |
| **UKL 8 retest 2** | 2-4 | | | 0 | 0 | 0 | 0 | | | | | | | | | | | | | | | | | 250 | 1000 |
| **UKB 9** | 6-24 | 24 | 20 | 24 | 18 | 24 | 18 | 22 | 22 | 16 | 20 | 12 | 12 | 22 | 20 | 22 | 18 | 24 | 14 | 10 | 6 | 12 | 12 | 500 | 1000 |
| **UKB 10** | 2-10 | 8 | 4 | 4 | 2 | 2 | 10 | 8 | 8 | 8 | 10 | 2 | 8 | 2 | 0 | 2 | 4 | 0 | 10 | 4 | 2 | 6 | 2 | 310 | 1000 |
| **UKB 11** | 30-46 | 44 | 34 | 54 | 30 | 30 | 38 | 34 | 36 | 34 | 34 | 38 | 30 | 44 | 34 | 38 | 36 | 34 | 34 | 36 | 32 | 30 | 36 | 500 | 1000 |
| **UKB 12** | 32-48 | 32 | 36 | 36 | 40 | 34 | 34 | 44 | 36 | 46 | 38 | 48 | 48 | 36 | 34 | 44 | 38 | 44 | 44 | 42 | 34 | 36 | 34 | 500 | 1000 |
| **UKB 13** | 28-38 | 28 | 30 | 38 | 30 | 38 | 38 | 30 | 36 | 36 | 32 | 34 | 34 | 36 | 30 | 28 | 32 | 30 | 36 | 34 | 30 | 36 | 38 | 500 | 1000 |
| **UKL 14** | 4-22 | 6 | 6 | 10 | 14 | 12 | 6 | 4 | 8 | 10 | 14 | 4 | 4 | 12 | 14 | 14 | 12 | 12 | 8 | 4 | 4 | 4 | 22 | 400 | 1000 |
| **UKL 15** | 6-12 | 4 | 10 | 10 | 8 | 0 | 4 | 14 | 12 | 10 | 10 | 0 | 8 | 14 | 12 | 6 | 2 | 12 | 14 | 8 | 10 | 8 | 8 | 246 | 1000 |
| **UKB 16** | 36-48 | 34 | 36 | 38 | 42 | 46 | 26 | 46 | 42 | 40 | 38 | 36 | 36 | 46 | 46 | 32 | 30 | 36 | 28 | 36 | 38 | 38 | 36 | 500 | 1000 |
| **UKB 17** | 8-12 | 4 | 6 | 6 | 12 | 12 | **24** | 4 | 4 | 8 | **20** | 6 | 6 | 10 | 2 | **20** | 6 | 8 | 6 | 0 | 6 | 8 | 4 | 350 | 1000 |
| **UKB 17 retest 1** | 10-12 | | | | | 10 | 12 | 12 | | 8 | 8 | 6 | | | 8 | 10 | 10 | | | | | | | 300 | 1000 |
| **UKB 17 retest 2** | 10-12 | | | | | 8 | 14 | 12 | | 8 | 10 | 8 | | | 6 | 6 | 8 | | | | | | | 332 | 1000 |
| **UKB 18** | 20-38 | 30 | 32 | 28 | 28 | 24 | 28 | 26 | 34 | 36 | 24 | 20 | 28 | 24 | 22 | 26 | 34 | 38 | 38 | 36 | 36 | 30 | 32 | 500 | 1000 |
| **UKB 19** | 30-44 | 42 | 36 | 36 | 34 | 30 | 32 | 32 | 30 | 38 | 40 | 38 | 42 | 38 | 44 | 34 | 34 | 34 | 36 | 36 | 30 | 44 | 42 | 500 | 1000 |
| **UKB 20** | 18-36 | 18 | 36 | 20 | 26 | 16 | 22 | 16 | 20 | 6 | 14 | 20 | 10 | 14 | 20 | 36 | 36 | 32 | 12 | 12 | 8 | 16 | 24 | 500 | 1000 |
| **UKL 21** | 0-16 | 0 | 16 | 14 | 4 | 10 | 14 | 16 | 6 | 2 | 4 | 0 | 6 | 4 | 10 | 6 | 6 | 16 | 0 | 10 | 2 | 2 | 2 | 500 | 1000 |
| **UKL 22** | 0-6 | 4 | 4 | 2 | 0 | 4 | 6 | 6 | 6 | 2 | 10 | 2 | 2 | 2 | 2 | 2 | 4 | 2 | 4 | 4 | 2 | 2 | 6 | 500 | 1000 |
| **UKB 23** | 2-14 | 4 | 6 | 6 | 10 | 10 | 14 | 6 | 0 | 4 | 2 | 10 | 2 | 6 | 8 | 6 | 2 | 14 | 4 | 8 | 8 | 14 | 6 | 500 | 1000 |
| **UKB 24** | 0-12 | 12 | 8 | 10 | 6 | 8 | 4 | 6 | 2 | 14 | 0 | 6 | 4 | 10 | 8 | 4 | 6 | 10 | 12 | 4 | 0 | 10 | 6 | 500 | 1000 |
| | | | | | | | | | | | | | | | | | | | | | | | | | |
| **UKL 25** | 2-16 | 12 | 10 | 12 | 6 | 12 | 16 | 14 | 14 | 16 | 10 | 10 | 4 | 16 | 16 | 4 | 8 | 14 | 2 | 8 | 8 | 8 | 6 | 16 | 1000 |

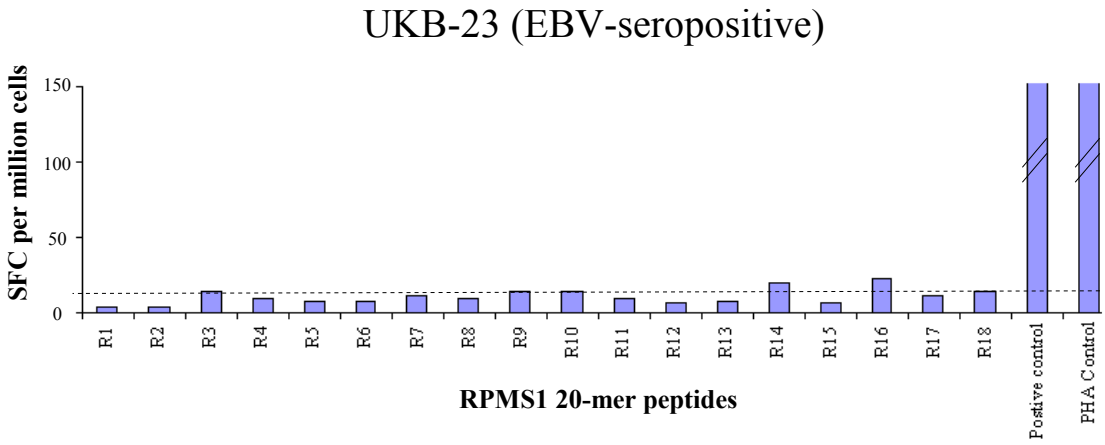
**Table 3.1.3: SUMMARY OF A73 PEPTIDE SCREENING**

UKL - Laboratory Donors
UKB - Buffy Coat Samples
**Positive ELISpot results are bolded
#All positive results are retested twice
(+) Wells that exceed countable by eye are given a value of 500 SFC per million cells
(-) ELISpot wells containing PHA show up as a dark purple well and are given the value of 1000 SFC per million cells

**A**


UKB-13 (EBV-seropositive)
SFC per million cells
150
100
50
0
R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
R11
R12
R13
R14
R15
R16
R17
R18
Postive control
PHA Control
RPMS1 20-mer peptides

**B**


UKB-23 (EBV-seropositive)
SFC per million cells
150
100
50
0
R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
R11
R12
R13
R14
R15
R16
R17
R18
Postive control
PHA Control
RPMS1 20-mer peptides

**C**

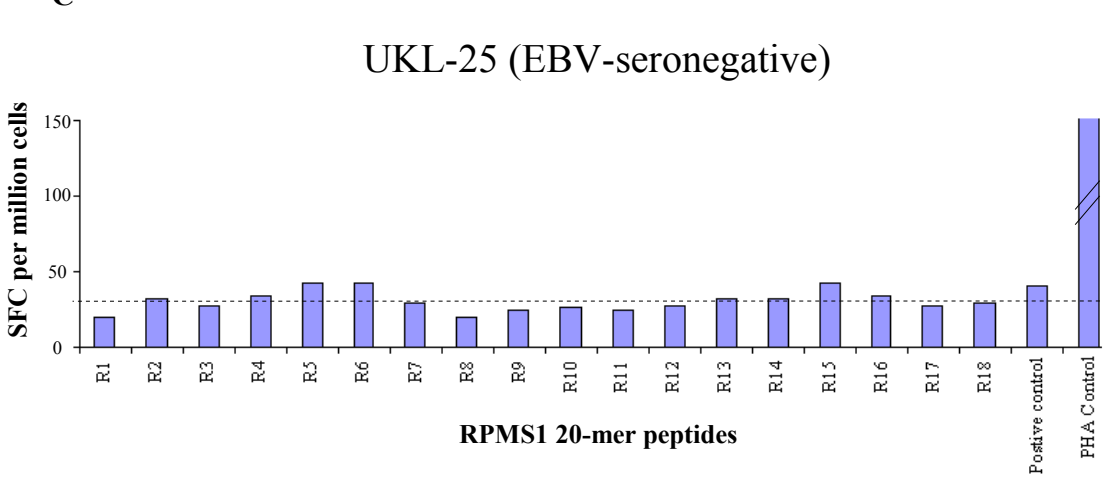

UKL-25 (EBV-seronegative)
SFC per million cells
150
100
50
0
R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
R11
R12
R13
R14
R15
R16
R17
R18
Postive control
PHA Control
RPMS1 20-mer peptides

**Figure 3.1.8: Screening for T cell responses to RPMS1 20-mer peptides**

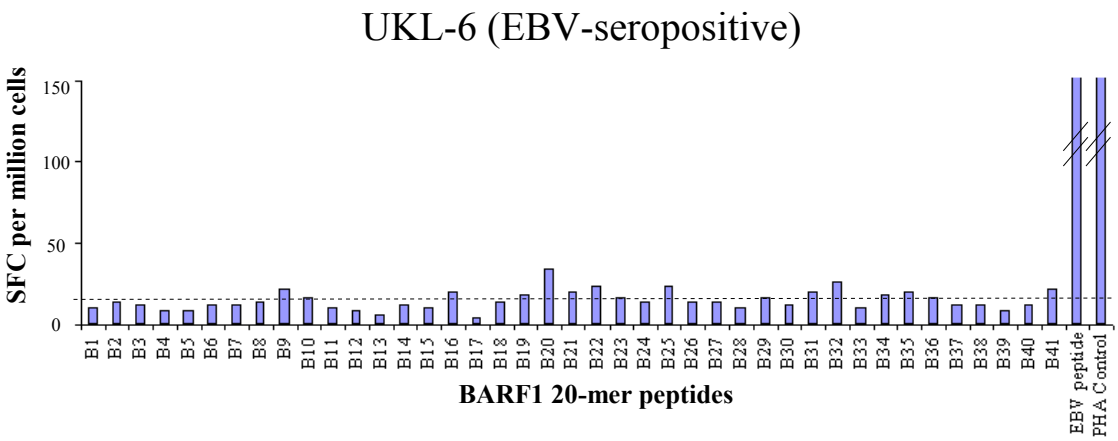
Result show three typical IFN-γ ELISpot assays using whole PBMCs from donors UKB-13 (A), UKB-23 (B) and UKL-25 (C) exposed to a panel of 18 B95.8 EBV strain predicted RPMS1 20-mer peptides. Results are shown as SFC per $10^6$ cells. Both EBV-seropositive donors, UKB-13 and UKB-23, responded to the EBV peptide pool control, whereas, EBV-seronegative donor (UKL-25) did not. All three donors responded against PHA. Note that there are no significant T cell responses to any of the RPMS1 peptides above the background (dotted line) level from any of the three donors tested.

| | | RPMS1 peptide panel | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Donor** | **Background** | **R1** | **R2** | **R3** | **R4** | **R5** | **R6** | **R7** | **R8** | **R9** | **R10** | **R11** | **R12** | **R13** | **R14** | **R15** | **R16** | **R17** | **R18** | **EBV peptide pool** | **PHA Control** |
| **UKL 1** | 38-46 | 34 | 46 | 44 | 38 | 42 | 46 | 44 | 40 | 40 | 46 | 38 | 46 | 46 | 38 | 44 | 40 | 38 | 42 | 500(+) | 1000(-) |
| **UKL 2** | 28-42 | 34 | 32 | 28 | 24 | 42 | 30 | 40 | 38 | 36 | 36 | 30 | 36 | 38 | 34 | 26 | 32 | 36 | 38 | 292 | 1000 |
| **UKL 3** | 20-30 | 20 | 26 | 22 | 30 | 26 | 30 | 20 | 22 | 30 | 24 | 28 | 28 | 24 | 26 | 24 | 20 | 22 | 30 | 500 | 1000 |
| **UKL 4** | 22-40 | 30 | 54 | 32 | 26 | 38 | ****68** | 26 | 40 | 22 | 32 | 40 | 24 | **58** | **64** | 32 | 34 | 36 | 28 | 500 | 1000 |
| **UKL 4 retest#** | 0-4 | 2 | 2 | 0 | 0 | 0 | 2 | 0 | 2 | 2 | 2 | 0 | 2 | 0 | 2 | 0 | 2 | 0 | 4 | 500 | 1000 |
| **UKL 5** | 4-24 | 12 | 16 | 16 | 12 | 14 | 24 | 14 | 14 | 6 | 12 | 4 | 18 | 12 | 18 | 22 | 18 | 24 | 18 | 266 | 1000 |
| **UKL 6** | 2-12 | 4 | 4 | 12 | 4 | 16 | 14 | 6 | 12 | 8 | 2 | 4 | 4 | 8 | 4 | 2 | 8 | 6 | 8 | 500 | 1000 |
| **UKL 6 retest** | 0-16 | 0 | 8 | 6 | 0 | 4 | 8 | 8 | 0 | 6 | 0 | 2 | 8 | 4 | 0 | 0 | 8 | 2 | 16 | 500 | 1000 |
| **UKL 7** | 0-12 | 6 | 0 | 0 | 12 | 12 | 2 | 10 | 12 | 8 | 6 | 10 | 2 | 4 | 8 | 6 | 6 | 12 | 10 | 214 | 1000 |
| **UKL 8** | 10-24 | 4 | 12 | 6 | 2 | 6 | 6 | 12 | 10 | 10 | 8 | 6 | 12 | 20 | 2 | 8 | 18 | 24 | 8 | 500 | 1000 |
| **UKB 9** | 2-16 | 8 | 6 | 2 | 10 | 6 | 14 | 14 | 12 | 10 | 16 | 10 | 16 | 8 | 16 | 10 | 6 | 4 | 10 | 500 | 1000 |
| **UKB 10** | 0-10 | 0 | 0 | 2 | 2 | 2 | 2 | 4 | 2 | 6 | 4 | 10 | 0 | 0 | 0 | 0 | 0 | 2 | 2 | 340 | 1000 |
| **UKB 11** | 20-38 | 28 | 30 | 28 | 28 | 32 | 32 | 34 | 20 | 22 | 34 | 20 | 38 | 32 | 38 | 28 | 36 | 34 | 22 | 500 | 1000 |
| **UKB 12** | 14-30 | 25 | 28 | 26 | 30 | 28 | 26 | 20 | 30 | 24 | 24 | 28 | 22 | 18 | 14 | 18 | 16 | 16 | 14 | 500 | 1000 |
| **UKB 13** | 14-34 | 34 | 34 | 32 | 34 | 22 | 24 | 30 | 18 | 26 | 24 | 20 | 22 | 22 | 24 | 30 | 34 | 24 | 14 | 500 | 1000 |
| **UKL 14** | 16-34 | 14 | 24 | 34 | 12 | 30 | 26 | 20 | 30 | 24 | 22 | 26 | 32 | 18 | 32 | 34 | 24 | 30 | 34 | 400 | 1000 |
| **UKL 15** | 20-38 | 28 | 28 | 22 | 22 | 20 | 30 | 26 | 34 | 20 | 24 | 22 | 34 | 30 | 28 | 38 | 24 | 22 | 22 | 318 | 1000 |
| **UKB 16** | 22-48 | 22 | 36 | 48 | 42 | 28 | 36 | 36 | 46 | 30 | 36 | 22 | 20 | 30 | 44 | 34 | 34 | 42 | 36 | 500 | 1000 |
| **UKB 17** | 28-48 | 40 | 44 | 24 | 40 | 44 | 48 | 40 | 46 | 42 | 40 | 30 | 46 | 34 | 28 | 30 | 38 | 48 | 40 | 400 | 1000 |
| **UKB 18** | 20-32 | 24 | 22 | 20 | 20 | 30 | 28 | 20 | 24 | 30 | 26 | 22 | 20 | 24 | 22 | 32 | 24 | 30 | 24 | 500 | 1000 |
| **UKB 19** | 16-34 | 22 | 20 | 26 | 22 | 24 | 16 | 24 | 22 | 30 | 34 | 22 | 22 | 34 | 22 | 30 | 24 | 34 | 22 | 500 | 1000 |
| **UKB 20** | 16-34 | 34 | 24 | 20 | 28 | 34 | 18 | 18 | 22 | 18 | 28 | 20 | 16 | 30 | 32 | 24 | 20 | 24 | 30 | 500 | 1000 |
| **UKL 21** | 20-38 | 34 | 34 | 22 | 20 | 30 | 24 | 38 | 20 | 30 | 32 | 22 | 38 | 30 | 32 | 30 | 26 | 28 | 22 | 500 | 1000 |
| **UKL 22** | 20-38 | 30 | 26 | 32 | 32 | 38 | 20 | 30 | 22 | 38 | 38 | 32 | 22 | 26 | 36 | 38 | 22 | 30 | 30 | 500 | 1000 |
| **UKB 23** | 4-22 | 4 | 4 | 14 | 10 | 8 | 8 | 12 | 10 | 14 | 14 | 10 | 6 | 8 | 20 | 6 | 22 | 12 | 14 | 500 | 1000 |
| **UKB 24** | 10-28 | 12 | 18 | 12 | 22 | 28 | 24 | 20 | 10 | 12 | 14 | 12 | 28 | 26 | 10 | 24 | 18 | 28 | 12 | 500 | 1000 |
| | | | | | | | | | | | | | | | | | | | | | |
| **UKL 25** | 34-42 | 20 | 32 | 28 | 34 | 42 | 42 | 30 | 20 | 24 | 26 | 24 | 28 | 32 | 32 | 42 | 34 | 28 | 30 | 40 | 1000 |

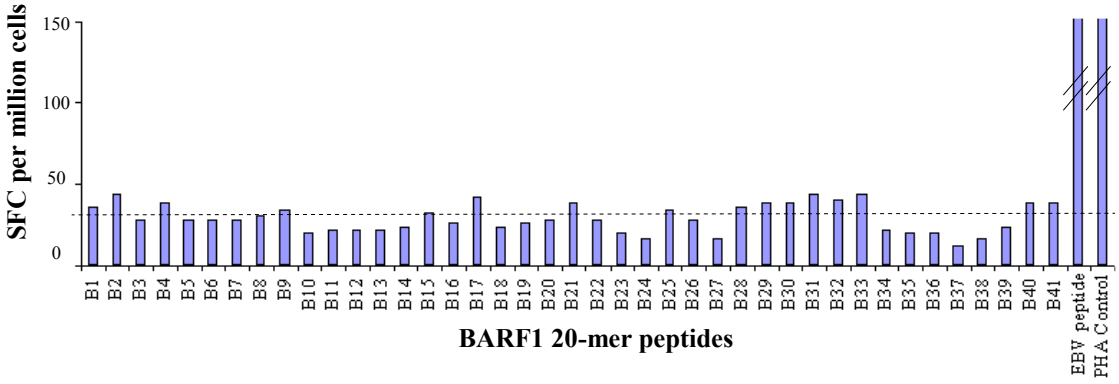
**Table 3.1.4: SUMMARY OF RPMS1 PEPTIDE SCREENING**

UKL - Laboratory Donors
UKB - Buffy Coat Samples
**Positive ELISpot results are bolded
#All positive results are retested once
(+) Wells that exceed countable by eye are given a value of 500 SFC per million cells
(-) ELISpot wells containing PHA show up as a dark purple well and are given the value of 1000 SFC per million cells

**A**


UKL-6 (EBV-seropositive)
SFC per million cells
150
100
50
0
B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15 B16 B17 B18 B19 B20 B21 B22 B23 B24 B25 B26 B27 B28 B29 B30 B31 B32 B33 B34 B35 B36 B37 B38 B39 B40 B41 EBV peptide PHA Control
BARF1 20-mer peptides

**B**


UKB-19 (EBV-seropositive)
SFC per million cells
150
100
50
0
B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15 B16 B17 B18 B19 B20 B21 B22 B23 B24 B25 B26 B27 B28 B29 B30 B31 B32 B33 B34 B35 B36 B37 B38 B39 B40 B41 EBV peptide PHA Control
BARF1 20-mer peptides

**C**

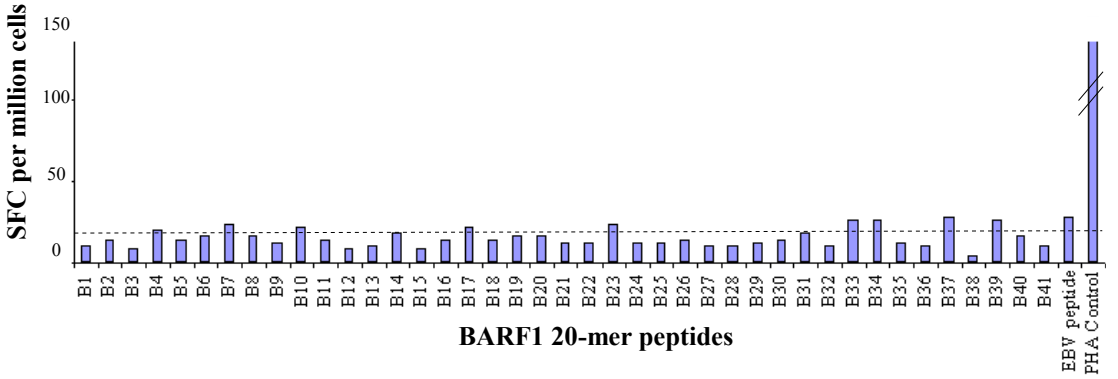

UKL-25 (EBV-seronegative)
SFC per million cells
150
100
50
0
B1 B2 B3 B4 B5 B6 B7 B8 B9 B10 B11 B12 B13 B14 B15 B16 B17 B18 B19 B20 B21 B22 B23 B24 B25 B26 B27 B28 B29 B30 B31 B32 B33 B34 B35 B36 B37 B38 B39 B40 B41 EBV peptide PHA Control
BARF1 20-mer peptides

**Figure 3.1.9: Screening for T cell responses to BARF1 20-mer peptides**

Result show three typical IFN-γ ELISpot assays using whole PBMCs from donors UKL-6 (A), UKB-19 (B) and UKL-25 (C) exposed to a panel of 42 B95.8 EBV strain BARF1 20-mer peptides. Results are shown as SFC per $10^6$ cells. Both EBV-seropositive donors, UKL-6 and UKB-19, responded to the EBV peptide pool control, whereas, EBV-seronegative donor (UKL-25) did not. All three donors responded against PHA. Note that there are no significant T cell responses to any of the BARF1 peptides above the background (dotted line) level from any of the three donors tested.

| | | BARF1 peptide panel | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Donor | Background | B1 | B2 | B3 | B4 | B5 | B6 | B7 | B8 | B9 | B10 | B11 | B12 | B13 | B14 | B15 | B16 | B17 | B18 | B19 | B20 | B21 | B22 | B23 | B24 | B25 | B26 | B27 | B28 | B29 | B30 | B31 | B32 | B33 | B34 | B35 | B36 | B37 | B38 | B39 | B40 | B41 | EBV peptide pool | PHA Control |
| UKL 1 | 4-24 | 18 | 20 | 18 | 8 | 24 | 4 | 30 | 16 | 8 | 16 | 16 | 14 | 24 | 18 | 16 | 20 | 14 | 22 | 12 | 8 | 16 | 14 | 8 | 6 | 24 | 8 | 20 | 20 | 8 | 20 | 18 | 6 | 14 | 24 | 8 | 14 | 18 | 16 | 12 | 14 | 16 | 500(+) | 1000(-) |
| UKL 2 | 10-36 | 14 | 26 | 14 | 32 | 38 | 34 | 36 | 36 | 32 | 32 | 34 | 36 | 24 | 28 | 36 | 26 | 28 | 30 | 32 | 34 | 28 | 24 | 10 | 36 | 20 | 32 | 34 | 34 | 36 | 22 | 36 | 32 | 32 | 34 | 28 | 34 | 18 | 18 | 22 | 36 | 36 | 106 | 1000 |
| UKL 3 | 10-40 | 10 | 24 | 26 | 30 | 28 | 40 | 30 | 24 | 24 | 38 | 40 | 38 | 20 | 40 | 40 | 30 | 34 | 34 | 32 | 40 | 40 | 38 | 32 | 42 | 10 | 30 | 40 | 40 | 40 | 40 | 34 | 42 | 22 | 40 | 10 | 18 | 22 | 28 | 40 | 30 | 26 | 500 | 1000 |
| UKL 4 | 10-36 | 14 | 26 | 14 | 32 | 36 | 34 | 36 | 36 | 32 | 32 | 34 | 36 | 24 | 28 | 36 | 26 | 28 | 30 | 32 | 34 | 28 | 24 | 10 | 36 | 20 | 32 | 34 | 34 | 42 | 22 | 36 | 32 | 32 | 34 | 28 | 34 | 18 | 18 | 22 | 36 | 66 | 500 | 1000 |
| UKL 5 | 32-36 | 20 | 46 | 38 | 22 | 34 | 36 | 24 | 26 | 38 | **70 | **72** | 48 | 38 | 36 | **72** | **64** | **68** | 42 | 48 | **72** | 34 | 46 | 50 | 22 | 56 | 46 | 52 | 40 | 32 | 34 | 26 | 36 | 48 | 48 | 52 | 52 | 38 | 30 | 28 | 28 | **64** | 138 | 1000 |
| UKL 5 retest 1# | 34-38 | | | | | | | | | 36 | 34 | 38 | 36 | | 36 | 38 | 34 | 34 | 38 | 34 | 34 | 36 | | | | | | | | | | | | | | | | | | | 32 | 30 | 122 | 1000 |
| UKL 5 retest 2 | 38-64 | | | | | | | | | 34 | 34 | 36 | 34 | | 34 | 34 | 34 | 34 | 34 | 34 | 36 | 38 | | | | | | | | | | | | | | | | | | | 36 | 36 | 120 | 1000 |
| UKL 6 | 10-34 | 10 | 14 | 12 | 8 | 8 | 12 | 12 | 14 | 22 | 16 | 10 | 8 | 6 | 12 | 10 | 20 | 4 | 14 | 18 | 34 | 20 | 24 | 16 | 14 | 24 | 14 | 14 | 10 | 16 | 12 | 20 | 26 | 10 | 18 | 20 | 16 | 12 | 12 | 8 | 12 | 22 | 500 | 1000 |
| UKL 7 | 0-10 | 0 | 2 | 0 | 4 | 4 | 0 | 6 | 4 | 6 | 8 | 8 | 0 | 2 | 4 | 4 | 2 | 2 | 4 | 6 | 2 | 12 | 0 | 10 | 2 | 2 | 4 | 2 | 0 | 2 | 4 | 2 | 6 | 0 | 0 | 4 | 0 | 4 | 8 | 2 | 2 | 2 | 110 | 1000 |
| UKL 8 | 2-28 | 2 | 10 | 14 | 0 | 16 | 8 | 26 | 8 | 10 | 8 | 4 | 2 | 12 | 18 | 14 | 18 | 12 | 14 | 12 | 12 | 10 | 18 | 8 | 2 | 2 | 14 | 22 | 20 | 22 | 24 | 22 | 20 | 12 | 10 | 6 | 10 | 14 | 2 | 10 | 28 | 18 | 500 | 1000 |
| UKB 9 | 2-20 | 12 | 8 | 14 | 10 | 8 | 6 | 20 | 6 | 12 | 6 | 14 | 8 | 14 | 2 | 20 | 6 | 14 | 10 | 8 | 8 | 4 | 10 | 6 | 4 | 6 | 10 | 8 | 12 | 4 | 4 | 8 | 4 | 8 | 6 | 10 | 6 | 6 | 6 | 4 | 6 | 6 | 500 | 1000 |
| UKB 10 | 2-16 | 4 | 4 | 10 | 8 | 6 | 12 | 10 | 8 | 2 | 6 | 10 | 24 | 10 | 8 | 12 | 12 | 10 | 6 | 8 | 2 | 8 | 10 | 0 | 6 | 4 | 8 | 6 | 8 | 8 | 6 | 8 | 6 | 14 | 10 | 6 | 4 | 10 | 14 | 10 | 14 | 10 | 166 | 1000 |
| UKB 11 | 0-12 | 0 | 0 | 4 | 10 | 8 | 10 | 6 | 2 | 12 | 0 | 0 | 10 | 0 | 2 | 0 | 2 | 2 | 2 | 6 | 4 | 0 | 0 | 12 | 6 | 0 | 4 | 2 | 0 | 0 | 6 | 6 | 0 | 4 | 6 | 2 | 8 | 4 | 4 | 6 | 0 | 4 | 500 | 1000 |
| UKB 12 | 0-10 | 2 | 4 | 2 | 0 | 2 | 10 | 4 | 8 | 0 | 4 | 2 | 2 | 6 | 6 | 6 | 8 | 0 | 4 | 6 | 8 | 8 | 6 | 2 | 6 | 2 | 2 | 6 | 8 | 8 | 2 | 4 | 4 | 10 | 6 | 8 | 2 | 10 | 2 | 4 | 8 | 2 | 500 | 1000 |
| UKB 13 | 14-40 | 18 | 20 | 32 | 18 | 30 | 36 | 34 | 26 | 14 | 40 | 38 | 40 | 40 | 36 | 26 | 40 | 24 | 36 | 20 | 32 | 26 | 30 | 32 | 14 | 22 | 20 | 28 | 32 | 32 | 34 | 24 | 34 | 14 | 38 | 22 | 24 | 38 | 16 | 40 | 20 | 26 | 500 | 1000 |
| UKL 14 | 22-32 | 32 | 26 | 22 | 24 | 22 | 28 | 32 | 24 | 24 | 30 | 30 | 22 | 22 | 22 | 30 | 30 | 28 | 26 | 24 | 30 | 30 | 32 | 30 | 28 | 32 | 30 | 30 | 30 | 30 | 32 | 28 | 28 | 32 | 32 | 32 | 28 | 28 | 24 | 28 | 28 | 32 | 220 | 1000 |
| UKL 15 | 4-28 | 16 | 8 | 24 | 8 | 10 | 14 | 10 | 16 | 8 | 10 | 16 | 10 | 18 | 6 | 22 | 12 | 4 | 6 | 18 | 16 | 12 | 10 | 14 | 10 | 12 | 8 | 14 | 12 | 6 | 6 | 16 | 10 | 12 | 6 | 10 | 22 | 28 | 16 | 6 | 8 | 10 | 148 | 1000 |
| UKB 16 | 14-32 | 20 | 18 | 28 | 14 | 18 | 32 | 32 | 32 | 22 | 32 | 24 | 24 | 12 | 26 | 26 | 22 | 26 | 16 | 30 | 32 | 6 | 18 | 20 | 20 | 18 | 24 | 28 | 32 | 32 | 26 | 18 | 30 | 14 | 16 | 28 | 12 | 10 | 22 | 22 | 30 | 16 | 500 | 1000 |
| UKB 17 | 20-40 | 28 | 20 | 24 | 34 | 34 | 30 | 36 | 36 | 20 | 34 | 30 | 34 | 34 | 36 | 34 | 36 | 38 | 26 | 36 | 38 | 26 | 40 | 24 | 38 | 40 | 28 | 32 | 40 | 40 | 38 | 36 | 24 | 22 | 32 | 24 | 40 | 40 | 36 | 40 | 20 | 20 | 192 | 1000 |
| UKB 18 | 10-32 | 16 | 18 | 32 | 24 | 24 | 32 | 26 | 16 | 14 | 24 | 22 | 12 | 20 | 24 | 30 | 30 | 32 | 20 | 30 | 32 | 32 | 26 | 32 | 10 | 16 | 18 | 24 | 28 | 32 | 30 | 24 | 20 | 32 | 32 | 28 | 20 | 12 | 16 | 32 | 18 | 14 | 500 | 1000 |
| UKB 19 | 20-44 | 36 | 44 | 28 | 38 | 28 | 28 | 28 | 30 | 34 | 20 | 22 | 22 | 22 | 24 | 32 | 26 | 42 | 24 | 26 | 28 | 38 | 28 | 20 | 16 | 34 | 28 | 16 | 36 | 38 | 38 | 44 | 40 | 44 | 22 | 20 | 20 | 12 | 16 | 24 | 38 | 38 | 500 | 1000 |
| UKB 20 | 8-28 | 8 | 16 | 22 | 22 | 20 | 14 | 28 | 28 | 20 | 12 | 28 | 18 | 30 | 28 | 24 | 24 | 28 | 26 | 28 | 28 | 28 | 28 | 24 | 22 | 28 | 22 | 18 | 20 | 16 | 26 | 26 | 22 | 28 | 24 | 24 | 22 | 12 | 16 | 22 | 16 | 18 | 500 | 1000 |
| UKL 21 | 8-20 | 8 | 8 | 20 | 12 | 10 | 6 | 8 | 10 | 6 | 8 | 12 | 18 | 10 | 20 | 14 | 12 | 10 | 12 | 18 | 14 | 20 | 10 | 12 | 20 | 16 | 26 | 20 | 12 | 14 | 8 | 14 | 12 | 18 | 8 | 12 | 16 | 20 | 14 | 18 | 18 | 12 | 500 | 1000 |
| UKL 22 | 6-24 | 12 | 8 | 10 | 12 | 14 | 18 | 12 | 24 | 10 | 14 | 8 | 10 | 12 | 24 | 20 | 6 | 10 | 8 | 20 | 10 | 18 | 24 | 16 | 20 | 20 | 12 | 10 | 24 | 20 | 8 | 18 | 14 | 16 | 6 | 16 | 12 | 16 | 18 | 10 | 24 | 24 | 500 | 1000 |
| UKB 23 | 12-26 | 26 | 22 | 28 | 24 | 20 | 26 | 26 | 26 | 18 | 20 | 20 | 14 | 24 | 20 | 18 | 14 | 18 | 24 | 26 | 20 | 18 | 26 | 16 | 24 | 16 | 16 | 18 | 12 | 12 | 22 | 18 | 20 | 24 | 20 | 22 | 16 | 12 | 22 | 26 | 24 | 18 | 500 | 1000 |
| UKB 24 | 4-24 | 22 | 16 | 24 | 22 | 18 | 24 | 20 | 16 | 12 | 4 | 10 | 8 | 18 | 10 | 24 | 12 | 10 | 18 | 22 | 24 | 16 | 20 | 24 | 10 | 18 | 12 | 20 | 20 | 18 | 14 | 22 | 20 | 22 | 10 | 16 | 12 | 16 | 22 | 22 | 14 | 14 | 500 | 1000 |
| | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| UKL 25 | 8-26 | 10 | 14 | 8 | 20 | 14 | 16 | 24 | 16 | 12 | 22 | 14 | 8 | 10 | 18 | 8 | 14 | 22 | 14 | 16 | 16 | 12 | 12 | 24 | 12 | 12 | 14 | 10 | 10 | 12 | 14 | 18 | 10 | 26 | 26 | 12 | 10 | 28 | 4 | 26 | 16 | 10 | 28 | 1000 |

**Table 3.1.5: SUMMARY OF BARF1 PEPTIDE SCREENING**

UKL - Laboratory Donors
UKB - Buffy Coat Samples
**Positive ELISpot results are bolded
#All positive results are retested twice
(+) Wells that exceed countable by eye are given a value of 500 SFC per million cells
(-) ELISpot wells containing PHA show up as a dark purple well and are given the value of 1000 SFC per million cells

Indeed perhaps the strongest evidence to date for their existence is the finding in a single healthy Caucasian donor of a response to one of the predicted BARF0-derived peptides, an HLA-A*0201 restricted epitope LLWAARPRL (Kienzle *et al*, 1998). In that work, this response was observed in vitro as a $CD8^+$ cytotoxic T cell clone which recognised cells transfected with a BARF0 expression construct (Keinzle *et al*, 1998). More importantly, the authors showed that such response could be detected in other EBV-seropositive carriers but not in EBV-seronegative individuals. This particular 9-mer epitope (RK13) was therefore incorporated in the ELISpot screening along with the panel of 20-mer peptides. Interestingly, by chance, we were able to include in this present work the very donor whom the clone response was cloned by Kienzle *et al*. No response was seen in this donor by ELISpot (UKL25) and more importantly we found that this donor is EBV sero-negative. This suggests that the findings by Kienzle *et al* (1998 and 1999) do not reflect a specific EBV-induced response to the BARF0 peptide but the existence within UKL25 T cell repertoire of rare cells fortuitously cross-reactive with the peptide.

One of the major differences between the study by Kienzle *et al* (1998) and the work presented in this thesis is in the methods used in the detection for T cell responses. In the study by Kienzle *et al* (1998), the authors were using an epitope prediction program to determine possible HLA-restricted epitopes within the amino acid sequence of BARF0. At the time of this study, these epitope prediction softwares are limited only to the prediction of well defined HLA molecules. More importantly, these softwares do occasionally miss-out well defined HLA-restricted epitopes and often give high scores to epitopes that do not induce T cell responses in *in vitro* studies (Kotturi *et al*, 2007). To further demonstrate this point, the A73 amino acid sequence was analysed by a well-developed epitope prediction software for HLA-A*0201-restricted epitope, SYFPEITHI (http://www.syfpeithi.de). The SYFPEITHI software predicts an HLA-A*0201 epitope RLLNKPPTV with a score of 26 out of a possible maximum score of 36. Note that the maximum score for a well defined HLA-A*0201 epitope GILGFVFTL from the influenza matrix protein is 30/36. This RLLNKPPTV sequence (see Figure 3.1.7) lies in the A2 15-mer peptide and no CD8+ T

cell responses were ever detected, above background IFN-γ release, from any of the 25 donors using IFN-γ ELISpot assay.

A second important difference between this work and work reported by Kienzle *et al* (1998) is the method of reading T cell response. In the study by Kienzle *et al* (1998), the authors measured cytotoxicity by $^{52}$Cr release of BARF0-specific T cell clones, while in this study, IFN-γ production was measure by ELISpot assay of whole PBMC. There are two points discussed here; (1) the study clones versus whole PBMC and (2) measuring cytotoxicity versus IFN-γ production. Firstly, the study of responses in whole PBMC reflects more closely to responses seen *in vivo* as a whole. Studies to date strongly suggest that the number of virus-specific T cells play an important role in the control of infection and closely reflects antigen expression. For example, EBV lytic antigen-specific CD8+ T cell responses are high in IM patients which accounts for 25% of the total CD8+ T cell population (Callan *et al*, 1998). This is because during primary infection, lytic cycle antigens are expressed therefore there is a high number of circulating EBV lytic antigen-specific CD8+ T cells. If responses could only be detected by cloning and not by IFN-γ ELISpot assay, this may reflect that these BART products and BARF1 protein are not expressed or are expressed only at very low levels, and more importantly, responses are very weak.

Secondly, the ELISpot assay is a sensitive method in detecting IFN-γ secretion by a single cell, since theoretically, each spot that are present on a ELISpot well represents IFN-γ secretion by a single cell. The reason why IFN-γ ELISpot assay was chosen for the studies in this thesis is because CD4+ T cells of Th1 phenotype secrete IFN-γ in response to antigen stimulation and have been demonstrated to have cytotoxic functions (Wilson *et al*, 2001). More importantly, IFN-γ ELISpot assay was demonstrated to be an effective method of detecting EBNA1-sepcific CD8+ T cell responses to viral antigens where cytotoxicity assays have failed to do so(Blake *et al*, 1997), and these IFN-γ producing EBNA1-specific CD8+ T cells when cloned have also been shown to inhibit outgrowth of LCLs. While other studies have shown that IFN-γ ELISpot assay is good for detecting T cell specificity

to epitopes and the number of T cells that make response, it often does not correlate to anti-viral activity (Loffredo *et al*, 2007; Yang, 2003). Keeping in mind the objective of this section of work, the IFN-γ ELISpot assay is ideal for screening, in large scale studies, for antigen-specific T cell responses in initial studies. Functional studies will ultimately require cloning of antigen-specific T cells.

The consistently negative results seen in the present work cannot prove that there are no T-cell responses against the BART and BARF1 peptides. However, they do suggest that, if such responses do exist, they are present at very low frequency. The central objective to this study was to determine, using the BART and BARF1 peptides, the potential usefulness of BART products and/or BARF1 as T cell targets. Clearly, if T cell responses are rare, these proteins (if expressed) will have little or no therapeutic value. The ELISpot assay has its limitations, not least because such responses need to be seen over the background that frequently complicates the assay. We have set our criteria that a significant response must be +3SD reproducibly above the background. However, we could improve the sensitivity of the assay screening separately for CD4 and CD8 T cell responses, i.e. by depleting whole PBMC of CD16 and CD4 populations to enhance detection for CD8 T cell responses and depleting whole PBMC of CD16 and CD8 populations to enhance detection CD4 T cell responses. However, such a procedure demands large blood samples from laboratory donors and is very time consuming.

This work has been dealing with these predicted BART polypeptides as potential targets for T cell responses. The fact that such responses were never observed does not necessarily mean that these polypeptides are never expressed. It must be stressed that the predicted BART polypeptides are all relatively small, with BARF0 being the largest of the three at 174 amino acids long. It may be possible that these polypeptides induce responses only very rarely because they are so small and therefore provide a very limited repertoire of potential T cell epitopes; a parallel example could be the rarity of $CD8^+$ and $CD4^+$ T cell responses among Caucasians to the smallest EBNAs, EBNA-LP, which has 111 amino acids of unique sequences.

Unlike the predicted BART polypeptides, BARF1 is a known early lytic protein that is repeatedly expressed in NPC and gastric carcinoma in the absence of other lytic proteins (Seto *et al*, 2005). But, more interestingly, BARF1 shared 18% amino acid sequence homology with human CD80. This is shown in Figure 3.1.10 highlighted in red with the BARF1 sequence at the top aligned with the human CD80 sequence at the bottom and the numbering represents the coordinates in relation to the BARF1 amino acids sequence.

$CD8^+$ T cell responses against the BARF1 protein had been looked at before in the context of acute viral infection in IM patients. Pudney *et al* (2005) reported $CD8^+$ T cell responses in IM patients to the two immediate early proteins, against seven early lytic proteins and three late lytic proteins. However no BARF1-specific $CD8^+$ responses were found testing on vaccinia BARF1-expressing target cells. The present findings extend this by showing that virus carriers have no detectable $CD4^+$ and $CD8^+$ T cell memory to BARF1 by *ex vivo* ELISpot analysis on peptide panels. This lack of responses to BARF1 could be partly explained by its limited homology to a cellular protein (CD80), by its immune-evasive properties or by the fact that the protein is again reasonably small and may only rarely provide immunogenic T cell epitopes.

Taken at face value, the present results imply that BART polypeptides (if they exist) and BARF1 have little or no immunotherapeutic value in the context of NPC. However, this work has been conducted using Caucasian donors while our main interest is directed at the Chinese population where NPC occurrence is high. Extrapolations from one to the other population need to be made with care since there is (1) geographic polymorphisms in EBV latent gene sequences that may well extend to BARTs and to BARF1 (Midgley *et al*, 2003), and (2) differences in HLA allele distribution (Chang and Hawkins, 1997) between Caucasians and Chinese populations. Both could affect the immunogenecity of these potential target antigens.

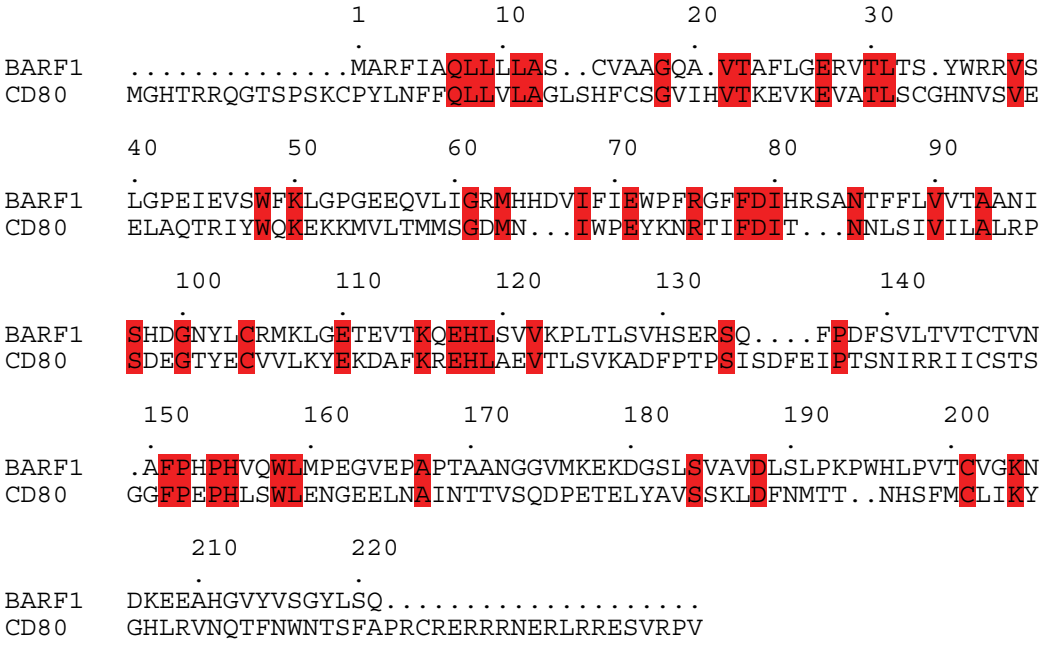

1 10 20 30
BARF1 .....MARFIAQLLLLAS..CVAAGQA.VTAFLGERVTLTS.YWRRVS
CD80 MGHTRRQGTSPSKCPYLNFFQLLVLAGLSHFCSGVIHVTKEVKEVATLSCGHNVSVE
40 50 60 70 80 90
BARF1 LGPEIEVSWFKLGPGEEQVLIGRMHHDVIFIEWPFRGFFDIHRSANTFFLVVTAANI
CD80 ELAQTRIYWQKEKKMVLTMMSGDMN...IWPEYKNRTIFDIT...NNLSIVILALRP
100 110 120 130 140
BARF1 SHDGNYLCRMKLGETEVTKQEHLSVVKPLTLSVHSERSQ....FPDFSVLTVTCTVN
CD80 SDEGTYECVVLKYEKDAFKREHLAEVTLSVKADFPTPSISDFEIPTSNIRRIICSTS
150 160 170 180 190 200
BARF1 .AFPHPHVQWLMPEGVEPAPTAANGGVMKEKDGSLSVAVDLSLPKPWHLPVTCVGKN
CD80 GGFPEPHLSWLENGEELNAINTTVSQDPETELYAVSSKLDFNMTT..NHSFMCLIKY
210 220
BARF1 DKEEAHGVYVSGYLSQ.....................
CD80 GHLRVNQTFNWNTSFAPRCRERRRNERLRRESVRPV

**Figure 3.1.10: Amino acid sequence of BARF1 aligned with human CD80**

This Figure shows the amino acid sequence alignment of BARF1 alongside human CD80. The numbers located at the top of the figure are the amino acid coordinates in relation to the BARF1 sequence. Highlighted in red are the amino acids that are shared between BARF1 and human CD80. The (.) represents empty gaps so that the two sequences can form the best homology match. There are 47 amino acids that are shared (highlighted red) between BARF1 and human CD80 out of a total of 256 amino acids (the total number of amino acids from human CD80), making BARF1 approximately 18% homologous to human CD80.

As a preliminary step to assess 'geographic' sequence polymorphisms in EBV, the BARF1 gene was PCR amplified and sequenced from EBV DNA extracted from the Caucasian type I virus B95.8, the African type II virus Ag876 and two representative Chinese isolate CKL (Type I Chinese EBV isolate from Chinese NPC patient) and C5 (type I EBV isolate from a healthy Chinese donor as reported by Midgley *et al* (2003)). Figure A3.1.1 shows the results. Underlined is the B95.8 BARF1 sequence (used as a reference point) starting at B95.8 nucleotide 165504 and ending at B95.8 nucleotide 166164. The B95.8 and Ag876 virus strains were found to have identical BARF1 nucleotide sequences. Both Chinese isolates, CKL and C5, showed one nucleotide change (T to C) marked in red at position. This does not lead to a coding change and therefore the BARF1 amino acid sequence is fully conserved between the different virus strains tested. Work on geographic polymorphism in BART sequences is still ongoing; the BARTs are heavily spliced across many distantly arranged exons making them more difficult to sequence and analyse.

A second factor, HLA allele distributions are different in Chinese compared to Caucasians (Chang and Hawkins, 1997) and so epitope choice may be radically different. There is therefore really no alternative other than to re-do the ELISpot screening in Chinese donors using appropriate peptide panels. However, a judgment had to be made and it was felt that the present finding of non-responsiveness to these polypeptides in the UK population made it unlikely that the same polypeptides would be highly immunogenic in another population. For these reasons, it was decided that the project would return to studying T cell responses to EBNA1, the only EBV latent protein definitely known to be expressed in all NPC tumours and one which is known to elicit T cell responses, in particular $CD4^+$ T cell responses, in Caucasian populations.

# Section 3.2

# Screening Chinese donors for CD4+ T cell memory to EBNA1

### 3.2.0 Screening Chinese Donors for $CD4^+$ T cell Memory to EBNA1

### 3.2.1 Introduction

In this section of work, I describe the ELISpot screening of $CD4^+$ T cell response to Chinese sequence EBNA1 peptides. This was felt to be potentially more important than $CD8^+$ responses. Note that, although, $CD8^+$ T cell responses against EBNA1, as described in the Introduction, had been identified (Blake *et al*., 1997; Blake *et al*., 2000), the biological effectiveness, such as the inhibition of LCL outgrowth, of such responses has only recently been demonstrated (Tellam *et al*, 2001; Voo *et al*, 2004; Lee *et al*, 2004; Tellam *et al*, 2004). More importantly, such EBNA1-specific $CD8^+$ T cell responses remain relatively rare and linked to a small subset of HLA class I alleles. If EBNA1 were to be as immunogenic to $CD4^+$ T cells in Chinese donors as it was seen to be in Caucasians, the long term aim of exploiting EBNA1-specific responses in targeting of NPC tumours is therefore likely to be heavily reliant on such $CD4^+$ T cells.

### 3.2.2 EBNA 1 peptide panel

Since earlier literature reported that Chinese EBV strains carried a slightly different EBNA1 allelic sequence (the I allele) to both of the alleles (the V and T allele) present in Caucasian viruses, a first step was to sequence the EBNA 1 gene from a representative Chinese virus isolate. The virus strain selected was CKL, an isolate originally made from the blood of a Chinese NPC patient that earlier studies in this laboratory had shown to carry typical Chinese virus sequences at the EBNA 2, 3A, 3B and 3C loci (Midgley *et al*, 2000). The EBNA 1 gene of CKL was PCR amplified using sets of primers flanking the entire EBNA1 gene. Figure 3.2.1 shows the amino acid sequence, relative to the B95.8 prototype sequence that was determined in parallel in the present work. There were 14 substitutions in all, 3 in N-terminus upstream of the Gly/ala repeat domain and 11 amino acid changes in the downstream C-terminal half. To ensure that ELISpot screening was conducted using appropriate peptides, 36 new 20-mer peptides were made accommodating all the changes unique to the CKL sequence and these were used


1 10 11 20 21 30 31 40 41 50 51 60 61 70 71 80 81 90
B95.8 EBNA1 MSDEGPGTGP GNGLGEKGDT SGPEGSGGSG PQRRGGDNHG RGRGRGRGRG GGRPGAPGGS GSGPRHRDGV RRPQKRPSCI GCKGTHGGTG
Chinese EBNA1 MSDEGPGTGP GNGLGQKEDS SGPEGSGGSG PQRRGGDNHG RGRGRGRGRG GGRPGAPGGS GSGPRHRDGV RRPQKRPSCI GCKGTHGGTG
91 358 361 370 371 380 381 390 391 400 401 410 411 420 421 430
B95.8 EBNA1 GLY/ALA REPEAT DOMAINER ARGRSRERAR GRGRGRGEKR PRSPSSQSSS SGSPPRRPPP GRRPFFHPVG EADYFEYHQE GGPDGEPDVP
Chinese EBNA1 GLY/ALA REPEAT DOMAINER ARGRSRERAR GRGRGRGEKR PRSPSSQSSS SGSPPRRPPP GRRPFFHPVG DADYFEYLQE GGPDGEPDVP
431 440 441 450 451 460 461 470 471 480 481 490 491 500 501 510 511 520
B95.8 EBNA1 PGAIEQGPAD DPGEGPSTGP RGQGDGGRRK KGGWFGKHRG QGGSNPKFEN IAEGLRALLA RSHVERTTDE GTWVAGVFVY GGSKTSLYNL
Chinese EBNA1 PGAIEQGPTD DPGEGPSTGP RGQGDGGRRK KGGWFGKHRG QGGSNPKFEN IAEGLRVLLA RSHVERTTEE GNWVAGVFVY GGSKTSLYNL
521 530 531 540 541 550 551 560 561 570 571 580 581 590 591 600 601 610
B95.8 EBNA1 RRGTALAIPQ CRLTPLSRLP FGMAPGPGPQ PGPLRESIVC YFMVFLQTHI FAEVLKDAIK DLVMTKPAPT CNIRVTVCSF DDGVDLPPWF
Chinese EBNA1 RRGIALAVPQ CRITPLSRLP FGMAPGPGPQ PGPLRESIVC YFMVFLQTHI FAEVLKDAIK DLVMTKPAPT CNIKVTVCSF DDGVDLPPWF
611 620 621 630 631 640 641
B95.8 EBNA1 PPMVEGAAAE GDDGDDGDEG GDGDEGEEGQ E
Chinese EBNA1 PPMVEGAAAE GDDGDDGDEG GDGDEGEEGQ E

**Figure 3.2.1: EBNA1 amino acid sequence from B95.8 and Chinese virus strains**
The Figure shows the EBNA1 amino acid sequence from both the B95.8 and CKL EBV strains. The numbers located at the top of the sequences are the amino acid coordinates of the protein relative to the B95.8 EBNA1 sequence. In blue is the glycine-alanine repeat domain. In bold is the amino acid sequence which differs between the B95.8 and CKL EBV strains. There were 14 substitutions in all, 3 in N-terminus upstream of the Gly/ala repeat domain and 11 amino acid changes in the downstream C-terminal half.

alongside the remaining 45 EBNA 1 20-mer peptides from the existing B95.8 panel which represented sequences in common with the CKL allele. This is shown in appendix Table A3.2.1.

### 3.2.3 ELISpot screening with full EBNA 1 peptide panel

PBMC samples from healthy Chinese donors were either tested immediately on the day of bleeding or were cryopreserved as PBMCs until testing at a later date. Immediate assays were conducted at The Chinese University of Hong Kong by Lin Xiaorong using protocols developed at the University of Birmingham as part of the present thesis work; assays on cryopreserved samples were conducted at The University of Birmingham using the same panel of peptides and the same experimental protocol. In every assay, PBMCs were first depleted of $CD8^+$ T-cells by CD8 magnetic Dynabeads. This step serves both to enrich the tested population for $CD4^+$ T cells and to remove any possible contamination from EBNA 1-specific $CD8^+$ T cell memory. The quality of the depletion was assessed by staining with FITC-labelled IgG monoclonal antibody CD8 and as controls, with similarly labelled $IgG_1$ antibodies to CD4 and an irrelevant antibody antigen. The FACS profiles of the assay are shown in Figure 3.2.2; after CD8-depletion, the $CD4^+$ T cell population is still present whereas the $CD8^+$ population is completely removed.

Assays were then conducted on CD8-depleted PBMCs using each of the 81 individual EBNA 1 20-mer peptides separately, rather than in pools of 3 adjacent peptides as described in earlier work by Leen *et al* (2001). This was possible because the present assays were conducted on large blood samples obtained from buffy coat donations rather than from the usual 60ml bleeds donated by laboratory volunteers. Figure 3.2.3 and 3.2.4 present the data obtained from fresh cells isolated from four representative donors, HK control 201, 215, 228, and 233. The donors are represented as HK controls (healthy EBV carriers) and would serve in later studies for comparison with data from NPC patients. Responses to individual peptides are shown as histograms and are expressed as the number of spot forming cells (SFC) per $10^6$ CD8-depleted PBMCs. Clear positive responses above background values (mean SFC count ± 3 standard deviation from control wells; see dotted

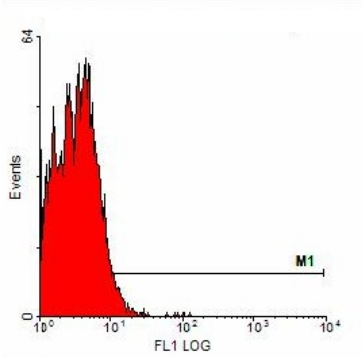
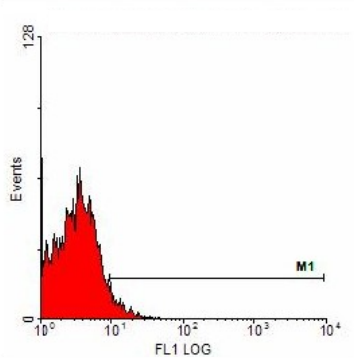
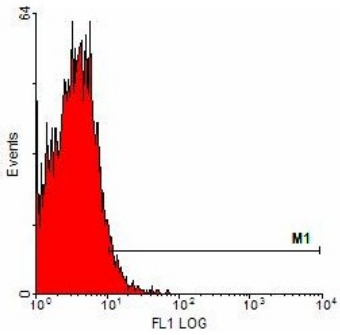
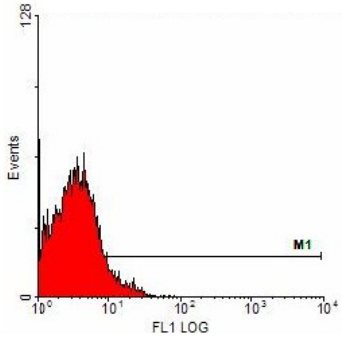
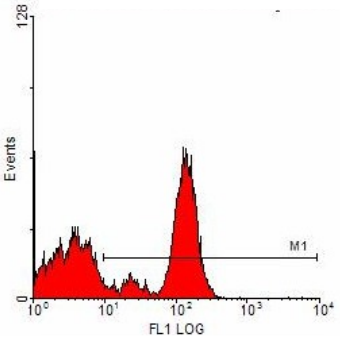
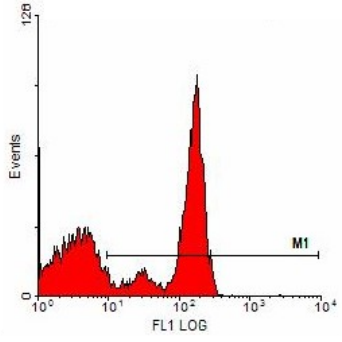
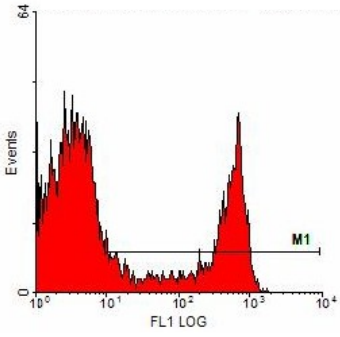
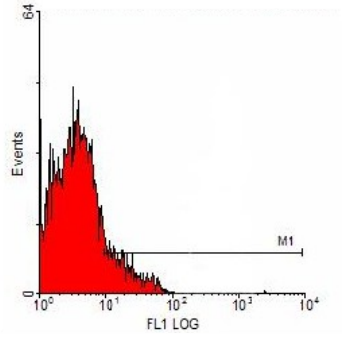
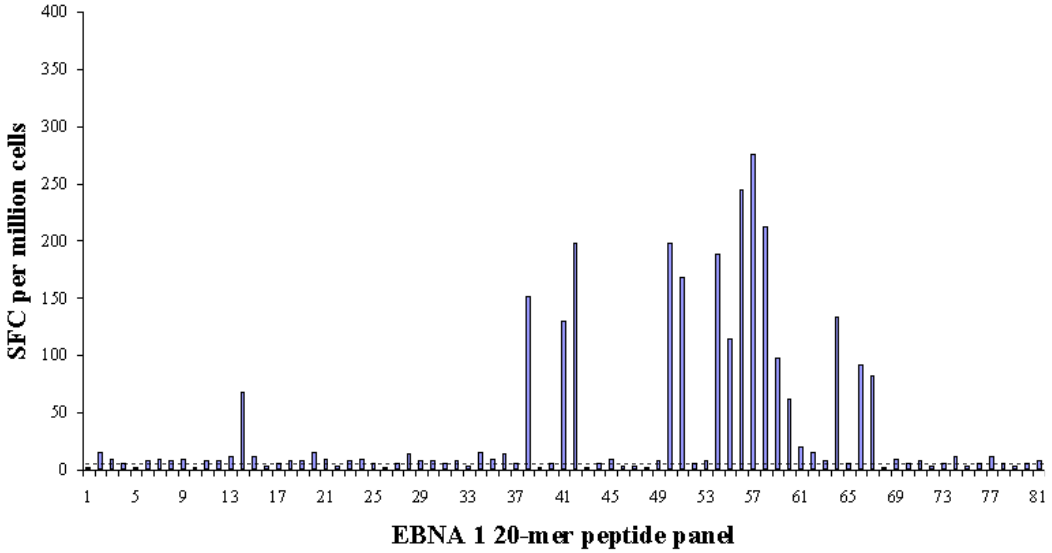
| | Before CD8 depletion | After CD8 depletion |
|---|---|---|
| **No Antibodies** |  |  |
| **IgG1 FITC** |  |  |
| **CD4 FITC** |  |  |
| **CD8 FITC** |  |  |

**Figure 3.2.2: FACS analysis of CD8-depletion**

Result show the FACS analysis before (left column) and after (right column) CD8-depletion. Before CD8-depletion (left column), both $CD4^+$ and $CD8^+$ T cell subpopulation could be clearly seen in FACS chart. After $CD8^+$-depletion (right column), the $CD4^+$ subpopulation is unaffected by the depletion assay and the $CD8^+$ subpopulation is completely removed.

## HK Control 201




## HK Control 215

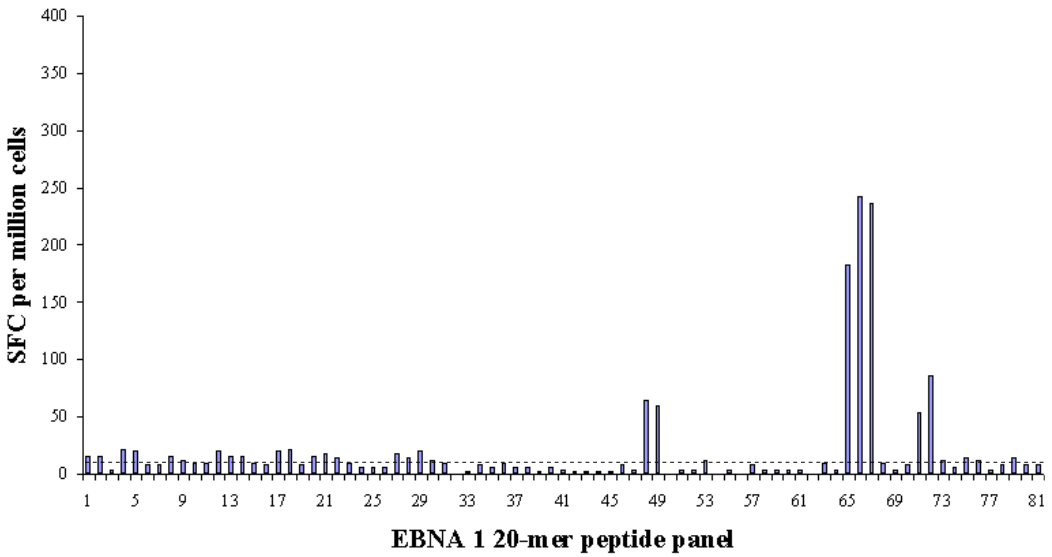
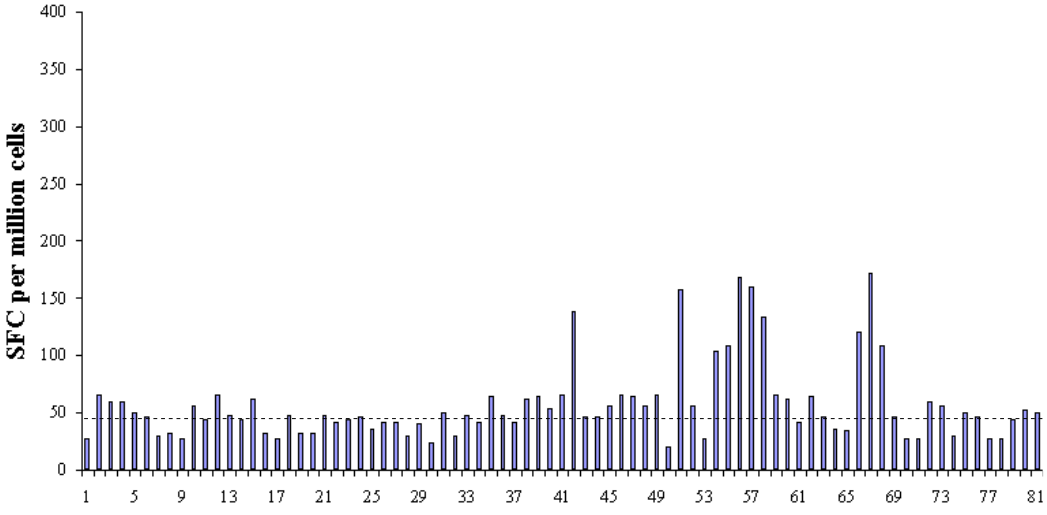


**Figure 3.2.3: IFN-γ ELISpot screening for EBNA1 response**

Result shows two typical IFN-γ ELISpot assays of two Hong Kong donor PBMC samples (HK Control 201 and 215) exposed to 81 individual EBNA1 20-mer peptides derived from the CKL EBV strain (this wok was conducted in Hong Kong by Lin Xiaorong). The results are expressed as the number of spot forming cells (SFC) per $10^6$ CD8-depleted PBMCs. HK Control 201 (top) show responses to multiple EBNA1 20-mer peptides (peptides 14, 38, 41, 42, 50, 51, 54-62, 64, 66 and 67) above the back ground reading (dotted line). HK Control 215 (bottom) also shows responses to multiple EBNA1 20-mer peptides (peptides 48, 49, 65-67, 71 and 72). Both donors gave strong responses to PHA control with the entire ELISpot well covered (data not shown). Note that the two donors show a completely different pattern of response against the EBNA1 peptide panel. HK Control 201 has a very broad response spanning throughout the entire EBNA1 protein, whereas, HK 215 responds only to a few peptides. The HLA typing of these two donors were not available at the time of this work discussed in this section and will be discussed later in the next section.

## HK Control 228




## HK Control 233

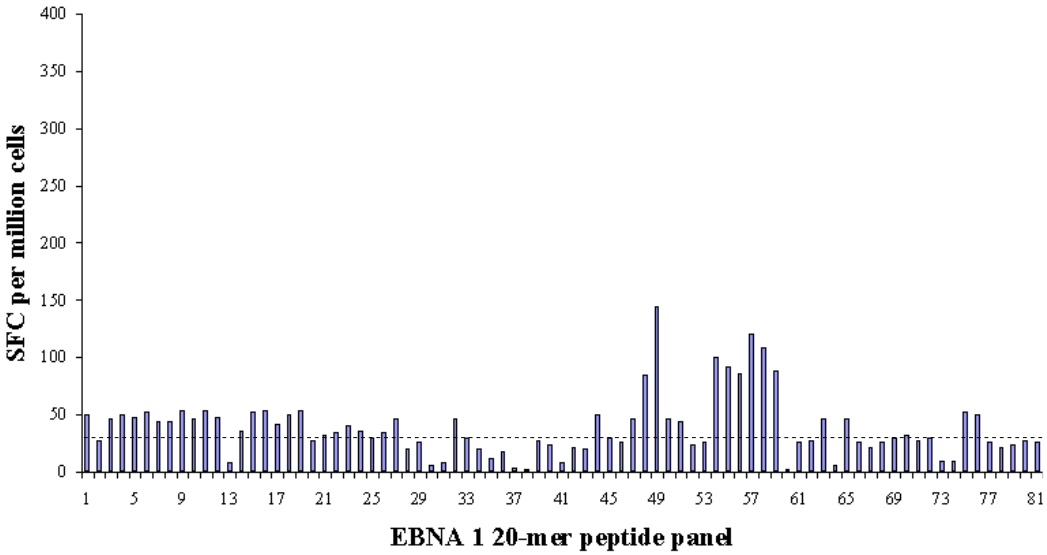


**Figure 3.2.4: More IFN-γ ELISpot screening of EBNA1 response**

Result shows two more typical IFN-γ ELISpot assays of two Hong Kong donor PBMC samples (HK Control 228 and 233) exposed to 81 individual EBNA1 20-mer peptides derived from the CKL EBV strain (this work was conducted by Lin Xiaorong in Hong Kong). The results are expressed as the number of spot forming cells (SFC) per $10^6$ CD8-depleted PBMCs. HK Control 228 (top) show responses to multiple EBNA1 20-mer peptides (peptides 42, 51, 54-58, 66-68) above the back ground reading (dotted line). HK Control 233 (bottom) also shows responses to multiple EBNA1 20-mer peptides (peptides 48, 49, 54-59). Both responders gave strong responses to PHA control (data not shown). Note that the two donors also show a completely different pattern of response against the EBNA1 peptide panel. But, there is also a pattern of response that is commonly observed in both donors, peptides 54-58. Also note that both donors have a high back ground reading, approximately 40-50 SFC per million cells.

line) were observed in all four donors, and had strong responses to PHA (data not shown). It is important to note that the level of background on ex vivo fresh samples varied significantly between individual donors such that the mean background could be as low as 20 SFC/$10^6$ and as high as 70 SFC/$10^6$. Any assays giving backgrounds over 100 SFC/$10^6$ in the initial screening were discarded because, at that time, the NK cell depletion assay (described in section 3.1) was not set up in Hong Kong.

From the initial results illustrated in Figure 3.2.3 and 3.2.4 it can be seen that responses varied in size up to a maximum of 300 SFC/$10^6$ above background. All samples were then checked by re-screening on cryopreserved samples from the same bleeds. Re-screening assays were carried out focusing either on all peptides which gave positive responses in the initial assay or, where cryopreserved cell numbers were limiting, only on peptides that had produced weak positive or borderline responses. To illustrate results obtained in such re-screening, Table 3.2.1 shows the original data alongside the re-test results. The latter are expressed again as SFC/$10^6$ cells and responses significantly above background (i.e. > mean background +3 SD) are identified by yellow shading. Note that Table 3.2.1 only includes the EBNA1 peptides against which one or more donors showed a response in the initial assay. Generally, these repeat assays confirmed the original findings even though the absolute size of the response could vary by a factor of (at most) three fold between the two assays. However, there were occasional examples where a response identified in the original ex vivo screen did not repeat in either of the duplicate re-test wells, such as HK control 201 to peptides 14 and 38. Such non-reproducible responses were omitted in the overall compilation of the data. Table 3.2.1 nevertheless does show the reproducibility of the assay in most cases, for example HK control 201 responses to peptides 41/42, 50/51, 54-60, 64, and 66/67.

In total, 47 healthy Chinese donors were screened on the full panel of EBNA 1 peptides and, where appropriate, re-screened for positive peptide responses. Four of the 47 donors were discarded because background IFNγ response levels were unacceptably high in the initial screening. The data were therefore compiled from 43 informative donors, and the

| | | EBNA 1 Peptide Number | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Donor** | **Background** | **1** | **2** | **3** | **14** | **38** | **41** | **42** | **47** | **48** | **49** | **50** | **51** | **52** | **53** | **54** | **55** | **56** | **57** | **58** | **59** | **60** | **62** | **63** | **64** | **65** | **66** | **67** | **68** | **69** | **70** | **72** | **73** |
| **HK Control 201 initial** | 2-16 | | | | 68 | 152 | 130 | 198 | | | | 198 | 168 | | | 188 | 114 | 244 | 276 | 212 | 98 | 62 | | | 134 | | 92 | 82 | | | | | |
| **HK Control 201 retest 1** | 2-10 | | | | 4 | 2 | 130 | 198 | | | | 118 | 118 | | | 188 | 114 | 162 | 244 | 212 | 98 | 36 | | | 134 | | 92 | 82 | | | | | |
| **HK Control 201 retest 2** | 2-10 | | | | 4 | 6 | 114 | 206 | | | | 124 | 118 | | | 180 | 88 | 172 | 280 | 190 | 88 | 46 | | | 118 | | 96 | 64 | | | | | |
| **HK Control 202 initial** | 2-8 | | | | | | | | | | | | | | | | 34 | 52 | | | | | | | | 34 | 40 | 34 | | | | | |
| **HK Control 202 retest** | 2 | | | | | | | | | | | | | | | 48 | 52 | 56 | 80 | 58 | | | | | 60 | 62 | 62 | 68 | 74 | | | | |
| **HK Control 212 initial** | 4-16 | | | | | | | | | | | | | | | 40 | 30 | | | | | | | | | | | | 44 | | | | |
| **HK Control 212 retest** | 16 | | | | | | | | | | | | | | 4 | 14 | 28 | 36 | 34 | | | | | | | | | | | | | | |
| **HK Control 215 initial** | 4-16 | | | | | | | | | 64 | 60 | | | | | | | | | | | | | | | | 182 | 242 | 236 | | | 54 | 86 |
| **HK Control 215 retest 1** | 6-10 | | | | | | | | | 48 | 52 | | | | | | | | | | | | | | | | 178 | 180 | 102 | | | 54 | 70 |
| **HK Control 215 retest 2** | 6-10 | | | | | | | | | 60 | 50 | | | | | | | | | | | | | | | | 184 | 190 | 86 | | | 66 | 82 |
| **HK Control 221 initial** | 4-16 | | 124 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 221 retest** | 46 | 50 | 48 | 62 | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 222 initial** | 6-26 | | | | | | | | | | | | | | | | 50 | 52 | 36 | | | | | 34 | 36 | 82 | 90 | 42 | | | | | |
| **HK Control 222 retest** | 0 | | | | | | | | | | | | | | | 8 | 18 | 22 | 18 | 28 | | | 8 | 6 | 2 | 12 | 46 | 18 | 10 | | | | |
| **HK Control 224 initial** | 10-22 | | | | | | | | | 104 | 96 | | | | | | | | 40 | 46 | 50 | | | | | | | 44 | 46 | | | | |
| **HK Control 224 retest** | 0 | | | | | | | | | | | | | | | | | | 14 | 22 | 22 | 6 | | | | | 10 | 24 | 28 | 4 | | | |
| **HK Control 228 initial** | 28-66 | | | | | | | 138 | | | | | 158 | | | 104 | 108 | 168 | 160 | 134 | | | | | | | 120 | 172 | 108 | | | | |
| **HK Control 228 retest 1** | 2-24 | | | | | | | 64 | | | | | 102 | | | 102 | 64 | 90 | 98 | 100 | | | | | | | 74 | 98 | 48 | | | | |
| **HK Control 228 retest 2** | 2-24 | | | | | | | 60 | | | | | 116 | | | 98 | 80 | 74 | 88 | 104 | | | | | | | 76 | 114 | 72 | | | | |
| **HK Control 229 initial** | 14-28 | | | | | | | | | | | | | | | | | | | | | | | | | | 48 | | | | | | |
| **HK Control 229 retest** | 24 | | | | | | | | | | | | | | | | | | | | | | | | | 4 | 18 | 14 | 30 | 18 | | | |
| **HK Control 230 initial** | 0-10 | | | | | | | | | | | *56 | | | | | 32 | 52 | | | | | | | | 42 | 64 | 92 | 74 | | | | |
| **HK Control 230 retest** | 4 | | | | | | | | | | **12 | 44 | 50 | | | 36 | 70 | 90 | 26 | | | | | | 20 | 14 | 90 | 166 | 106 | 64 | | | |
| **HK Control 232 initial** | 40-78 | | | | | | | | | | | | | | | | | | | | | | | | | | 120 | 136 | | | | | |
| **HK Control 232 retest** | 4 | | | | | | | | | | | | | | | | | | | | | | | | | 2 | 12 | 18 | 18 | 4 | | | |
| **HK Control 233 initial** | 2-54 | | | | | | | | | 84 | 144 | | | | | 100 | 92 | 86 | 120 | 108 | 88 | | | | | | | | | | | | |
| **HK Control 233 retest 1** | 0-4 | | | | | | | | | 64 | 50 | | | | | 48 | 32 | 84 | 44 | 76 | 28 | | | | | | | | | | | | |
| **HK Control 233 retest 2** | 0-4 | | | | | | | | | 58 | 64 | | | | | 46 | 44 | 80 | 58 | 64 | 30 | | | | | | | | | | | | |
| **HK Control 234 initial** | 34-54 | | | | | | | | | | | | | | | | 120 | | | | | | | | | | 96 | 112 | | | | | |
| **HK Control 234 retest** | 176 | | | | | | | | | | | | | | | 152 | 172 | 238 | | | | | | | | 190 | 210 | 164 | 162 | | | | |
| **HK Control 240 initial** | 14-36 | | | | | | | | | | | | | | | | | 48 | | 56 | | | | | | | | 76 | 62 | | | | |
| **HK Control 240 retest** | 4 | | | | | | | | | | | | | | | | 18 | 42 | 44 | 16 | 44 | | | | | | 28 | 56 | 68 | 22 | | | |
| **HK Control 241 initial** | 34-64 | | | | | | | | | | | | | | | | | | 300 | 132 | 100 | | | | | | | 108 | 90 | | | | |
| **HK Control 241 retest** | 10 | | | | | | | | | | | | | | | | 32 | 120 | 98 | 46 | 36 | | | | | | 38 | 40 | 22 | 14 | | | |
| **HK Control 242 initial** | 26-32 | | | | | | | | | | | | | | | | | | 46 | 46 | | | | | | | | | | | | | |
| **HK Control 242 retest** | 1 | | | | | | | | | | | | | | | | 4 | 18 | 26 | 14 | 20 | | | | | | | | | | | | |
| **HK Control 244 initial** | 68-80 | | | | | | | | | | | 120 | 122 | | | | | | | | 260 | | | | | | 162 | | | | | | |
| **HK Control 244 retest** | 92 | | | | | | | | | | 52 | 160 | 144 | 76 | | | | | 104 | 88 | 136 | 176 | | | | 134 | 174 | 162 | 110 | | | | |
| **HK Control 247 initial** | 0 | | | | | | | | | 110 | 124 | 20 | | | | | | | | | 62 | | | | | | | | | | | | |
| **HK Control 247 retest** | 82 | | | | | | | | 130 | 214 | 214 | 236 | 128 | | | | | | 48 | 102 | 128 | 216 | | | | | | | | | | | |
| **HK Control 248 initial** | 2-10 | | | | | | | | | | | | | | | | | 98 | 70 | 38 | | | | | | | 32 | 38 | | | | | |
| **HK Control 248 retest** | 2 | | | | | | | | | | | | | | | | 4 | 28 | 20 | 14 | 6 | | | | | 4 | 8 | 20 | 16 | | | | |
| **HK Control 249 initial** | 10-16 | | | | | | | | | | | | | | | | | | | | | | | | | | 34 | 106 | 90 | 38 | | | |
| **HK Control 249 retest** | 98 | | | | | | | | | | | | | | | | | | | | | | | | | 74 | 182 | 180 | 172 | 118 | 176 | | |

**Table 3.2.1: SUMMARY OF EBNA1 PEPTIDE RE-TEST FROM FIRST COHORT STUDY**

* highlighted yellow is the peptide response that needs retesting
** neighboring peptide that were included in the rest along side the retest peptide

| | | EBNA 1 Peptide Number | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Donor** | **Background** | **2** | **14** | **38** | **41** | **42** | **48** | **49** | **50** | **51** | **54** | **55** | **56** | **57** | **58** | **59** | **60** | **63** | **64** | **65** | **66** | **67** | **68** | **69** | **72** | **73** |
| **HK Control 201** | 2-16 | | *68 | 152 | 130 | 198 | | | 198 | 168 | 188 | 114 | 244 | 276 | 212 | 98 | 62 | | 134 | | 92 | 82 | | | | |
| **HK Control 202** | 2-8 | | | | | | | | | | | 34 | 52 | | | | | | | 34 | 40 | 34 | | | | |
| **HK Control 203** | 28-40 | | | | | | | | | | | | | | | 80 | | | | 70 | 64 | 82 | 66 | | | |
| **HK Control 204** | 12-24 | | | | | | 44 | 30 | | | | | | | | | | | | | | 34 | 42 | | | |
| **HK Control 205** | 0-4 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 206** | 4-12 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 207** | 0-8 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 208** | 14-30 | | | | | | | | | | | | | | | | | | | | | 44 | 58 | | | |
| **HK Control 209** | 4-16 | | | | | | | | | | | | | | | | | | | | 30 | 48 | 26 | | | |
| **HK Control 210** | 42-60 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 211** | 4-20 | | | | | | | | | | | | | | | | | | | | 40 | 72 | 56 | 28 | | |
| **HK Control 212** | 4-18 | | | | | | | | | | 40 | 30 | | | | | | | | | | | 44 | | | |
| **HK Control 213** | 4-24 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 215** | 4-16 | | | | | | 64 | 60 | | | | | | | | | | | | | 182 | 242 | 236 | | 54 | 86 |
| **HK Control 218** | 54-80 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 219** | 4-32 | | | | | | 112 | 130 | | | | | | | | | | | | | | 60 | 42 | | | |
| **HK Control 220** | 12-46 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 221** | 4-16 | 124 | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 222** | 6-26 | | | | | | | | | | | 50 | 52 | 36 | | | | 34 | 36 | 82 | 90 | 42 | | | | |
| **HK Control 223** | 6-16 | | | | | | | | | | | | | | | | | | | | 44 | 46 | 34 | | | |
| **HK Control 224** | 10-22 | | | | | | 104 | 96 | | | | | | 40 | 46 | 50 | | | | | | 44 | 46 | | | |
| **HK Control 225** | 62-84 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 226** | 12-56 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 227** | 30-50 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 228** | 28-66 | | | | | 138 | | | | 158 | 104 | 108 | 168 | 160 | 134 | | | | | | 120 | 172 | 108 | | | |
| **HK Control 229** | 14-28 | | | | | | | | | | | | | | | | | | | | 48 | | | | | |
| **HK Control 230** | 0-10 | | | | | | | | 56 | | | | 32 | 52 | | | | | | 42 | 64 | 92 | 74 | | | |
| **HK Control 231** | 38-74 | | | | | | | | | | | | | | | | | | | | | 100 | 108 | | | |
| **HK Control 232** | 40-78 | | | | | | | | | | | | | | | | | | | | 120 | 136 | | | | |
| **HK Control 233** | 2-54 | | | | | | 84 | 144 | | | 100 | 92 | 86 | 120 | 108 | 88 | | | | | | | | | | |
| **HK Control 234** | 34-58 | | | | | | | | | | | 120 | | | | | | | | | 96 | 112 | | | | |
| **HK Control 236** | 8-30 | | | | | | | | | | | | | | | | | | | | | 62 | | | | |
| **HK Control 237** | 26-44 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 240** | 14-36 | | | | | | | | | | | | 48 | | 56 | | | | | | | 76 | 62 | | | |
| **HK Control 241** | 34-64 | | | | | | | | | | | | | 300 | 132 | 100 | | | | | | 108 | 90 | | | |
| **HK Control 242** | 26-32 | | | | | | 58 | 52 | | | | | | 46 | 46 | | | | | | | | | | | |
| **HK Control 243** | 50-56 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 244** | 68-80 | | | | | | | | 120 | 122 | | | | | | 260 | | | | | 162 | | | | | |
| **HK Control 245** | 62-90 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 247** | 0 | | | | | | 110 | 124 | 20 | | | | | | | 62 | | | | | | | | | | |
| **HK Control 248** | 2-10 | | | | | | | | | | | | 98 | 70 | 38 | | | | | | 32 | 38 | | | | |
| **HK Control 249** | 10-16 | | | | | | | | | | | | | | | | | | | | 34 | 106 | 90 | 38 | | |
| **HK Control 250** | 0-2 | | | | | | | | | | | | | | | | | | | | | | | | | |

**Table 3.2.2: SUMMARY OF FULL-PANEL EBNA 1 PEPTIDE SCREENING**

Note: only positive data are shown in the table
* results are expressed as spot forming cells (SFC) per million cells

overall results are shown in Table 3.2.2; again only those peptides against which one or more donors made a response are included in the Table. Of the 43 informative donors, 29 gave a positive response to one or more EBNA 1 peptides, whereas 14 donors did not respond to any peptides. Scanning the Table immediately shows that some peptides (e.g. in the region 66-68) were seen by several different individuals, whereas other peptides (e.g. 41/42 and 72/73) were seen by one or two responders only. Levels of response ranged from 30 SFC/$10^6$ (i.e. the minimum considered significant in such assays) to a maximum of 300 SFC/$10^6$ above background. Further comments on the data are made later after presenting the results from more limited screening on a second cohort of donors.

A second cohort of 43 healthy Chinese donors was screened on a mini-panel of 18 EBNA1 peptides that were observed to be most frequently recognised in the initial cohort assay. Table 3.2.3 shows the 18 peptides in the mini-panel screening. Some 8 donors gave unacceptably high backgrounds in this assay and so informative results were obtained from 35 individuals. Representative results from four informative donors are shown as histograms in Figure 3.2.5 (for HK controls 263 and 280) and Figure 3.2.6 (for HK controls 285 and 291). Again clear IFN-γ responses could be observed to particular sets of overlapping peptides. Responses to some of these donors were re-tested and the data are shown in Table 3.2.4 and again non-reproducible responses were omitted in the overall compilation of the data. Overall results from screening on the EBNA 1 mini-panel are summarised in Table 3.2.5. Of the 35 informative donors, 25 gave a positive response to one or more peptides and 10 were uniformly negative. Responses ranged in size from 30 SFC/$10^6$ to, in this case, a maximum of 380 SFC/$10^6$ cells.

### 3.2.4 Provisional identification of epitope regions

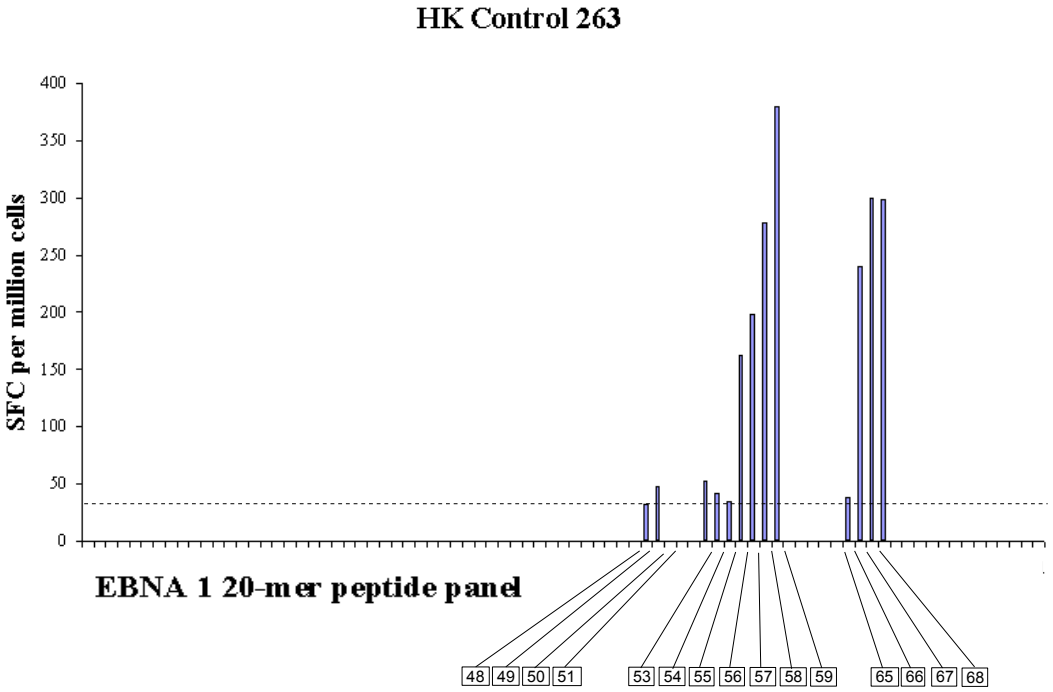
Table 3.2.6 summarises the results of both the full-panel and mini-panel screening. Identifying individual epitopes from ELISpot data of this kind is problematic because of the nature of the assay. Responding cells are exposed to a high peptide concentration and therefore might be able to respond to adjacent pairs or even adjacent triplets of 20-mer peptides (i.e. shared sequences 15 or even 10 amino acids in length) even though the

| | Peptide sequence | Coordinates | Virus type | Peptide no. |
|---|---|---|---|---|
| **EBNA1** | WFGKHRGQGGSNPKFENIAE | 464-483 | B95-8 | 47 |
| **EBNA1** | *RGQGGSNPKFENIAEGLR**V**L | 469-488 | **Chinese | 48 |
| **EBNA1** | SNPKFENIAEGLR**V**LLARSH | 474-493 | Chinese | 49 |
| **EBNA1** | ENIAEGLR**V**LLARSHVERTT | 479-498 | Chinese | 50 |
| **EBNA1** | VERTT**E**EG**N**WVAGVFVYGGS | 494-513 | Chinese | 53 |
| **EBNA1** | **E**EG**N**WVAGVFVYGGSKTSLY | 499-518 | Chinese | 54 |
| **EBNA1** | VAGVFVYGGSKTSLYNLRRG | 504-523 | B95-8 | 55 |
| **EBNA1** | VYGGSKTSLYNLRRGIA**L**A**V** | 509-528 | Chinese | 56 |
| **EBNA1** | KTSLYNLRRGIA**L**A**V**PQCR**I** | 514-533 | Chinese | 57 |
| **EBNA1** | NLRRGIA**L**A**V**PQCR**I**TPLSR | 519-538 | Chinese | 58 |
| **EBNA1** | IA**L**A**V**PQCR**I**TPLSRLPFGM | 524-543 | Chinese | 59 |
| **EBNA1** | PQCR**I**TPLSRLPFGMAPGPG | 529-548 | Chinese | 60 |
| **EBNA1** | PQPGPLRESIVCYFMVFLQT | 549-568 | B95-8 | 64 |
| **EBNA1** | LRESIVCYFMVFLQTHIFAE | 554-573 | B95-8 | 65 |
| **EBNA1** | VCYFMVFLQTHIFAEVLKDA | 559-578 | B95-8 | 66 |
| **EBNA1** | VFLQTHIFAEVLKDAIKDLV | 564-583 | B95-8 | 67 |
| **EBNA1** | HIFAEVLKDAIKDLVMTKPA | 569-588 | B95-8 | 68 |
| **EBNA1** | VLKDAIKDLVMTKPAPTCNI | 574-593 | B95-8 | 69 |

**Table 3.2.3: Mini-panel EBNA1 peptides**

***** In bold is the amino acid substitution that is found in the CKL sequence

** Unique to the Chinese CKL EBV strain


HK Control 263
SFC per million cells
400
350
300
250
200
150
100
50
0
EBNA 1 20-mer peptide panel
48 49 50 51 53 54 55 56 57 58 59 65 66 67 68

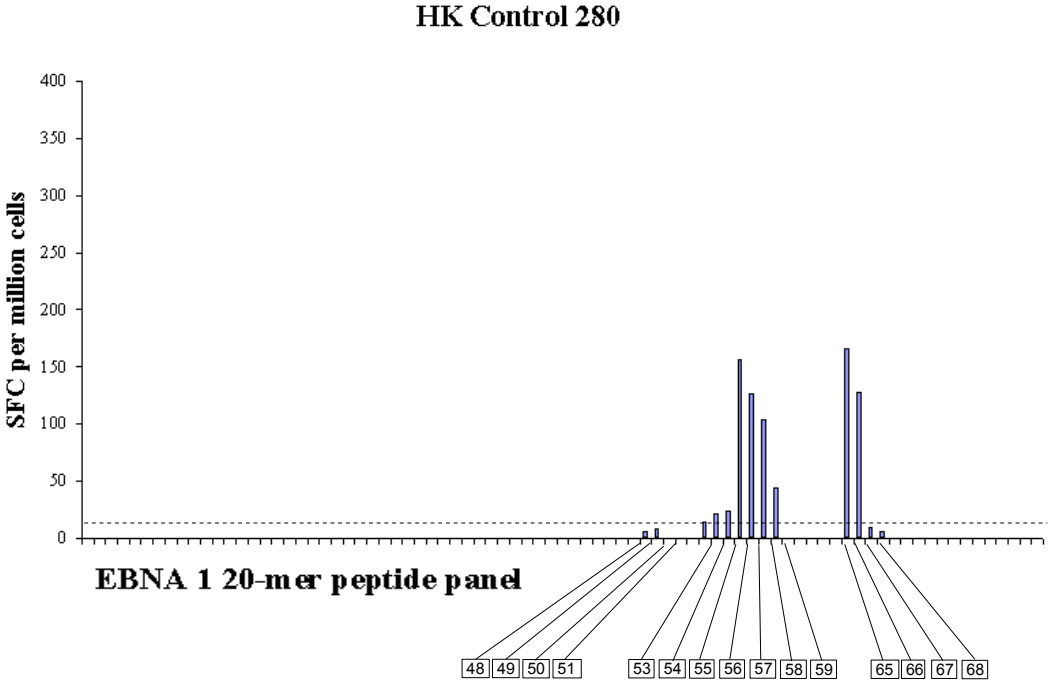
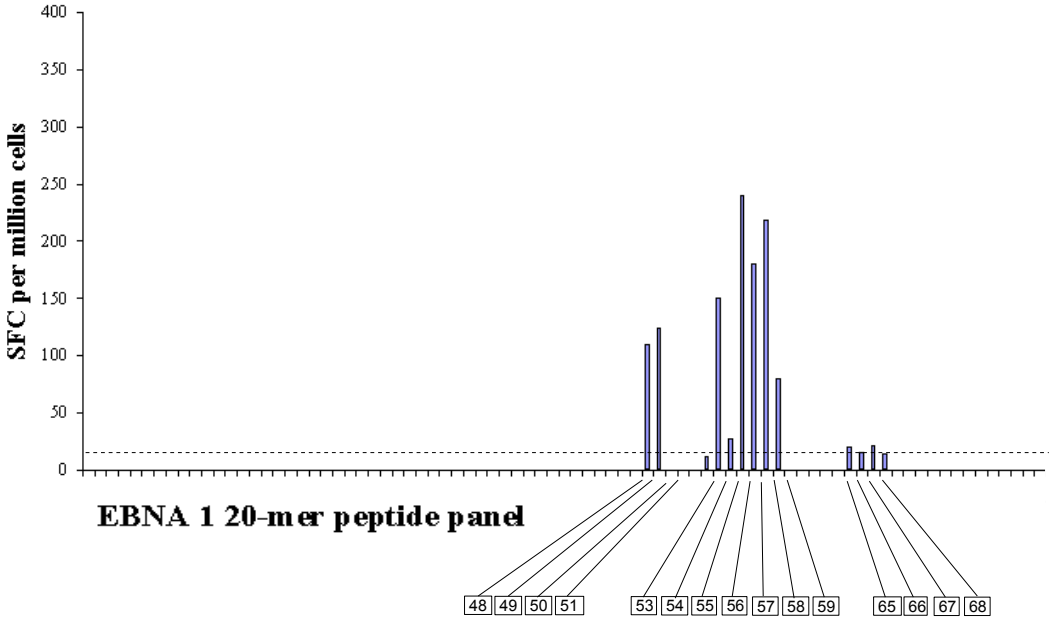

HK Control 280
SFC per million cells
400
350
300
250
200
150
100
50
0
EBNA 1 20-mer peptide panel
48 49 50 51 53 54 55 56 57 58 59 65 66 67 68

**Figure 3.2.5: IFN-γ ELISpot screening of mini-panel EBNA1 peptide**

Result shows two typical IFN-γ ELISpot assays of two Hong Kong donor PBMC samples (HK Control 263 and 280) exposed to 18 individual mini-panel EBNA1 20-mer peptides derived from the CKL EBV strain. The results are expressed as the number of spot forming cells (SFC) per $10^6$ CD8-depleted PBMCs. HK Control 263 (top) show responses to multiple mini-panel EBNA1 20-mer peptides (peptides 55-58 and 66-68) above the back ground reading (dotted line). HK Control 280 (bottom) also shows responses to multiple EBNA1 20-mer peptides (peptides 55-58 and 65-66). Note that the two donors have a common response to peptides 55-58.

## HK Control 285




## HK Control 291

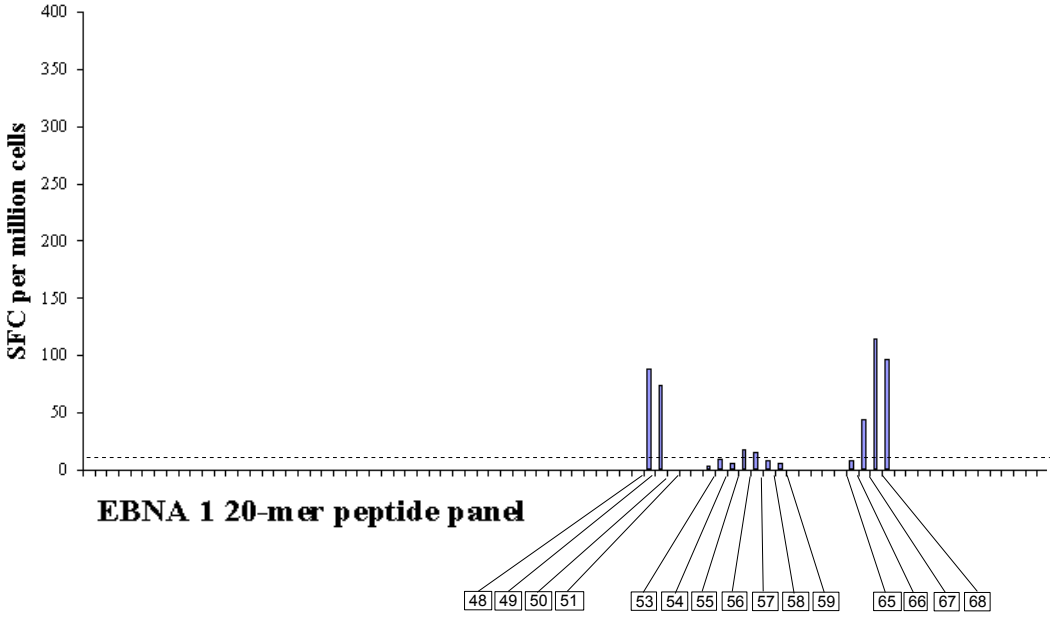


**Figure 3.2.6: IFN-γ ELISpot screening of mini-panel EBNA1 peptide continued**
Result shows two typical IFN-γ ELISpot assays of two Hong Kong donor PBMC samples (HK Control 285 and 291) exposed to 18 individual mini-panel EBNA1 20-mer peptides derived from the CKL EBV strain. The results are expressed as the number of spot forming cells (SFC) per $10^6$ CD8-depleted PBMCs. HK Control 285 (top) show responses to multiple mini-panel EBNA1 20-mer peptides (peptides 48, 49, 53, and 55-58) above the back ground reading (dotted line). HK Control 291 (bottom) also shows responses to multiple EBNA1 20-mer peptides (peptides 48, 49 and 66-68). Note that the two donors have a common response to peptides 48 and 49.

| | | EBNA 1 Peptide Number | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Donor** | **Background** | **1** | **2** | **3** | **14** | **38** | **41** | **42** | **47** | **48** | **49** | **50** | **51** | **52** | **53** | **54** | **55** | **56** | **57** | **58** | **59** | **60** | **62** | **63** | **64** | **65** | **66** | **67** | **68** | **69** | **70** | **72** | **73** |
| **HK Control 252 initial** | 1-10 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | 158 | | n.d | n.d | | 162 | 148 | 88 | 42 | | n.d | n.d | n.d |
| **HK Control 252 retest** | 40 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | 188 | 156 | 266 | 292 | n.d | n.d | 258 | 166 | 370 | 168 | | | n.d | n.d | n.d |
| **HK Control 256 initial** | 4-22 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | | | n.d | n.d | | 96 | 98 | 96 | 46 | | n.d | n.d | n.d |
| **HK Control 256 retest** | 188 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | | | n.d | n.d | 180 | 190 | 164 | 122 | 226 | 150 | n.d | n.d | n.d |
| **HK Control 257 initial** | 1-12 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | *96 | | n.d | n.d | | | | | | 92 | 82 | | n.d | n.d | | | | | | | n.d | n.d | n.d |
| **HK Control 257 retest** | 24 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | **20 | 40 | 60 | 106 | n.d | n.d | | | | | | | | | n.d | n.d | | | | | | | n.d | n.d | n.d |
| **HK Control 262 initial** | 2-12 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | 74 | | n.d | n.d | | 68 | 90 | 68 | 70 | | n.d | n.d | n.d |
| **HK Control 262 retest** | 30 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | 40 | 62 | 70 | 44 | n.d | n.d | 40 | 54 | 74 | 86 | 58 | | n.d | n.d | n.d |
| **HK Control 263 initial** | 32-52 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | 162 | 198 | 278 | 380 | | n.d | n.d | | | 240 | 300 | 298 | | n.d | n.d | n.d |
| **HK Control 263 retest** | 80 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | 122 | 198 | 170 | 282 | | n.d | n.d | | | | | | | n.d | n.d | n.d |
| **HK Control 266 initial** | 16-38 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | 94 | | n.d | n.d | | 86 | 96 | 80 | 68 | | n.d | n.d | n.d |
| **HK Control 266 retest** | 20 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | 46 | 108 | 90 | n.d | n.d | | | | | | | n.d | n.d | n.d |
| **HK Control 268 initial** | 4-14 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | 112 | 100 | | n.d | n.d | | | | | | | | | n.d | n.d | | 70 | | | | | n.d | n.d | n.d |
| **HK Control 268 retest** | 114 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | | | n.d | n.d | 130 | 106 | 126 | 102 | | | n.d | n.d | n.d |
| **HK Control 276 initial** | 2-12 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | 26 | | n.d | n.d | | 124 | 144 | 96 | | | n.d | n.d | n.d |
| **HK Control 276 retest** | 18 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | | | n.d | n.d | 42 | 80 | 94 | 80 | 40 | | n.d | n.d | n.d |
| **HK Control 280 initial** | 6-24 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | 156 | 126 | 104 | 44 | | n.d | n.d | | 166 | 128 | | | | n.d | n.d | n.d |
| **HK Control 280 retest** | 58 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | 48 | 104 | 124 | 54 | 38 | 58 | n.d | n.d | 40 | 72 | 122 | 48 | 56 | | n.d | n.d | n.d |
| **HK Control 282 initial** | 2-10 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | | | n.d | n.d | | 58 | 50 | | | | n.d | n.d | n.d |
| **HK Control 282 retest** | 150 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | | | | n.d | n.d | | | | | | | | | n.d | n.d | 72 | 72 | 88 | 202 | 224 | | n.d | n.d | n.d |
| **HK Control 285 initial** | 12-28 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | | 110 | 124 | | n.d | n.d | | 150 | | 240 | 180 | 218 | 80 | | n.d | n.d | | | | | | | n.d | n.d | n.d |
| **HK Control 285 retest** | 40 | n.d | n.d | n.d | n.d | n.d | n.d | n.d | 60 | 114 | 124 | 120 | n.d | n.d | 108 | 108 | 28 | 136 | 132 | 76 | 62 | 50 | n.d | n.d | | | | | | | n.d | n.d | n.d |

**Table 3.2.4: SUMMARY OF EBNA1 PEPTIDE RE-TEST FROM SECOND COHORT STUDY**

n.d - not determined
* highlighted yellow is the peptide response that needs retesting
** neighboring peptide that were included in the rest along side the retest peptide

| | | EBNA 1 Peptide Number | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Donor** | **Background** | **2** | **14** | **38** | **41** | **42** | **48** | **49** | **50** | **51** | **54** | **55** | **56** | **57** | **58** | **59** | **60** | **63** | **64** | **65** | **66** | **67** | **68** | **69** | **72** | **73** |
| **HK Control 251** | 4±2 | n.d | n.d | n.d | n.d | n.d | *116 | 118 | n.d | n.d | | | | | 40 | 30 | n.d | n.d | n.d | | 38 | 130 | 74 | n.d | n.d | n.d |
| **HK Control 252** | 3±1 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | 158 | n.d | n.d | n.d | 162 | 148 | 88 | 42 | n.d | n.d | n.d |
| **HK Control 253** | 4±2 | n.d | n.d | n.d | n.d | n.d | 56 | 48 | n.d | n.d | | | | | | | n.d | n.d | n.d | 64 | 24 | 56 | 50 | n.d | n.d | n.d |
| **HK Control 254** | 4±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 255** | 3±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 256** | 6±3 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | 96 | 98 | 96 | 46 | n.d | n.d | n.d |
| **HK Control 257** | 4±3 | n.d | n.d | n.d | n.d | n.d | | 96 | n.d | n.d | | | | | 92 | 82 | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 258** | 7±3 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | 136 | 196 | 172 | n.d | n.d | n.d |
| **HK Control 259** | 3±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 260** | 2±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | 20 | n.d | n.d | n.d | 128 | 158 | 138 | 66 | n.d | n.d | n.d |
| **HK Control 261** | 5±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 262** | 4±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | 74 | n.d | n.d | n.d | 68 | 90 | 68 | 70 | n.d | n.d | n.d |
| **HK Control 263** | 22±4 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | 162 | 198 | 278 | 380 | n.d | n.d | n.d | | 240 | 300 | 298 | n.d | n.d | n.d |
| **HK Control 264** | 12±3 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 266** | 9±3 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | 94 | n.d | n.d | n.d | 86 | 96 | 80 | 68 | n.d | n.d | n.d |
| **HK Control 267** | 8±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | 86 | 114 | 80 | n.d | n.d | n.d |
| **HK Control 268** | 4±1 | n.d | n.d | n.d | n.d | n.d | 112 | 100 | n.d | n.d | | | | | | | n.d | n.d | n.d | 70 | | | | n.d | n.d | n.d |
| **HK Control 269** | 4±1 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 276** | 3±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | 26 | n.d | n.d | n.d | 124 | 144 | 96 | | n.d | n.d | n.d |
| **HK Control 277** | 4±2 | n.d | n.d | n.d | n.d | n.d | 42 | 82 | n.d | n.d | | | | | | 40 | n.d | n.d | n.d | 78 | 302 | 352 | 320 | n.d | n.d | n.d |
| **HK Control 278** | 3±2 | n.d | n.d | n.d | n.d | n.d | 74 | 58 | n.d | n.d | | | | | | | n.d | n.d | n.d | | 40 | 90 | 68 | n.d | n.d | n.d |
| **HK Control 279** | 8±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | 86 | 90 | 116 | 86 | n.d | n.d | n.d |
| **HK Control 280** | 6±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | 156 | 126 | 104 | 44 | n.d | n.d | n.d | 166 | 128 | | | n.d | n.d | n.d |
| **HK Control 281** | 4±1 | n.d | n.d | n.d | n.d | n.d | 64 | 50 | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 282** | 4±1 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | 58 | 50 | | | n.d | n.d | n.d |
| **HK Control 283** | 5±3 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | 40 | 60 | 40 | n.d | n.d | n.d |
| **HK Control 284** | 4±2 | n.d | n.d | n.d | n.d | n.d | 80 | 54 | n.d | n.d | | | | | | | n.d | n.d | n.d | 38 | 52 | 32 | 32 | n.d | n.d | n.d |
| **HK Control 285** | 9±3 | n.d | n.d | n.d | n.d | n.d | 110 | 124 | n.d | n.d | 150 | | 240 | 180 | 218 | 80 | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 286** | 14±3 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | 122 | 106 | 88 | | n.d | n.d | n.d | | | 86 | 60 | n.d | n.d | n.d |
| **HK Control 287** | 9±1 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | 68 | 104 | 96 | n.d | n.d | n.d |
| **HK Control 288** | 8±3 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | 74 | 124 | 108 | n.d | n.d | n.d |
| **HK Control 289** | 3±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | 46 | 32 | | n.d | n.d | n.d |
| **HK Control 290** | 2±1 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 291** | 6±2 | n.d | n.d | n.d | n.d | n.d | 88 | 74 | n.d | n.d | | | | | | | n.d | n.d | n.d | | 44 | 114 | 96 | n.d | n.d | n.d |
| **HK Control 293** | 7±1 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |

**Table 3.2.5: SUMMARY OF MIN-PANEL EBNA 1 PEPTIDE SCREENING**

n.d - not determined

***** results are shown as spot forming cells (SFC) per million cells

| | | EBNA 1 Peptide Number | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Donor** | **Background** | **2** | **14** | **38** | **41** | **42** | **48** | **49** | **50** | **51** | **54** | **55** | **56** | **57** | **58** | **59** | **60** | **63** | **64** | **65** | **66** | **67** | **68** | **69** | **72** | **73** |
| ***HK Control 201** | 4±2 | | 68 | 152 | (+)130 | 198 | | | 198 | 168 | 188 | 114 | 244 | 276 | 212 | 98 | 62 | | 134 | | 92 | 82 | | | | |
| **HK Control 202** | 2±1 | | | | | | | | | | | 34 | 52 | | | | | | | 34 | 40 | 34 | | | | |
| **HK Control 203** | 16±2 | | | | | | | | | | | | | | | 80 | | | | 70 | 64 | 82 | 66 | | | |
| **HK Control 204** | 9±2 | | | | | | 44 | 30 | | | | | | | | | | | | | | 34 | 42 | | | |
| ****HK Control 205** | 1±1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 206** | 4±1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 207** | 2±1 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 208** | 10±3 | | | | | | | | | | | | | | | | | | | | | 44 | 58 | | | |
| **HK Control 209** | 4±2 | | | | | | | | | | | | | | | | | | | | 30 | 48 | 26 | | | |
| **HK Control 210** | 27±3 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 211** | 4±2 | | | | | | | | | | | | | | | | | | | | 40 | 72 | 56 | 28 | | |
| **HK Control 212** | 5±2 | | | | | | | | | | 40 | 30 | | | | | | | | | | | 44 | | | |
| **HK Control 213** | 7±3 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 215** | 5±2 | | | | | | 64 | 60 | | | | | | | | | | | | | 182 | 242 | 236 | | 54 | 86 |
| **HK Control 218** | 33±4 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 219** | 6±4 | | | | | | 112 | 130 | | | | | | | | | | | | | | 60 | 42 | | | |
| **HK Control 220** | 13±5 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 221** | 4±2 | 124 | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 222** | 7±3 | | | | | | | | | | | 50 | 52 | 36 | | | | 34 | 36 | 82 | 90 | 42 | | | | |
| **HK Control 223** | 6±2 | | | | | | | | | | | | | | | | | | | | 44 | 46 | 34 | | | |
| **HK Control 224** | 7±2 | | | | | | 104 | 96 | | | | | | 40 | 46 | 50 | | | | | | 44 | 46 | | | |
| **HK Control 225** | 33±9 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 226** | 13±6 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 227** | 18±4 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 228** | 23±7 | | | | | 138 | | | | 158 | 104 | 108 | 168 | 160 | 134 | | | | | | 120 | 172 | 108 | | | |
| **HK Control 229** | 10±2 | | | | | | | | | | | | | | | | | | | | 48 | | | | | |
| **HK Control 230** | 1±1 | | | | | | | | 56 | | | | 32 | 52 | | | | | | 42 | 64 | 92 | 74 | | | |
| **HK Control 231** | 28±6 | | | | | | | | | | | | | | | | | | | | | 100 | 108 | | | |
| **HK Control 232** | 28±6 | | | | | | | | | | | | | | | | | | | | 120 | 136 | | | | |
| **HK Control 233** | 16±11 | | | | | | 84 | 144 | | | 100 | 92 | 86 | 120 | 108 | 88 | | | | | | | | | | |
| **HK Control 234** | 22±5 | | | | | | | | | | | 120 | | | | | | | | | 96 | 112 | | | | |
| **HK Control 236** | 10±3 | | | | | | | | | | | | | | | | | | | | | 62 | | | | |
| **HK Control 237** | 17±3 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 240** | 13±4 | | | | | | | | | | | | 48 | | 56 | | | | | | | 76 | 62 | | | |
| **HK Control 241** | 24±4 | | | | | | | | | | | | | 300 | 132 | 100 | | | | | | 108 | 90 | | | |
| **HK Control 242** | 16±5 | | | | | | 58 | 52 | | | | | | 46 | 46 | | | | | | | | | | | |
| **HK Control 243** | 31±4 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 244** | 44±10 | | | | | | | | 120 | 122 | | | | | | 260 | | | | | 162 | | | | | |
| **HK Control 245** | 35±9 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 247** | 1±2 | | | | | | 110 | 124 | 20 | | | | | | | 62 | | | | | | | | | | |
| **HK Control 248** | 4±4 | | | | | | | | | | | | 98 | 70 | 38 | | | | | | 32 | 38 | | | | |
| **HK Control 249** | 7±4 | | | | | | | | | | | | | | | | | | | | 34 | 106 | 90 | 38 | | |
| **HK Control 250** | 2±2 | | | | | | | | | | | | | | | | | | | | | | | | | |
| **HK Control 251** | 4±2 | n.d | n.d | n.d | n.d | n.d | 116 | 118 | n.d | n.d | | | | | 40 | 30 | n.d | n.d | n.d | | 38 | 130 | 74 | n.d | n.d | n.d |
| **HK Control 252** | 3±1 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | 158 | n.d | n.d | n.d | 162 | 148 | 88 | 42 | n.d | n.d | n.d |
| **HK Control 253** | 4±2 | n.d | n.d | n.d | n.d | n.d | 56 | 48 | n.d | n.d | | | | | | | n.d | n.d | n.d | 64 | 24 | 56 | 50 | n.d | n.d | n.d |
| **HK Control 254** | 4±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 255** | 3±2 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | | | | | n.d | n.d | n.d |
| **HK Control 256** | 6±3 | n.d | n.d | n.d | n.d | n.d | | | n.d | n.d | | | | | | | n.d | n.d | n.d | 96 | 98 | 96 | 46 | n.d | n.d | n.d |

optimal epitope peptide may be fully contained within just one of the 20-mer sequences. We looked for examples of such adjacent peptide pairs or triplets. Instances where responses were found to more than three adjacent peptides were deemed to reflect the existence of more than one epitope in this region, since full 20-mer peptides n and n+3 could only share 5 amino acids in common and would be very unlikely to detect the same epitope response. Based on these criteria, we provisionally identified the following epitope regions; peptides 41/42 are seen by two responders to one or both peptides (HK control 201 and 228); peptides 48/49 are seen by multiple donors all but one of whom responded to both peptides; peptides 50/51 are seen by four donors responding to one or both peptides; peptides 54/55 are seen by two donors responding to one or both peptides (HK control 201 and 285); peptides 56/57 are seen by multiple donors responding to one or both peptides at levels higher than seen against adjacent peptides 55 and 58; peptides 58/59 are seen by multiple donors responding to one or both peptides at levels higher than seen against adjacent peptides 57 and 60; peptide 64 is seen by a single donor HK control 201; peptides 65-67 are seen by multiple donors responding to one, two, or three peptides at levels higher than seen against peptides 64 and 68; peptides 66-68 are seen by multiple donors responding to one, two, or three peptides at levels higher than seen against adjacent peptides 65 and 69; and peptides 72/73 are seen by a single donor HK control 215.

Based on this interpretation, Table 3.2.7 identifies the ten provisional epitope regions with their coordinates, the primary EBNA 1 sequence and with adjacent 20-mer peptides aligned so as to display their 15-mer sequence of overlap. The donors who responded to these epitope regions are summarised in Table 3.2.8. From Tables 3.2.6 and 3.2.7, the frequency of response and response sizes was calculated to yield Table 3.2.9 A, B and C respectively. Table 3.2.9A shows the number of donors tested and the number who responded to any of the EBNA1 peptides in the two cohorts. Overall, the screening indicated that almost 70% donors screened have detectable $CD4^+$ T cell memory to one or more EBNA1 peptide. The actual frequency of positive responders may have been even higher if the mini-panel cohort of donors had been screened on the full panel of EBNA1 peptides.

| Peptide no. | Coordinate | Chinese epitope sequence |
|---|---|---|
| 41 | 434-453 | IEQGP**T**DDPGEGPSTGPRGQ |
| 42 | 439-458 | **T**DDPGEGPSTGPRGQGDGGR |
| 48 | 469-488 | RGQGGSNPKFENIAEGLR**V**L |
| 49 | 474-493 | SNPKFENIAEGLR**V**LLARSH |
| 50 | 479-498 | ENIAEGLR**V**LLARSHVERTT |
| 51 | 484-503 | GLR**V**LLARSHVERTT**E**EG**N**W |
| 54 | 499-518 | **E**EG**N**WVAGVFVYGGSKTSLY |
| 55 | 504-523 | VAGVFVYGGSKTSLYNLRRG |
| 56 | 509-528 | VYGGSKTSLYNLRRG**I**ALA**V** |
| 57 | 514-533 | KTSLYNLRRG**I**ALA**V**PQCR**I** |
| 58 | 519-538 | NLRRG**I**ALA**V**PQCR**I**TPLSR |
| 59 | 524-543 | **I**ALA**V**PQCR**I**TPLSRLPFGM |
| 64 | 549-568 | PQPGPLRESIVCYFMVFLQT |
| 65 | 554-573 | LRESIVCYFMVFLQTHIFAE |
| 66 | 559-578 | VCYFMVFLQTHIFAEVLKDA |
| 67 | 564-583 | VFLQTHIFAEVLKDAIKDLV |
| 68 | 569-588 | HIFAEVLKDAIKDLVMTKPA |
| 72 | 589-608 | PTCNI**K**VTVCSFDDGVDLPP |
| 73 | 594-613 | **K**VTVCSFDDGVDLPPWFPPM |

**Table 3.2.7: 10 provisional epitope regions**

| Responders (HK Control) | Epitope regions | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 41/42 | 48/49 | 50/51 | 54/55 | 56/57 | 58/59 | 64 | 65/66 | 67/68 | 72/73 |
| 201 | √ | | √ | √ | √ | √ | √ | √ | | |
| 202 | | | | | | | | √ | | |
| 203 | | | | | | √ | | √ | √ | |
| 204 | | √ | | | | | | | √ | |
| 208 | | | | | | | | | √ | |
| 209 | | | | | | | | | √ | |
| 211 | | | | | | | | | √ | |
| 215 | | √ | | | | | | | √ | √ |
| 219 | | √ | | | | | | | √ | |
| 222 | | | | | | | | √ | | |
| 223 | | | | | | | | | √ | |
| 224 | | √ | | | | √ | | | √ | |
| 228 | √ | | √ | √ | √ | | | | √ | |
| 230 | | | √ | | √ | | | | √ | |
| 231 | | | | | | | | | √ | |
| 233 | | √ | | √ | √ | √ | | | | |
| 234 | | | | | | | | √ | | |
| 236 | | | | | | | | | √ | |
| 240 | | | | | √ | | | | √ | |
| 241 | | | | | √ | | | | √ | |
| 242 | | √ | | | | | | | | |
| 244 | | | √ | | | √ | | √ | | |
| 247 | | √ | | | | √ | | | | |
| 248 | | | | | √ | | | | √ | |
| 249 | | | | | | | | | √ | |
| 251 | | √ | | | | √ | | | √ | |
| 252 | | | | | | √ | | √ | | |
| 253 | | √ | | | | | | √ | √ | |
| 257 | | | | | | √ | | | | |
| 258 | | | | | | | | | √ | |
| 260 | | | | | | √ | | √ | √ | |
| 262 | | | | | | √ | | √ | √ | |
| 263 | | | | | √ | √ | | | √ | |
| 266 | | | | | | √ | | √ | √ | |
| 267 | | | | | | | | | √ | |
| 268 | | √ | | | | | | | | |
| 276 | | | | | | √ | | √ | | |
| 277 | | √ | | | | √ | | | √ | |
| 278 | | √ | | | | | | | √ | |
| 279 | | | | | | | | | √ | |
| 280 | | | | | √ | | | √ | | |
| 281 | | √ | | | | | | | | |
| 283 | | | | | | | | | √ | |
| 284 | | √ | | | | | | √ | √ | |
| 285 | | √ | | √ | √ | √ | | | | |
| 286 | | | | | √ | | | | √ | |
| 287 | | | | | | | | | √ | |
| 288 | | | | | | | | | √ | |
| 291 | | √ | | | | | | | √ | |
| Responders | 2 | 16 | 4 | 4 | 11 | 16 | 1 | 14 | 34 | 1 |
| Total Tested | 43 | 78 | 43 | 78 | 78 | 78 | 43 | 78 | 78 | 43 |
| **Frequency (%)** | 5 | 21 | 9 | 5 | 14 | 21 | 2 | 18 | 44 | 2 |

**Table 3.2.8: Summary of epitope region analysis**

√ - there is a response to this epitope region

# The frequency of response to the 10 EBNA1 epitope regions

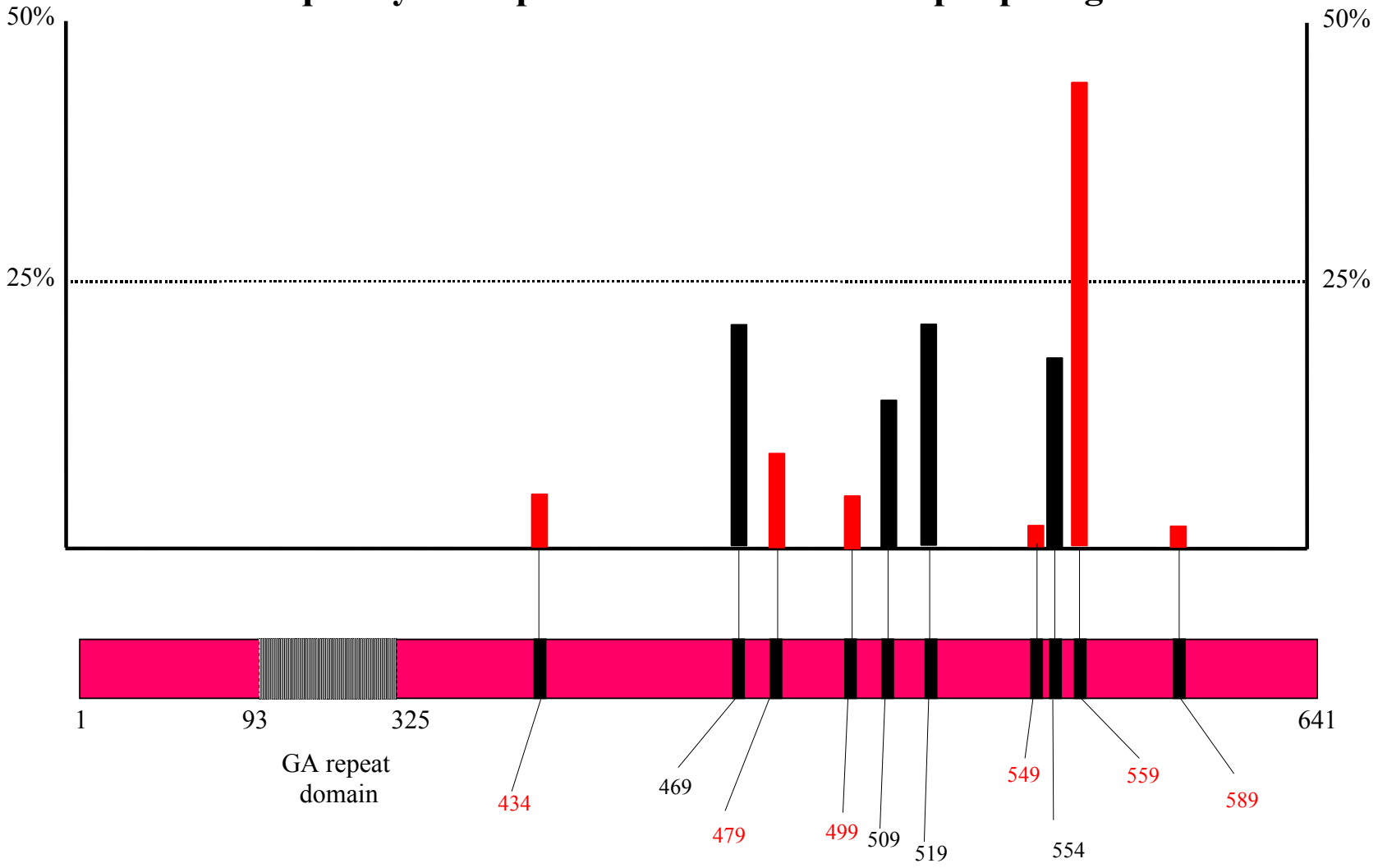


**Figure 3.2.7: The frequency of response to the 10 EBNA1 epitope regions**

The Figure shows the location of the ten epitope regions on the EBNA 1 molecule and, for each region, indicates the frequency of positive responders in the form of a histogram. The red histogram represent that the responses are novel epitopes (unique to the Chinese study), and black histogram represent that the responses found in this study and the Caucasian study. Looking at the figure immediately shows that the epitope regions 48/49 (469), 56/57(509), 58/59 (519), and 65-67 (554) were each recognised by 15-20% of individuals whereas epitope 66-68 (559) was recognised by a remarkable 44% of all individuals.

| | Cohort 1 | Cohort 2 | Total |
|---|---|---|---|
| **No. of donors tested** | 43 | 35 | 78 |
| **No. of responders** | 25 | 24 | 49 |
| **Frequency of response (%)** | 58 | 69 | 63 |

**Table 3.2.9A: Frequency of responses in the two studied cohort**

| peptide number | 41/42 | 48/49 | 50/51 | 54/55 | 56/57 | 58/59 | 64 | 65-67 | 66-68 | 72/73 |
|---|---|---|---|---|---|---|---|---|---|---|
| **No. of responders** | 2 | 16 | 4 | 4 | 11 | 16 | 1 | 14 | 34 | 1 |
| **No. donors tested** | 43 | 78 | 43 | 78 | 78 | 78 | 43 | 78 | 78 | 43 |
| **Frequency of response (%)** | 5 | 21 | 9 | 5 | 14 | 21 | 2 | 18 | 44 | 2 |

**Table 3.2.9B: Frequency of responses to the individual epitope regions**

| peptide number | 41/42 | 48/49 | 50/51 | 54/55 | 56/57 | 58/59 | 64 | 65-67 | 66-68 | 72/73 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Range of response** | 138-198 | 44-144 | 56-198 | 40-120 | 46-300 | 20-380 | 134 | 40-166 | 38-352 | 86 |
| **mean of response** | 168 | 92 | 134 | 87 | 133 | 110 | 134 | 96 | 111 | 86 |

**Table 3.2.9C: The response size to the individual epitope regions**

Figure 3.2.7 shows the location of these ten epitope regions on the EBNA 1 molecule and, for each region, indicates the frequency of positive responders. Using the values from summary Table 3.2.9B, while some epitope regions only showed occasional responses, it was clear that other regions were frequently recognised. The epitopes 48/49 (469), 56/57 (509), 58/59 (519), and 65-67 (554) were each recognised by 15-20% of individuals whereas epitope 66-68 (559) was recognised by a remarkable 44% of all individuals. Figure 3.2.7 also emphasises the concentration of the epitope regions within the C-terminal half of EBNA 1.

Table 3.2.9C shows the mean size and overall range of the response observed and all individual responses for each epitope are plotted in Figure 3.2.8 above the map of the EBNA 1 molecule showing epitope location. The average mean response size ranges from 86-168 SFC per 10^6 cells however the individual response size range from 20-380 SFC per 10^6 cells. Looking at Figure 3.2.8, the majority of the SFC per 10^6 cells lies within a range of approximately 50-150 SFC per 10^6 cells with occasionally very weak and very strong responses also observed. There is no one epitope that consistently elicits very strong or very weak responses as measured in the ELISpot assay.

### 3.2.5 Discussion

#### 3.2.5.1 Limitations of ELISPOT screening

ELISPOT screening for epitope detection is an imprecise science and there are a few areas of concern when interpreting ELISPOT data. One concern is the use of a high peptide concentration does not represent antigen expression under physiological condition and may explain why CD4+ T cell responses are detected to adjacent peptides, and precise epitope mapping is made difficult. Arguably this reduces the chances of missing weak epitope-specific responses but, because adjacent peptides share significant overlapping sequences, the assay can only identify ‘epitope regions’ rather than precise epitope location. Where responses to 4-5 adjacent peptides are seen (such as seen in responses to peptides 54-60 in

# The response size to the 10 EBNA1 epitope regions

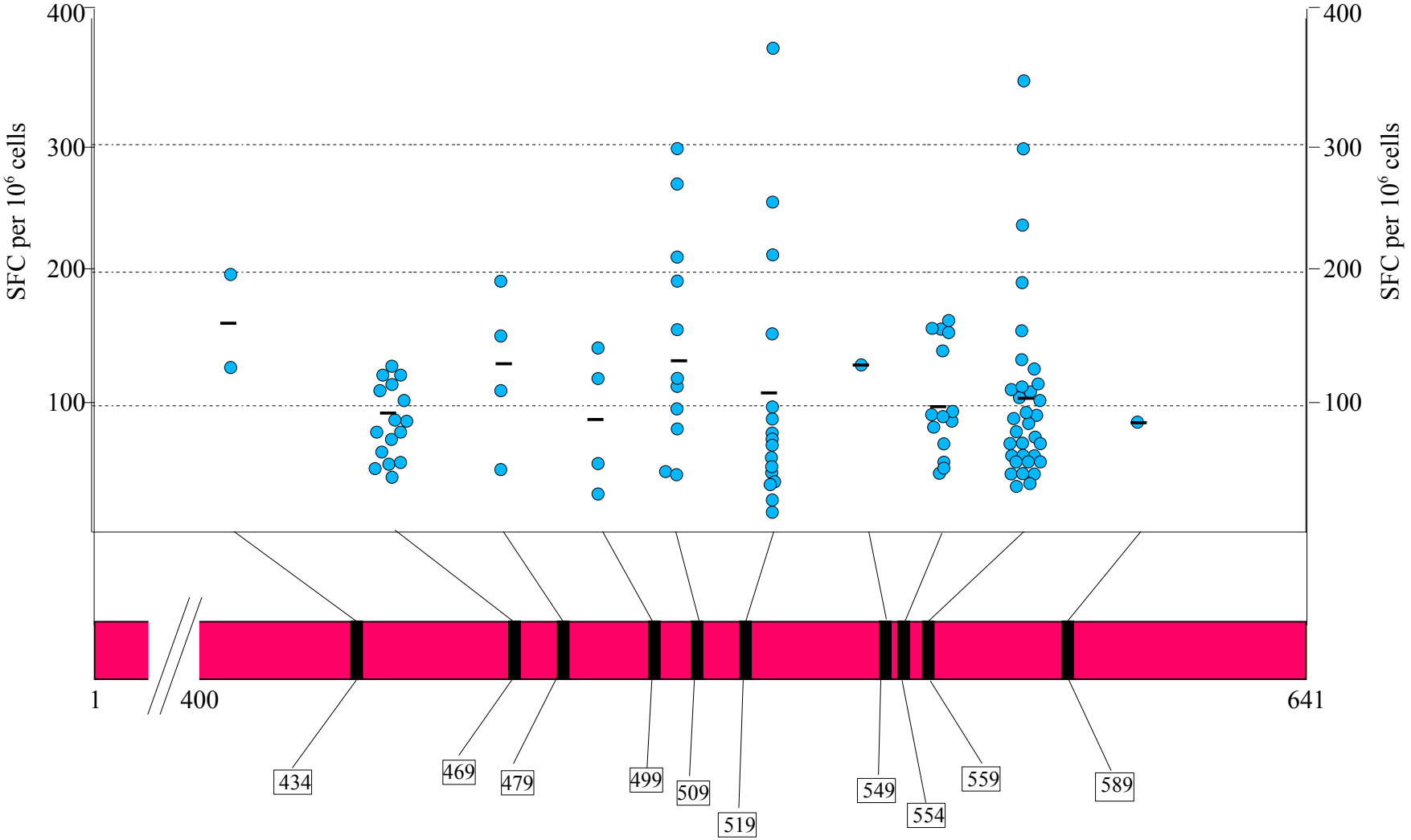


**Figure 3.2.8: The response size to the 10 EBNA1 epitope regions**

The Figure shows the location of the ten epitope regions on the EBNA 1 molecule and, for each region, the individual size of the response are represented by a blue dot. The scales located on both sides of the Figure are expressed as spot forming cells (SFC) per million cells. The black line represents the mean response size for that particular epitope region. Note that the range of response size may vary from as few as 20 SFC per million cells to as high as 380 SFC per million cells. However, the mean response size does not vary as much (86-168 SFC per million cells).

HK control 201), it raises a question as to how many epitopes are represented in this region of response? Defining 'epitope regions'this way is more likely to underestimate the true number of independent epitopes because an epitope restricted through an HLA II molecule is limited to a 15-mer peptide whereas 4-5 adjacent peptides covers 70-80 amino acids. Therefore, comparisons of response patterns in large numbers of different donors can give a clearer picture by identifying individuals that respond only to 'one end' of 4-5 peptide region and others responding to the other end. That is why we screened 79 donors in the present study. Hudgens *et al* (2004) recently introduced an ELISpot assay protocol allowing statistical analysis of results in order to establish 'unified' criteria for identifying a positive and a negative result. The aim is to allow the collation of results between laboratories, studies and populations. One of the most important requirements is to perform experiments with multiple repeats so that a statistical analysis could be performed. For example to study responses against one peptide, the experiment would need to be done in triplicate wells and repeated several times on different days. In practice, however, this is not feasible in the context of large scale screening on peptide panels, not least because of the volumes of blood required from each donor and the manpower required to screen a large range of individuals. Our approach was a compromise solution, using buffy coat samples that allowed an initial screen on the whole peptide panel and repeat assays to validate potential positive responses.

At the time when this work was initiated, the NK cell depletion assay (as described in the previous chapter) was not set up in Hong Kong. As a result, some donors were totally non-informative because of high background in the ELISpot assay and were omitted from the compilation of the results. We established a criteria in which a positive response is defined as +3SD over the mean background value. However, using these criteria underestimates the true frequency of responses since weak responses become undetectable especially when background values are high.

These assays used an ELISpot counter to read the spots on the ELISpot plates. This machine works by making a scan of the ELISpot plate and is assisted by software to

analyse and count the spots. Spots are counted according to criteria set by the user, for example the minimum size and intensity that defines a spot. The problem with counting the ELISpot using a machine is that dust particles can form spots that may match the user criteria and get counted as a spot; conversely true spots which do not match the criteria are missed. Therefore ELISpots that were counted by machine were routinely double-checked by eye to ensure the validity of results.

Even using our protocol rather than that by Hudgens *et al* (2004), the ELISpot assay has a high demand on cell numbers. Depleting the $CD8^+$ population from PBMC itself loses around 50% of the T-cells (those include around 25% due to loss of the $CD8^+$ population and further 25% non-specific losses in washing). However, $CD8^+$ depletion was deemed a necessary step. There must also be a balance between using too many cells (i.e. risking high background) and using too few cells (i.e. risking missing out responses) in the assay. The most appropriate cell numbers to use in the assay is between 200,000-500,000 cells/assay wells. Ideally, using 500,000 cells/assay wells is just enough cells to coat the bottom of the wells which reduces chance of missing out weak responses while preventing cells from overlapping each other. This kind of approach is suitable for initial screening on large peptide panels using buffy coats but not for screening on small patient samples. However, the matrix approach (Lauer *et al*, 2004) could solve the problem with large peptide panel screening using small patient samples. The matrix approach uses a system where peptide pools (containing no more than 10 peptides) are prepared in a matrix array such that each individual peptide could be found in two pools. A positive response in two peptide pools containing the same peptide would result in a true response against that peptide. This approach would use fewer cell numbers since the screening is done in peptide pools and it requires a response to be seen in two separate pools; therefore the chances of false response are rare. However there may be difficulties in screening the EBNA1 peptide panel in this way, since often a donor will respond to multiple epitopes and this opens up the possibility of competition between peptides in a large peptide pool.

### 3.2.5.2 EBNA1 responses in Chinese versus Caucasian

Leen *et al* (2001) described ELISpot screening on peptide pools using two peptide panels; 81 20-mer B95.8 EBNA1 peptides and 22 20-mer Q/T variant peptides. In total, 26 seropositive donors were screened against the B95.8 EBNA1 peptide panel of which 19 donors responded against a total of 15 epitope regions. These 7 non-responsive donors were subjected to screening using the 22 Q/T variant peptides in 3/7 responded to one or more of 3 Q/T-specific epitope regions; EBNA1 424-443, 514-533 and 589-608. More interestingly, the original 19 B95.8 EBNA1 peptide responders were also rescreened using the 22 Q/T variant peptides and in every case they showed cross-reactivity with the relevant epitope variant. It seems that, under the conditions of the ELISpot assay, differences of one or two amino acids in epitope sequences do not affect responsiveness. There were no additional responses that were specifically directed at the Q/T variant peptides. More recently (unpublished work) this laboratory has found two new B95.8 epitopes (EBNA1 56* and 379*) in a study of ELISpot responses in 10 more Caucasian donors; 1 in 10 donors screened responded to these two new epitopes.

In the present study, 43 donors were subjected to a complete screening and 35 donors to mini-panel screening using the CKL Chinese prototype virus peptide sequence. This detected responses in 53/78 donors and located 10 epitopes. The question is whether focusing on this one allelic sequence of EBNA 1 was justified. In this context, Midgley *et al* (2000) found that the non-CKL EBNA 1 sequences are found in only a very small percentage of Chinese virus strains. Interestingly, Midgley *et al* (2000) identified, by comparing DNA sequences of EBNA2 and the EBNA3s, two major viral strains designated the Li and Wu family isolates. In addition, Wu/Li recombinant viruses were identified such that the viruses show Wu-like EBNA2 and EBNA3A, recombinant EBNA3B and Li-like EBNA3C. In the context of EBNA1, all the Li family isolates follow the V-allele consensus (as described by Gutierrez *et al*., 1997) whereas, 3/7 Wu family virus isolates show a single codon change at position 585 from a Thr->Ile. Therefore, studying the CKL EBNA 1 sequence as representative of most Chinese strains is justified.

It is significant that in the present study of Chinese individuals, fewer EBNA1 epitope regions were found than in the earlier work on Caucasians, yet more donors were screened. It is of course possible that we have underestimated the true number of epitope regions using our current method because the epitopes lay close to or overlap each other, especially in the region EBNA1 494-548. This clustering of responses to the C-terminal half of the protein is also observed in the Caucasian study, thus it is likely that some of these epitope regions contain more than one epitope. Likewise we may be missing weak responses due to masking on to high backgrounds. However, there is no reason to believe that this is a problem unique to the present study. Likewise we may be missing rare responses, perhaps restricted through rare HLA II alleles in the Chinese population but, since we screened many more donors than Leen *et al* (2001), there is again no reason to believe that this is a problem that has affected our study only.

Figure 3.2.9 compares the epitope regions identified in this study and by Leen *et al* (2001) and re-emphasises the concentration of responses in the C-terminal half of the EBNA1-protein in both studies. This may reflect the possibility that the C-terminal half of the EBNA1 protein is highly immunogenic. However, further studies are required to determine the actual number of epitope in this region. There is clearly no global effect of gly-ala repeat domain on EBNA1 processing via the MHC class II pathway since there are so many epitopes in the C-terminal region, but could this suggest that the gly-ala repeat domain is inhibiting the processing of the N-terminal half of the protein from presentation to $CD4^+$ T cells? Significantly, the recent work on Caucasians has detected $CD4^+$ T cell responses to two epitopes located in the N-terminal half of the protein. The prediction is that some Chinese donors will also see N-terminal epitopes, although clearly if they do exist, such responses are quite rare.

As a corollary to the reduced number of epitopes detected from the study of Chinese donors, there were a correspondingly larger proportion of Chinese donors responding to particular regions. This is shown in Figure 3.2.10 comparing the two sets of results. The frequency is expressed as a percentage of the total number of responding donors tested

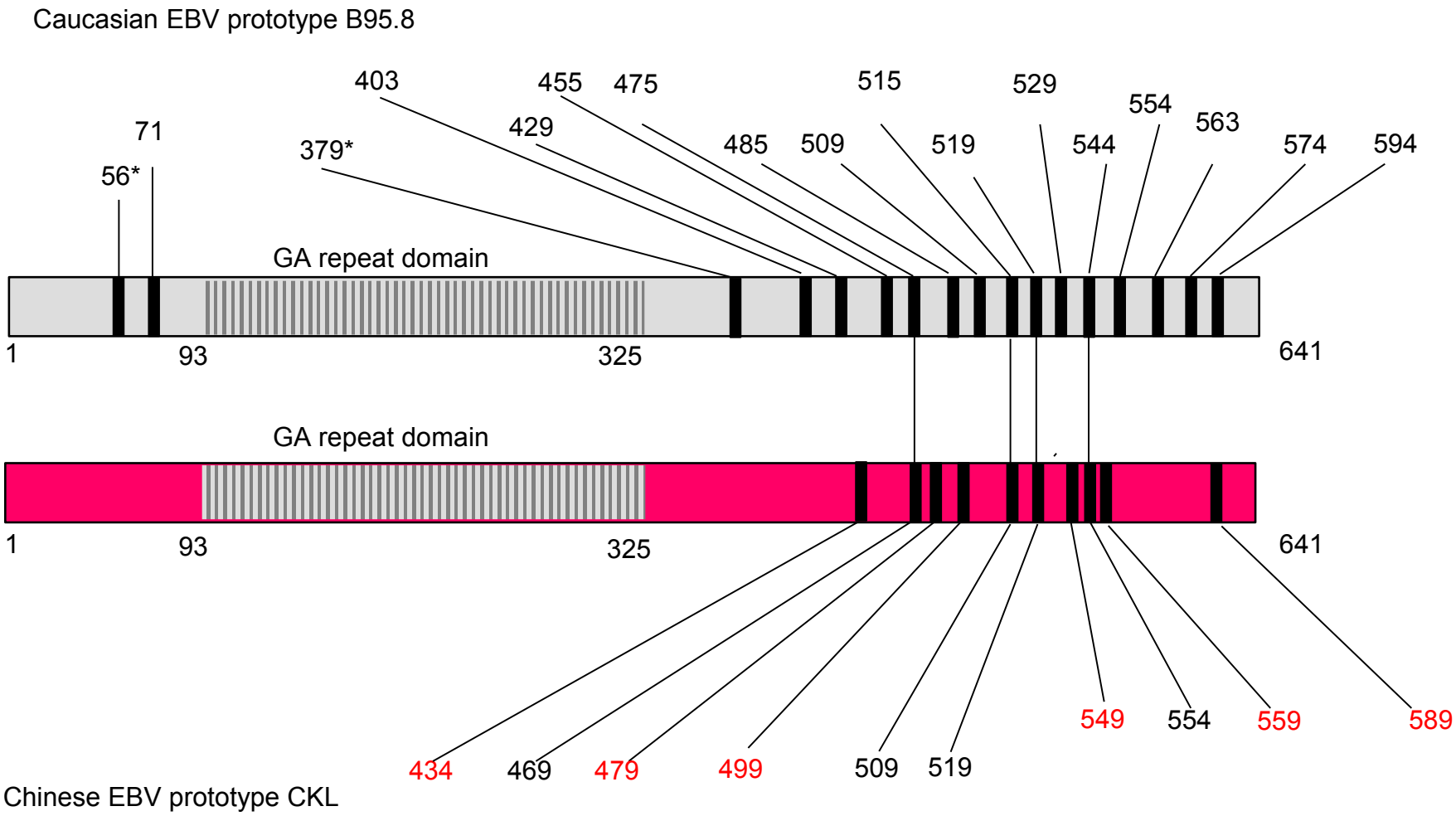
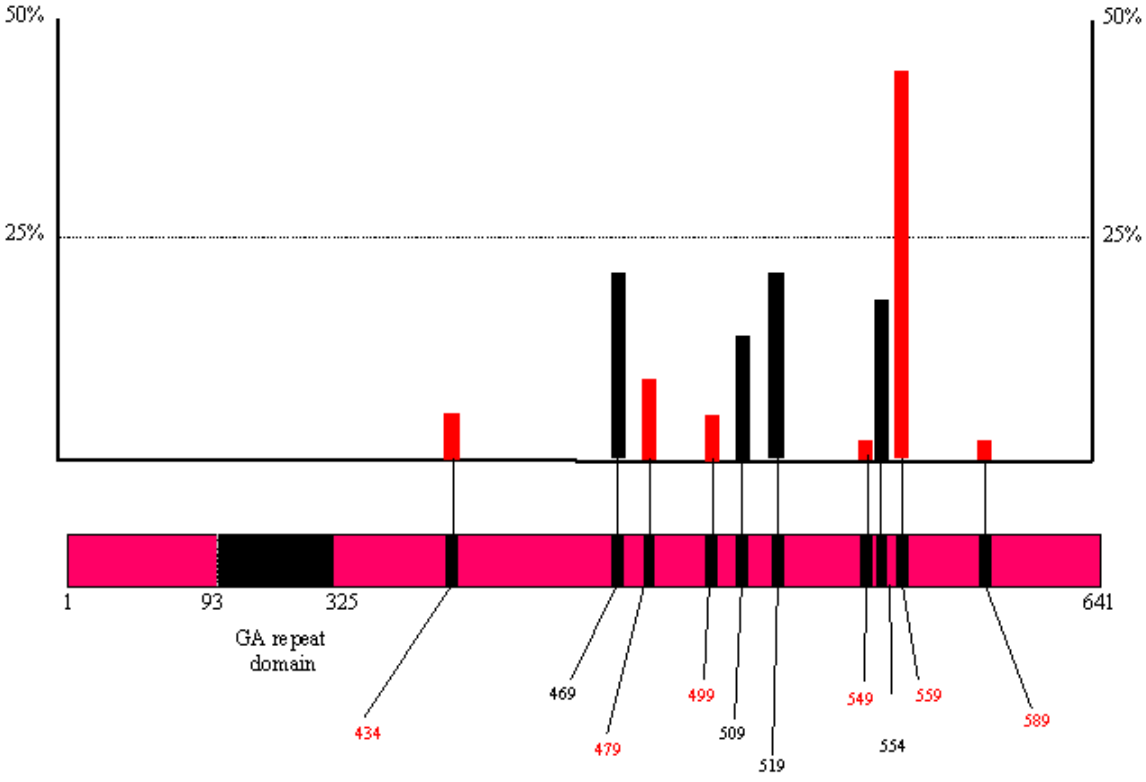

Caucasian EBV prototype B95.8
403
455
475
515
529
554
563
574
594
71
429
485
509
519
544
379*
56*
GA repeat domain
1
93
325
641
GA repeat domain
1
93
325
641
549
554
559
589
434
469
479
499
509
519
Chinese EBV prototype CKL

**Figure 3.2.9: $CD4^+$ T cell responses to EBNA1 in Caucasian and Chinese donors**
The Figure shows the location of $CD4^+$ T cell epitope/epitope regions in the EBNA1 molecule; the grey molecule represents the B95.8 EBV strain EBNA1 molecule, the molecule in red represents the CKL EBV strain EBNA1 molecule, the numbers located at the bottom of the molecule are the coordinate of the glycine-alanine repeat region within the molecule and the actual size of the EBNA1 molecule, and the number allocated to the bolded bars represent the epitope/epitope regions. There are a total of 17 $CD4^+$ epitopes identified in the Caucasian study, where two epitope (*) were recently found and are unpublished data. In our study, we have found 10 epitope regions of which 6 epitope regions (in red) are novel epitopes and 4 epitope regions are located in the same coordinate within the EBNA1 molecule as the ones described in the Caucasian work.

**Frequency of EBNA1 responses in the Chinese population**




**Frequency of EBNA1 responses in the Caucasian population**

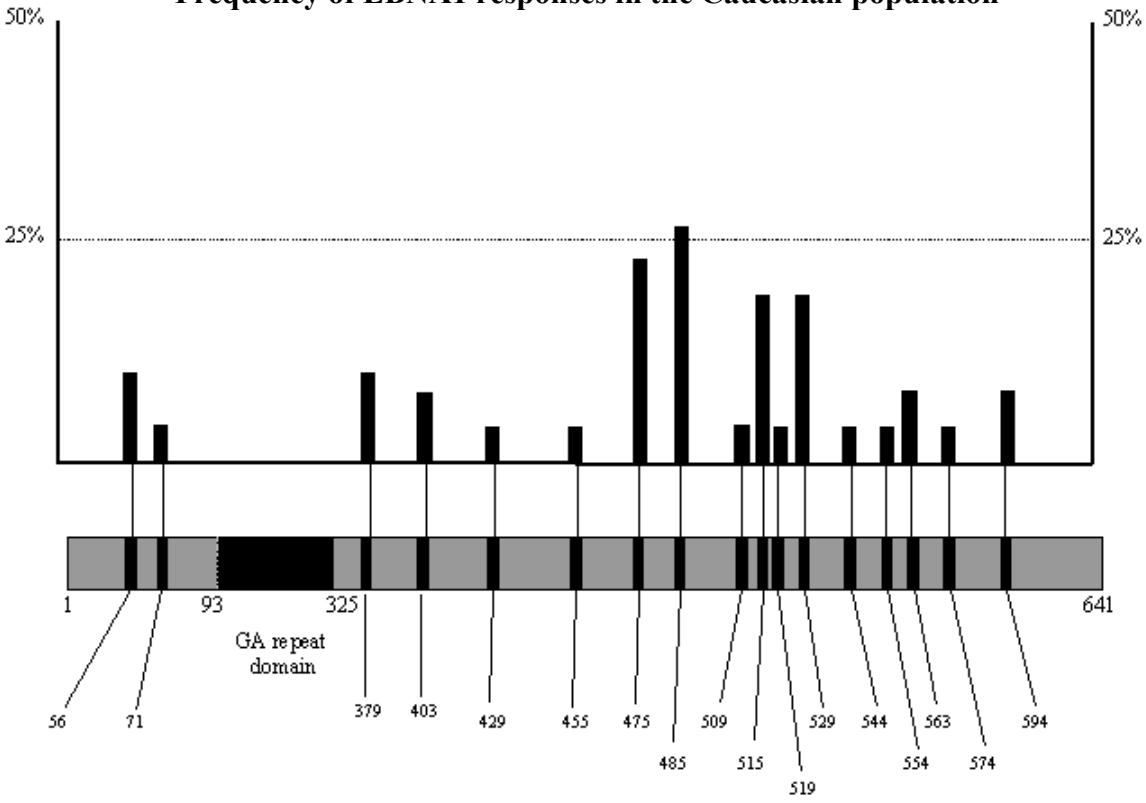


**Figure 3.2.10: Frequency of EBNA1 responses in Chinese vs. Caucasian**

This F igure shows the frequency of EBNA1 response in the Chinese population (top) and comparing the frequency of EBNA1 response in the Caucasian population (below). The frequency is expressed as a percentage of the total number of responding donors tested against that particular peptide(s). The Figure shows that there is a tendency towards higher frequencies in Chinese epitope regions and with one very frequent (EBNA1 559) response that is higher than anything in Caucasians.

| HK Control | Background | 41 | 42 | 50 | 51 | 54 | 55 |
|---|---|---|---|---|---|---|---|
| **201** | 2-20 | *102<br>**(130) | 236<br>(198) | 206<br>(198) | 188<br>(168) | 174<br>(188) | 96<br>(114) |
| **228** | 2-26 | n.d | 126<br>(138) | n.d | 116<br>(158) | 76<br>(104) | 94<br>(108) |
| **233** | 0-19 | n.d | n.d | n.d | n.d | 90<br>(100) | 138<br>(92) |

**Table 3.2.10: $CD4^+$ T cell responses to B95.8 equivalent EBNA1 peptides**

*$CD4^+$ T cell response to B95.8 equivalent of the EBNA1 peptide

** $CD4^+$ T cell response to the CKL EBNA1 peptide

n.d – not determined

against that particular peptide(s). The Figure shows that there is a tendency towards higher frequencies in Chinese epitope regions and with one very frequent (EBNA1 559) response that is higher than anything in Caucasians. However where such frequent responses are obtained, we do not yet know whether this is (i) HLA alleles being less polymorphic in the Chinese population or (ii) a larger proportion of promiscuous epitopes?

As shown in Figure 3.2.9, of the 10 epitope regions seen by Chinese donors, 6 are unique to the Chinese population. Three of the 6 Chinese-specific responses (i.e. EBNA 1 434, 479 and 499) are unique Chinese sequences and three were identical to the B95.8 sequences (i.e. EBNA 1 549, 559 and 589). The B95.8 equivalent of EBNA 1 434, 479 and 499 20-mer peptides were also tested on HK control 201 and 228 and EBNA 1 499 20-mer was tested on HK control 233 to see whether there is cross-reactivity of response. Table 3.2.10 shows the ELISpot data of the experiment, in red are the responses to the B95.8 equivalent of the epitope region along side (in black) are the responses to the Chinese peptides and the data are expressed as SFC/$10^6$ CD8-depleted PBMCs. The Table clearly shows that there are no significant differences between $CD4^+$ T cell responses against the Chinese or the B95.8 equivalent of the epitope regions. Therefore suggesting that EBNA 1 polymorphism, at least against these three epitope regions, is not the contributing factor as to why $CD4^+$ T cell responses are seen in Chinese but not in the Caucasian population. The existence of new epitopes is therefore likely to reflect differences in HLA polymorphism between the two populations. However, further studies are required to determine the factors that may influence the responses between Chinese and Caucasian donors.

The criteria that were used in mapping the number of epitope regions would have underestimated the number of true epitopes present, especially in the region EBNA 1 499-543. Eliciting a number of responses when mapping epitope regions, several factors were taken into account; (1) the pattern of response to individual peptides within the region, (2) the number of examples of this pattern within our studied population and (3) the size of the response to each individual peptide (designating weaker neighboring responses as overlap from the epitope response). There were some instances, such as that seen with HK control

240 response to peptide 56 but not 57 and HK control 242 response to peptide 57 but not 56, where both were categorized as a 56/57 epitope region response. However, such responses could represent rare responses to quite separate epitopes.

### 3.2.5.3 Immunodominance among EBNA 1 epitopes

There are several ways of defining immunodominance and consequently the term is used rather loosely in the literature. It may be used to refer (i) to epitopes that are commonly seen in assays on a population group; in this case immunodominance can be dramatically affected by the frequency of the restricting allele in the population or, for a promiscuous epitope, by the combined frequency of restricting alleles. Alternatively, it may be used to describe (ii) epitopes that in individuals with the relevant alleles, consistently induce strong responses i.e. responses that are larger than competing epitope responses seen through the same or different alleles in the individual responding donor.

According to definition (i), Figure 3.2.7 shows that; EBNA 1 559 is the most immunodominant epitope region because it is seen by 44% of the population studied followed by EBNA 1 469, EBNA 1 (509, 519 and 554), EBNA 1 479, EBNA 1 434, and EBNA 1 (499, 549 and 589). Where there are more than one epitope regions with the same frequency such as EBNA 1 (509, 519 and 554) and EBNA 1 (499, 549 and 589). However, according to definition (ii), from Figure 3.2.8 there is no clear hierarchy of immunodominance among the different epitopes according to response size. The mean response size for almost all response lies in a narrow range between 90 and 160 SFC per million cells. We will re-visit immunodominance after presenting the work to chapter 3.3.0.

## 3.2.6 Conclusion

Now that the work had identified 10 $CD4^+$ epitope regions in EBNA1, the next step was to characterise these epitope regions by cloning out the responding cells. This would allow definition of: (1) the optimal epitope sequence and (2) the restriction element(s) before re-visiting the question of immunodominance. Access to epitope specific clones would also allow us to address the possible therapeutic value of such responses, asking whether

EBNA1-specific $CD4^+$ T cells against particular epitopes are capable of recognising EBNA1-expressing latently-infected target cells.

# Section 3.3

# Generating and characterising EBNA1-specific T-cell clones

### 3.3.0 Generating and characterising EBNA1 specific CD4+ T-cell clones

### 3.3.1 Introduction

This section of work describes experiments designed to generate $CD4^+$ T cell clones from positive responders to EBNA1 (identified in section 3.2) using the epitope region peptides in an *in vitro* reactivation protocol. The objective was to determine whether the ELISpot findings reflect true EBNA1-specific memory responses and, if so, to study the restriction and functional capacity of such responses.

### 3.3.2 Optimisation of protocol

Preliminary experiments were conducted to determine the optimal conditions required for cloning from cryopreserved PBMC samples. In this case PBMCs from the EBV-seropositive donor UKSL (known to respond to a $CD4^+$ epitope region EBNA3A 649-666: see section 3.1) that had been cryopreserved for 6 months before thawing were compared with PBMCs freshly prepared from the same donor. Both thawed and fresh PBMCs were rested overnight before $CD8^+$ T cell depletion on day 2; this step was found to be optimal for cell number recovery post-depletion. CD8-depleted PBMC of both samples were then rested for a second night, and on day 3, both PBMC samples were stimulated with the two relevant EBNA3A peptides, EBNA3A 649-662 and 653-666, and cultured for seven days in serum free media to generate parallel polyclonal cell cultures (from frozen and fresh cells). In previous experiments (data not shown) PBMCs were reactivated in media containing different serum concentrations to generate polyclonal cell cultures. It was found that reactivation using serum free media generated polyclonal cell cultures that were antigen-specific whereas, polyclonal cultures generated from serum-containing media gave unspecific responses in IFN-γ ELISA assays.

Figure 3.3.1 summarises the culture protocol. From 7 post-stimulation (i.e. day 10 since the cells were thawed), the polyclonal cell cultures were maintained in culture media containing 10% pooled human serum. Also on day 7, both polyclonal cell cultures were

**EBNA 3A <u>649-666</u> cloning from UK-SL**

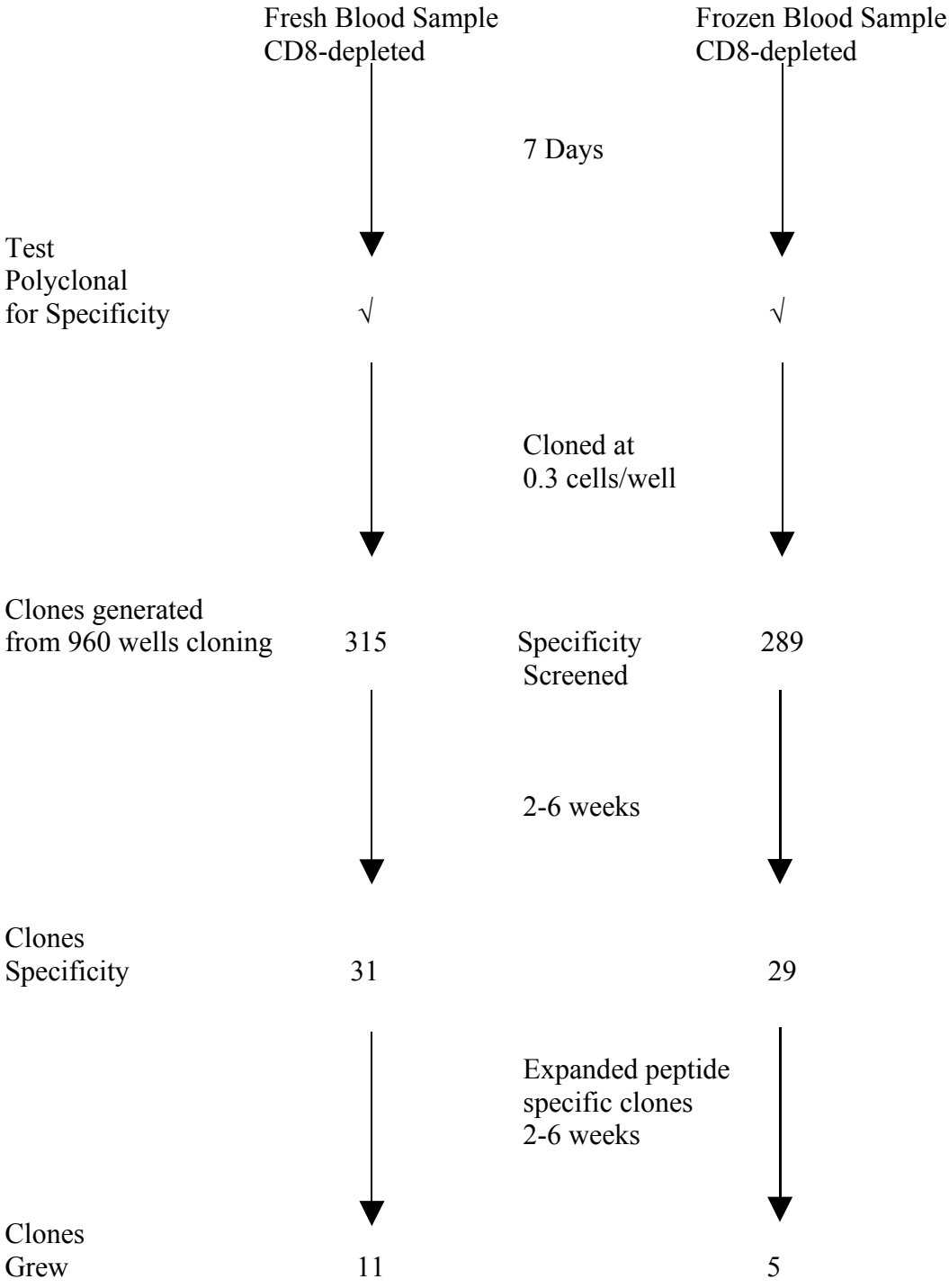
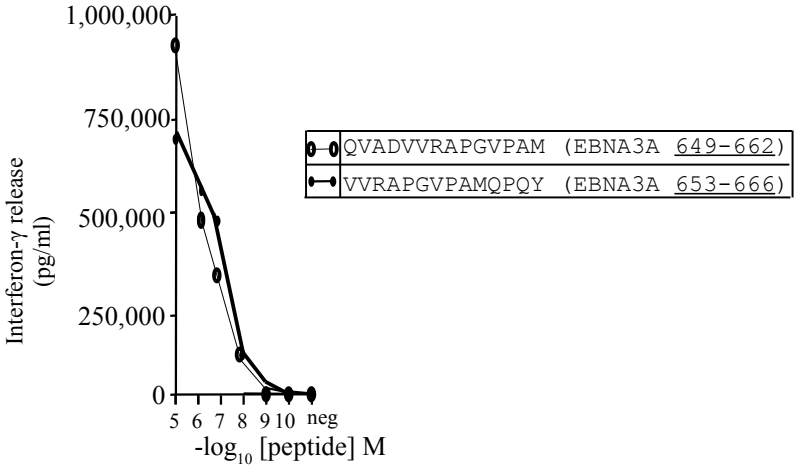


**Figure 3.3.1: Quality of UKSL cloning stages using frozen and fresh PBMCs**

This Figure summarises the cloning process of UK-SL, using fresh and frozen PBMC samples, to generate EBNA 3A 649-666-specific $CD4^+$ T cell clones. Fresh blood samples were taken on day 0, $CD8^+$ T cell depleted on day 2, before stimulation on day 3 with 2 relevant peptides EBNA 3A 649-662 and 653-666. Frozen PBMCs were kept in liquid nitrogen for 6 months before they were thawed on day 0 of the experiment, $CD8^+$ T cell depleted on day 2 and stimulated on day 3 with the same peptides. Both PBMC cultures were allowed to grow in culture for 7 days after stimulation. Both polyclonal cultures were then tested on day 7 by IFN-γ ELISA assay for peptide-specificity, and were (√) peptide-specific. The polyclonal cultures were also cloned on day 7 at 0.3 cells/well. The clones were left to expand in culture for 2-6 weeks before they were tested for peptide-specificity. 315/960 and 289/960 clones generated from fresh and frozen PBMCs respectively were tested peptide-specific. The peptide-specific clones were then transferred to 2ml culture wells for further expansion. After a further 2-6 weeks, the clones were re-rested for peptide-specificity to which only 31/315 (fresh PBMC) and 29/289 clones (frozen PBMCs) were peptide-specific. The peptide-specific clones were allowed a further 2-6 weeks to expand in culture before being retested for peptide-specificity. 11/31 (fresh) and 5/29 (frozen) clones were tested peptide-specific.

**A**

**EBNA 3A 649-666 UKL-SL polyclonal (Fresh PBMC)**




**B**

**EBNA 3A 649-666 UKL-SL polyclonal (Frozen PBMC)**

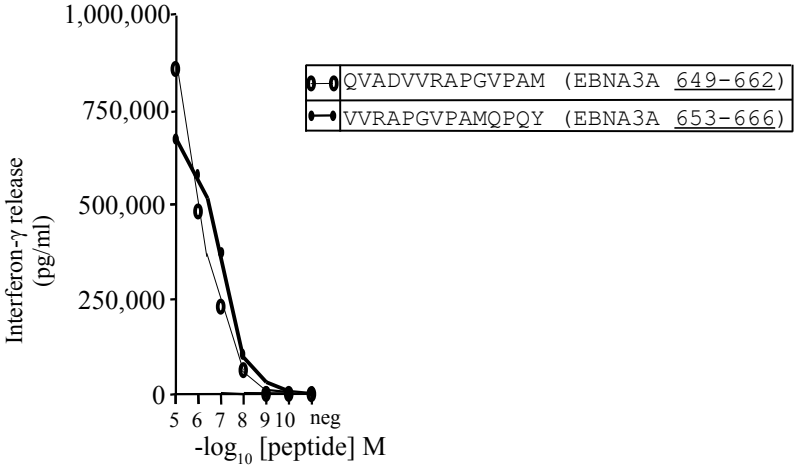


**Figure 3.3.2: IFN-γ ELISA assay to determine epitope-specificity of UKSL polyclonal cultures**

In this assay, 5000 polyclonal cells/well was cultured overnight with 50,000 autologous LCL/well pre-loaded with a range of concentrations (10-fold dilution) of the individual EBNA3A peptides from $10^{-5}$M to $10^{-10}$M or to DMSO alone (neg). Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Both fresh (A) and frozen (B) PBMC samples gave polyclonal cell cultures that specifically responded to the EBNA3A 649-662 and 653-666 peptides in a dose-dependent fashion, and gave very similar titration curves.

tested for reactivity to the two peptides and the data are shown in Figure 3.3.2. In this assay, 5000 polyclonal cells/well was cultured overnight with 50,000 autologous LCL/well pre-loaded with a range of concentrations (10-fold dilution) of the individual EBNA3A peptides from $10^{-5}$M to $10^{-10}$M or to DMSO alone (no peptide control) or an irrelevant peptide LMP2 340-349 at $10^{-5}$M. Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. Figure 3.3.2 shows the results as IFN-γ release expressed as pg/ml. Both frozen and fresh PBMC samples gave polyclonal cell cultures that specifically responded to the EBNA3A 649-662 and 653-666 peptides in a dose-dependent fashion, and gave very similar titration curves. For both polyclonal cultures, the peptide concentration that could induce half maximum response ($C_{1/2}$) was $10^{-7}$M for EBNA3A 649-662 and $10^{-7.5}$M for EBNA3A 653-666, suggesting that EBNA3A 653-666 is the optimal peptide.

As shown in Figure 3.3.1, on day 7 of culture both polyclonal cultures were seeded at 30, 3 and 0.3 cells/well in 10 x 96 well u-bottom tissue culture plates with a "clone mix" containing 50,000 γ-irradiated autologous LCL/ml (pre-loaded with the epitope region peptides) and 1 million γ-irradiated pooled PBMCs/ml (pre-treated with PHA). On day 13, the wells were fed with clone media (containing 60% MLA, 40U IL2 and 5% human serum) and re-fed every 2 weeks. After 2-6 weeks, all wells seeded at 30 and 3 cells/well had grown. Cloning at 0.3 cells/well had growth in 315 (fresh PBMC samples) and 289 (frozen PBMC samples) wells from 960 wells of cloning (see Figure 3.3.1). Because cloning from the polyclonal cell culture was setup at 0.3 cells/well and if all cells were to grow, it is expected that approximately 1/3 of 960 wells (320 wells) would have growth. Therefore, using the current protocol of cloning ~100% of positive wells had grown.

All clones were screened for epitope specificity in a simplified screening assay. In this assay, 50μl of resuspended cells from each growing well was cultured overnight with 50,000 autologous LCL/well pre-loaded with epitope region peptides or DMSO. Supernatant was tested for IFN-γ release by ELISA, identifying 31/315 and 29/289 clones as epitope region-specific from fresh and frozen PBMC samples respectively. These

epitope region-specific clones were transferred into 2ml tissue culture plates containing clone media, 50,000 γ-irradiated autologous LCL/ml (pre-loaded with epitope region peptides) and 1 million γ-irradiated pooled PBMCs/ml (pre-treated with PHA). After 2-3 weeks, 16 and 18 clones that had expanded well were re-tested for epitope region specificity. Of these, 11 and 5 clones from fresh and frozen PBMC samples respectively were still epitope region-specific.

The quality of the clones generated from fresh and frozen PBMC samples were assessed and representative data from one clone from fresh PBMCs (clone 8) and one clone from frozen PBMCs (clone 2) are shown in Figure 3.3.3. In this assay, 500 T cells/well were cultured overnight with 50,000 autologous LCL/well pre-loaded with diluting concentrations (10-fold) of epitope region peptides or with DMSO or LMP2 340-349 peptide controls as mentioned above. The Figure shows that clones generated from fresh and frozen PBMC samples responds similarly with the same $C_{1/2}$ values as observed in the polyclonal cell culture experiments, $10^{-7.5}$M for EBNA1 663-666. These results show that the polyclonal culture data could be reproduced using clones. Note that IFN-γ release (pg/ml) values are much lower in these assays than in those conducted using polyclonal cell cultures; this in part reflects the fact that fewer cells (10-fold) were used in the clonal assays.

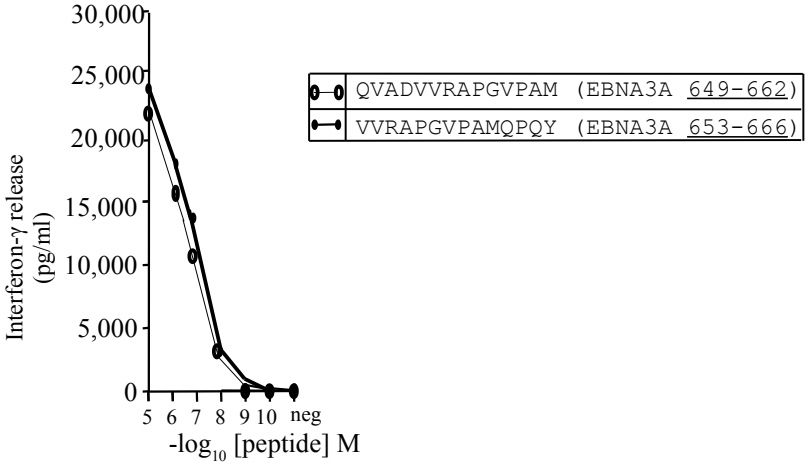
These preliminary experiments showed that this cloning protocol could yield epitope region-specific polyclonal cell cultures and clones with roughly similar efficiency from fresh and frozen PBMC preparations. This protocol was therefore employed in the following experiments conducted on frozen PBMC samples from HK control donors.

### 3.3.3 HLA typing of HK controls

Additional PBMC samples were available from 47 of the HK donors screened by ELIspot, of which 33 gave a response to one or more EBNA1 peptides. ELIspot responses from these 47 donors are summarised in Table 3.3.1; all 47 donors were HLA-DR and –DQ typed.

**A**

**EBNA 3A 649-666 UKL-SL Clone 8 (From Fresh PBMC)**




**B**

**EBNA 3A 649-666 UKL-SL Clone 2 (From Frozen PBMC)**

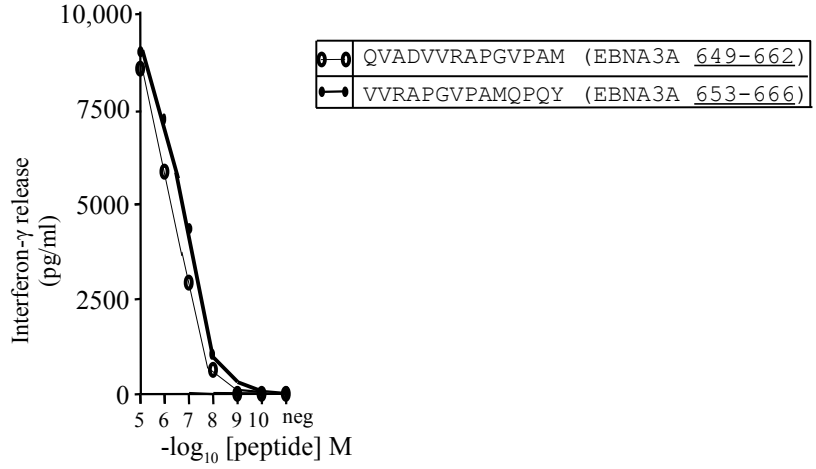


**Figure 3.3.3 IFN-γ ELISA assay to determine epitope-specificity of UKSL clones**
In this assay, 500 T cells/well were cultured overnight with 50,000 autologous LCL/well pre-loaded with diluting concentrations (10-fold) of epitope region peptides or with DMSO (neg). Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Both fresh (A) and frozen (B) PBMC samples generated clones that specifically responded to the EBNA3A 649-662 and 653-666 peptides in a dose-dependent fashion, also giving very similar titration curves.

| Responders | Epitope regions | | | | | | | | | | HLA Types | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (HK Control) | 41/42 | 48/49 | 50/51 | 54/55 | 56/57 | 58/59 | 64 | 65/66 | 67/68 | 72/73 | DR | DR | DR | DR | DQ | DQ | DP | DP |
| **201** | √ | | √ | √ | √ | √ | √ | √ | | | 11 | 13 | 52b | 52b | 7 | 7 | 2 | 13 |
| **202** | | | | | | | | √ | | | 8 | 12 | 52c | 52c | 7 | 6 | | |
| **203** | | | | | | √ | | √ | √ | | 4 | 12 | 52c | 53 | 7 | 7 | | |
| **204** | | √ | | | | | | | √ | | 15 | 16 | 51 | 51 | 5 | 6 | | |
| **208** | | | | | | | | | √ | | | | | | | | | |
| **209** | | | | | | | | | √ | | | | | | | | | |
| **211** | | | | | | | | | √ | | 4 | 9 | 53 | 53 | 8 | 9 | | |
| **215** | | √ | | | | | | | √ | √ | 9 | 16 | 53 | 51 | 9 | 5 | 5 | 5 |
| **219** | | √ | | | | | | | √ | | 9 | 15 | 53 | 51 | 6 | 9 | | |
| **222** | | | | | | | | √ | | | 8 | 12 | 52b | 52b | 7 | 6 | | |
| **223** | | | | | | | | | √ | | | | | | | | | |
| **224** | | √ | | | | √ | | | √ | | 15 | 16 | 51 | 51 | 5 | 6 | | |
| **228** | √ | | √ | √ | √ | | | | √ | | 17 | 11 | 52a | 52b | 2 | 7 | 2 | 5 |
| **230** | | | √ | | √ | | | | √ | | 11 | 14 | 52b | 52b | 7 | 5 | | |
| **231** | | | | | | | | | √ | | | | | | | | | |
| **233** | | √ | | √ | √ | √ | | | | | 11 | 15 | 52b | 51 | 7 | 6 | 3 | 13 |
| **234** | | | | | | | | √ | | | | | | | | | | |
| **236** | | | | | | | | | √ | | 16 | 12 | 52a | 51 | 5 | 7 | | |
| **240** | | | | | √ | | | | √ | | 9 | 11 | 52b | 53 | 7 | 9 | | |
| **241** | | | | | √ | | | | √ | | 11 | 14 | 52b | 52b | 5 | 7 | | |
| **242** | | √ | | | | | | | | | 8 | 16 | 51 | 51 | 4 | 5 | | |
| **244** | | | √ | | | √ | | √ | | | 17 | 12 | 52a | 52b | 2 | 7 | | |
| **247** | | √ | | | | √ | | | | | 12 | 15 | 52a | 51 | 6 | 7 | | |
| **248** | | | | | √ | | | | √ | | 9 | 11 | 52b | 53 | 7 | 9 | | |
| **249** | | | | | | | | | √ | | | | | | | | | |
| **251** | | √ | | | | √ | | | √ | | 15 | 16 | 51 | 51 | 5 | 6 | | |
| **252** | | | | | | √ | | √ | | | 15 | 12 | 52c | 51 | 6 | 7 | | |
| **253** | | √ | | | | | | √ | √ | | 7 | 15 | 53 | 51 | 2 | 6 | | |
| **257** | | | | | | √ | | | | | 14 | 14 | 52b | 52b | 5 | 5 | | |
| **258** | | | | | | | | | √ | | | | | | | | | |
| **260** | | | | | | √ | | √ | √ | | | | | | | | | |
| **262** | | | | | | √ | | √ | √ | | | | | | | | | |
| **263** | | | | | √ | √ | | | √ | | 11 | 14 | 52b | 52b | 7 | 5 | | |
| **266** | | | | | | √ | | √ | √ | | | | | | | | | |
| **267** | | | | | | | | | √ | | | | | | | | | |
| **268** | | √ | | | | | | | | | 15 | 15 | 51 | 51 | 6 | 6 | | |
| **276** | | | | | | √ | | √ | | | | | | | | | | |
| **277** | | √ | | | | √ | | | √ | | 12 | 15 | 52a | 51 | 6 | 7 | | |
| **278** | | √ | | | | | | | √ | | 15 | 15 | 51 | 51 | 6 | 6 | | |
| **279** | | | | | | | | | √ | | | | | | | | | |
| **280** | | | | | √ | | | √ | | | 11 | 12 | 52b | 52b | 7 | 7 | | |
| **281** | | √ | | | | | | | | | 15 | 13 | 52b | 51 | 6 | 7 | | |
| **283** | | | | | | | | | √ | | | | | | | | | |
| **284** | | √ | | | | | | √ | √ | | 12 | 15 | 51 | 51 | 7 | 6 | | |
| **285** | | √ | | √ | √ | √ | | | | | 11 | 15 | 52b | 51 | 7 | 6 | | |
| **286** | | | | | √ | | | | √ | | 10 | 15 | 51 | 51 | 5 | 5 | | |
| **287** | | | | | | | | | √ | | | | | | | | | |
| **288** | | | | | | | | | √ | | | | | | | | | |
| **291** | | √ | | | | | | | √ | | 8 | 16 | 51 | 51 | 5 | 6 | | |

**Table 3.3.1: Summary of epitope-specific responses to EBNA1 peptides and HLA Class II typing of 47 healthy HK donors**

√ - response was made to the epitope region

### 3.3.4 Selecting and cloning from HK Control donors

Based on Table 3.3.1, HK control 201, 215, 228 and 233 were chosen for cloning because they showed a wide range of epitope-region responses. *In vitro* reactivation and limiting dilution cultures were set up from thawed PBMCs using the protocol shown in Figure 3.3.1.

Figure 3.3.4 summarises a typical set of data, in this case from HK control 215, to illustrate the fact that the yield of amplifiable epitope-region-specific clones was always low from the HK samples. This donor responded to three epitope regions EBNA1 469-493, 564-588 and 589-613 in the ELISpot assay. Figure 3.3.5 shows the screening of epitope specificity on polyclonal cell cultures generated from the different peptide stimulations. Epitope-specific responses were detectable for two of the three peptide pairs tested. Interestingly, the polyclonal cell culture generated with the EBNA1 469-488 and 474-493 peptides only recognised peptide EBNA1 474-493 at a $C_{1/2}$ value of $10^{-7.5}$M and did not recognise EBNA1 469-488 (Figure 3.3.5A). The polyclonal cell culture generated using the EBNA1 564-583 and 569-582 peptides recognised both peptides 564-583 and 569-588 with $C_{1/2}$ values $10^{-8}$M and $10^{-6.5}$M respectively (Figure 3.3.5B). By contrast, stimulation with peptides 589-608 and 594-613 reproducibly produced cultures with a high spontaneous level of IFN-γ production that prevented any detection of epitope specificity (Figure 3.3.5C).

As in the established protocol, cloning was done on day 7 post-stimulation but in this case was also carried out from the polyclonal culture every week for six weeks. Typically, cultures seeded at 30 and 3 cells/well showed growth in all the wells and cultures seeded at 0.3 cells/well had growth in approximately 1/3 of all the wells. Clones from 0.3 cells/well were tested for peptide specificity, of which 28/318, 57/421 and 0/294 clones tested peptide specific to EBNA1 469-493, 564-588 and 589-613 respectively; the absence of EBNA1 589-613-specific clones was not surprising in view of the data already obtained from the polyclonal culture. However, most of those clones specific for the other two peptides did not expand and were lost at this stage. Overall only 10/28 and 8/57 clones generated against EBNA1 469-493 and 564-588 respectively expanded

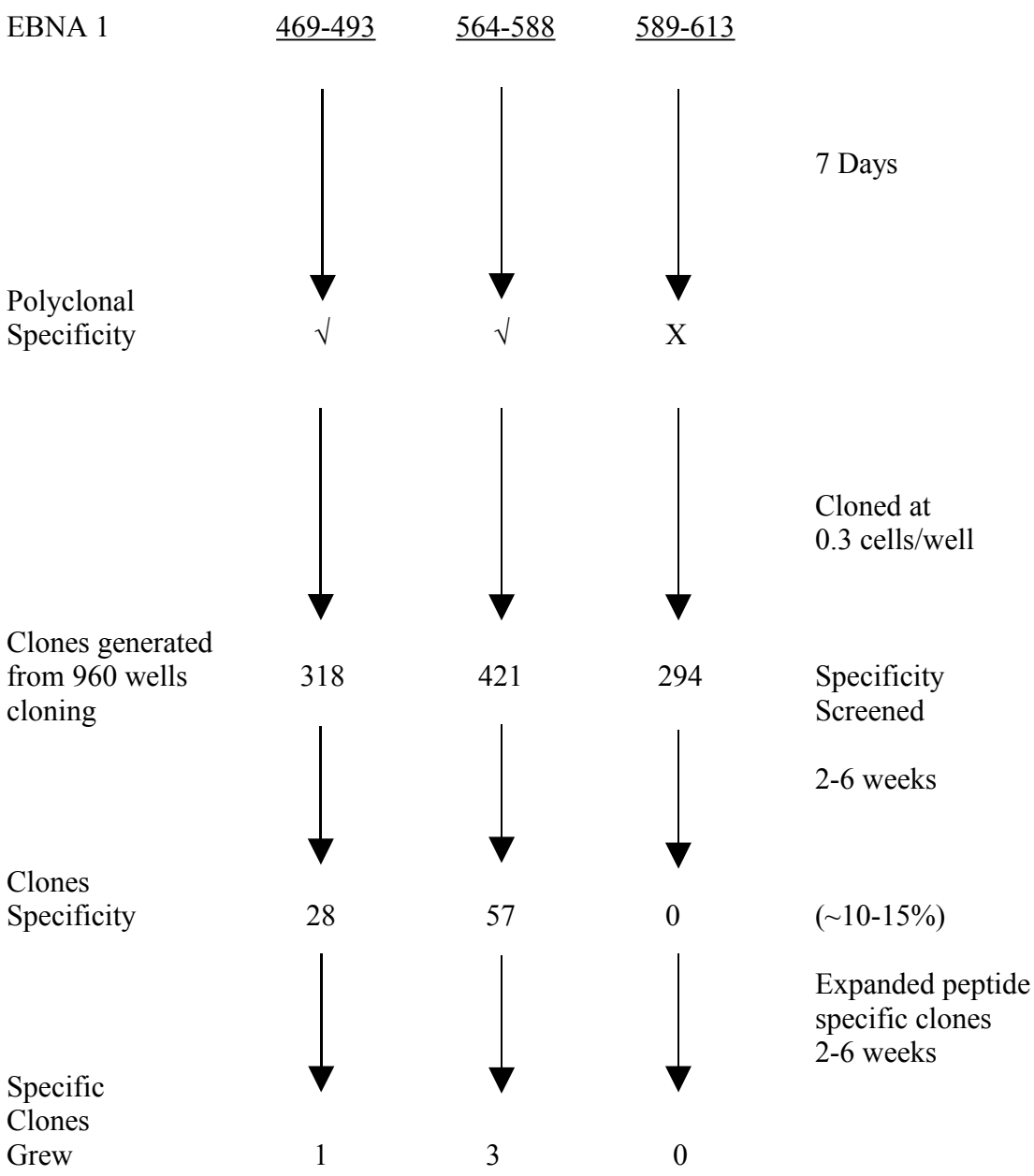
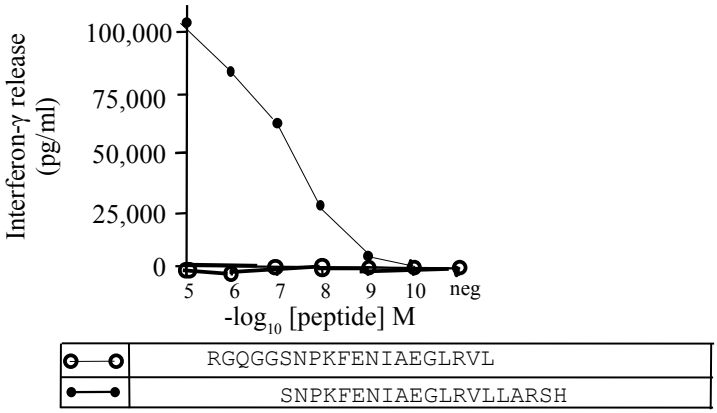

EBNA 1
469-493
564-588
589-613
7 Days
Polyclonal Specificity
√
√
X
Cloned at 0.3 cells/well
Clones generated from 960 wells cloning
318
421
294
Specificity Screened
2-6 weeks
Clones Specificity
28
57
0
(~10-15%)
Expanded peptide specific clones 2-6 weeks
Specific Clones Grew
1
3
0

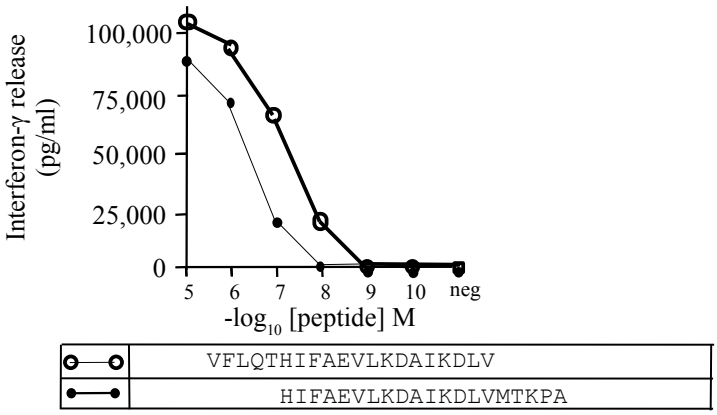
**Figure 3.3.4: Quality of HK Control 215 cloning stages**

This Figure summarises the cloning process of HK control 215, using frozen PBMC samples. Frozen PBMCs were thawed on day 0 of the experiment, $CD8^+$ T cell depleted on day 2 and stimulated on day 3 with the peptide pools EBNA1 469-493, 564-588, and 589-613. The PBMC cultures were allowed to grow in culture for 7 days after stimulation, and then tested on day 7 by IFN-γ ELISA assay for peptide-specificity. Polyclonal cultures generated from peptide pools EBNA1 469-493 and 564-588 were (√) peptide-specific, and polyclonal culture generated from peptide pool EBNA1 589-613 was (X) not peptide specific. The polyclonal cultures were also cloned on day 7 at 0.3 cells/well. The clones were left to expand in culture for 2-6 weeks before they were tested for peptide-specificity. 318/960, 421/960 and 294/960 clones generated from EBNA1 469-493, 564-588, and 589-613 peptide pool stimulation respectively were tested peptide-specific. The peptide-specific clones were then transferred to 2ml culture wells for further expansion. After a further 2-6 weeks, the clones were rerested for peptide-specificity to which 28/318 (EBNA1 469-493), 57/421 (EBNA1 564-588) and 0/294 clones (EBNA1 and 589-613) were peptide-specific. The peptide-specific clones were allowed a further 2-6 weeks to expand in culture before again being retested for peptide-specificity. 1/28 (EBNA1 469-493) and 3/57 (EBNA1 564-588) clones were tested peptide-specific.

**A** EBNA 1 469-493 HK 215 polyclonal




**B** EBNA 1 564-588 HK 215 polyclonal




**C** EBNA 1 589-613 HK 215 polyclonal

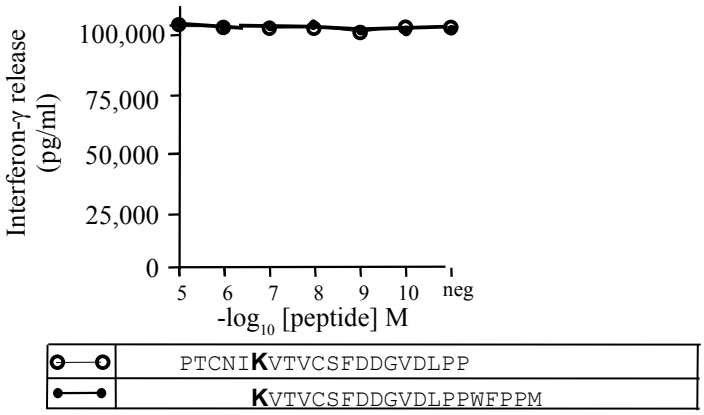


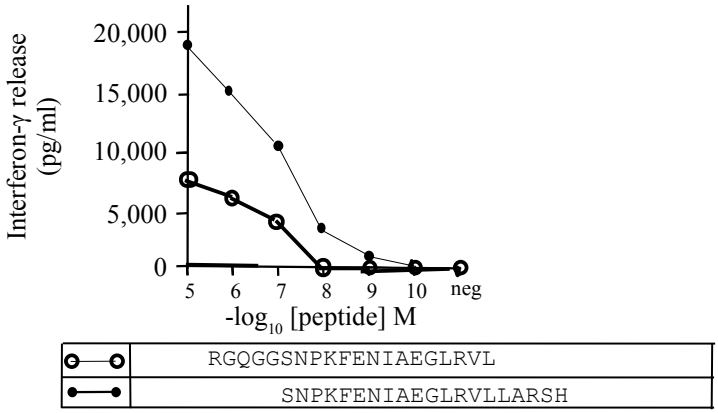
**Figure 3.3.5: IFN-γ ELISA assay to determine epitope-specificity of HK#215 polyclonal cultures**

In this assay, 5000 polyclonal cells/well was cultured overnight with 50,000 autologous LCL/well pre-loaded with a range of concentrations (10-fold dilution) of the individual EBNA1 peptides from $10^{-5}$M to $10^{-10}$M or to DMSO alone (neg). Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Interestingly, the polyclonal cell culture generated with the EBNA1 469-488 and 474-493 peptides only recognised peptide EBNA1 474-493 and did not recognise EBNA1 469-488 (A). The polyclonal cell culture generated using the EBNA1 564-583 and 569-582 peptides recognised both peptides 564-583 and 569-588 (B). By contrast, stimulation with peptides 589-608 and 594-613 reproducibly produced cultures with a high spontaneous level of IFN-γ production that prevented any detection of epitope specificity (C).

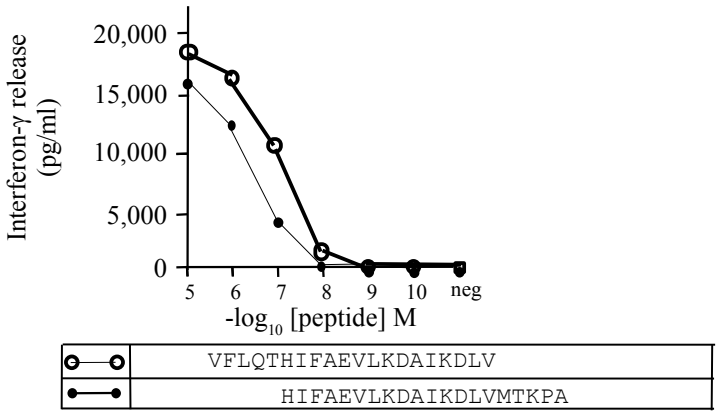
further and were available to be re-tested for epitope peptide-specificity. As summarised in Figure 3.3.4, 1 of these 10 and 3 of these 8 clones respectively had retained epitope specificity. Figure 3.3.6 shows examples of one epitope-specific clone of each type. Figure 3.3.6A shows that clone HK215c1 (EBNA1 469-493) responds to both EBNA1 peptides 469-488 and 474-493 in a dose-dependent manner, unlike the polyclonal cultures which it was derived from only responds to EBNA1 474-493. Whereas clone HK215c50 (EBNA1 564-588) responds to both EBNA1 peptides, 564-583 and 569-588, also in a dose-dependent manner similar to the polyclonal culture which it was derived from (Figure 3.3.6B). This possibly suggests that the epitope is contained between the overlap of the two 20-mer peptides. However, further tests are needed to confirm the exact epitope location. Figure 3.3.6C shows a typical response of clones generated from EBNA1 589-613 polyclonal cultures. The clone did not respond to any of the two EBNA1 peptides, 589-608 and 594-613. Note that the EBNA1 589-613 polyclonal cell line (Figure 3.3.5C) showed high spontaneous level of IFN-γ production. Initially it was believed that the polyclonal cell line was infected, so a new polyclonal cell line was made, but the same high spontaneous IFN- γ production was also observed. A possible explanation to this observation is that the polyclonal cell line was still producing IFN-γ as a result of peptide stimulation from day 0. This may explain why EBNA1 589-613 -specific clones, generated from the EBNA1 589-613 polyclonal cell line, loose their epitope-specificity over time, possible due to over stimulation. Note that responses from clones (pg/ml of IFN-γ release) are much lower than response seen in the polyclonal culture assays. This is because fewer cell numbers are used in the clone assay (500 clonal T cells) compared to the polyclonal assay (5000 polyclonal cells).

Table 3.3.2A summarises which epitope region-specific polyclonal cell culture and clones were successfully generated from the four donors tested, HK Control 201, 215, 228 and 233, and Table 3.3.2B shows the number of epitope region-specific clones obtained. Where there are multiple clones generated against one epitope region, these clones were cryopreserved for later testing while one clone was selected for immediate tests.

**A** EBNA 1 469-493 HK 215c1 clone




**B** EBNA 1 564-588 HK 215c50 clone




**C** EBNA 1 589-613 HK 215 c0 clone

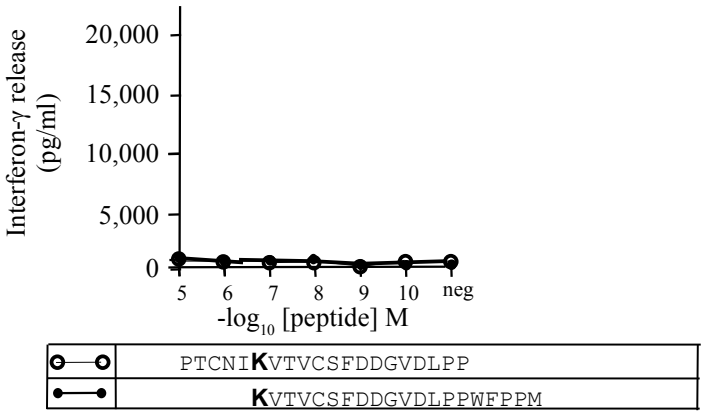


**Figure 3.3.6: IFN-γ ELISA assay to determine epitope-specificity of HK#215 clones**
In this assay, 500 T cells/well were cultured overnight with 50,000 autologous LCL/well pre-loaded with diluting concentrations (10-fold) of epitope region peptides or with DMSO (neg). Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Interestingly, clone HK215c1 generated with the EBNA1 469-488 and 474-493 peptides recognised both peptides in a dose-dependent manner (A). Clone HK215c50 generated using the EBNA1 564-583 and 569-582 peptides also recognised both peptides 564-583 and 569-588 in a dose-dependent manner (B). By contrast, stimulation with peptides 589-608 and 594-613 reproducibly produced clones which had lost peptide-specificity (C).

| Responders | Epitope regions | | | | | | |
|---|---|---|---|---|---|---|---|
| (HK Control) | 434-458 | 469-493 | 479-503 | 499-543 | 549-578 | 564-588 | 589-613 |
| **201** | X | | X | X | X | | |
| **215** | | √ | | | | √ | X |
| **228** | X | | X | X | | X | |
| **233** | | √ | | X | | | |

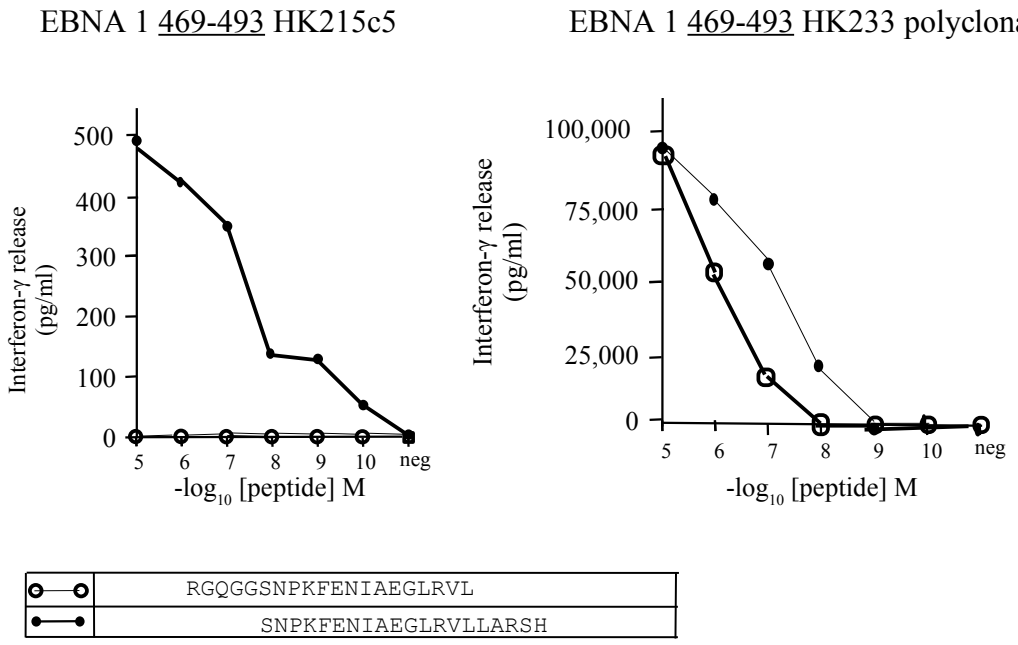
**Table 3.3.2A: Summary of data from polyclonal cultures generated**

√ - Epitope specificity detected
X – No specificity detected

| Responders | Epitope regions | | | | | | |
|---|---|---|---|---|---|---|---|
| (HK Control) | 434-458 | 469-493 | 479-503 | 499-543 | 549-578 | 564-588 | 589-613 |
| **201** | 0 | | 0 | 0 | 0 | | |
| **215** | | 1 | | | | 3 | 0 |
| **228** | 0 | | 12 | 0 | | 23 | |
| **233** | | 0 | | 1 | | | |

**Table 3.3.2B: Summary of epitope-specific clones established**

**A**


EBNA 1 469-493 HK215c5
EBNA 1 469-493 HK233 polyclonal
Interferon-γ release (pg/ml)
500
400
300
200
100
0
5 6 7 8 9 10 neg
-log10 [peptide] M
100,000
75,000
50,000
25,000
0
RGQGGSNPKFENIAEGLRVL
SNPKFENIAEGLRVLLARSH

**B**

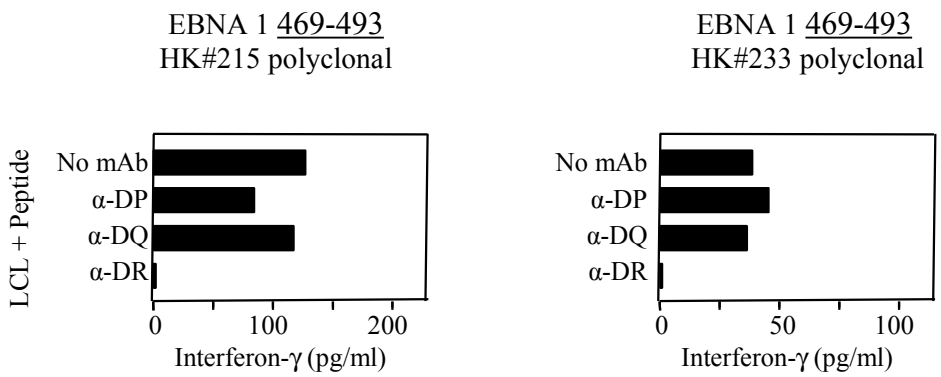

EBNA 1 469-493
HK#215 polyclonal
EBNA 1 469-493
HK#233 polyclonal
LCL + Peptide
No mAb
α-DP
α-DQ
α-DR
0 100 200
Interferon-γ (pg/ml)
0 50 100

**Figure 3.3.7: Characterisation of EBNA1 469-493 using clone HK215c5 and polyclonal cell lines generated from HK#215 and 233**

In the assay shown in (A), 500 T cells/well (clones) or 5000 cells/well (polyclonal cells) were cultured overnight with 50,000 autologous LCL/well pre-loaded with diluting concentrations (10-fold) of epitope region peptides or with DMSO (neg). In the blocking assay (B), autologous LCLs (50,000 LCL cells/well) were pre-loaded with $10^{-7}$ M peptide (EBNA1 474-493) and then treated with anti-DP, -DQ, –DR, or culture media with no antibodies for one hour before the cells were cultured with 5000 polyclonal cells/well in an overnight assay. Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Figure 3.3.7A shows a peptide titration curve of clone HK Control 215c5 and polyclonal culture HK Control 233 against peptides EBNA1 469-488 and 474-493. Clone HK Control 215c5 recognises peptide EBNA1 474-493 but not peptide EBNA1 469-488 while polyclonal culture HK Control 233 recognises peptide EBNA1 474-493 better than EBNA1 469-488. Figure 3.3.7B shows the blocking data from the two polyclonal cultures, HK215 and 233. There was no blocking of IFN-γ release when using anti-DQ and anti-DP monoclonal antibodies with IFN-γ release values whereas peptide recognition was completely blocked by anti-DR monoclonal antibody in both polyclonal cultures.

### 3.3.5 Analysis of responses to epitope region EBNA1 469-493

The following section describe assays conducted using the above clones and polyclonal cell cultures. The results are reported with respect to individual epitope regions, taking all the available data for each region from the four donors tested. One clone was generated from HK control 215 and two polyclonal cell cultures from HK control 215 and 233 that were specific for epitope region EBNA1 469-493. Figure 3.3.7A shows a peptide titration curve of clone HK Control 215c5 and polyclonal culture HK Control 233 against peptides EBNA1 469-488 and 474-493. This assay was conducted in the same manner as described above.

Clone HK Control 215c5 recognises peptide EBNA1 474-493 but not peptide EBNA1 469-488 while polyclonal culture HK Control 233 recognises peptide EBNA1 474-493 better than EBNA1 469-488. Note that in the initial ELISpot assay, HK control 215 responded to both overlapping peptides. However the clone only recognises one peptide, namely EBNA1 474-493. This suggest that there may be two possible epitopes within the two overlapping peptides, one epitope that is recognised by HK control 215c5 (EBNA1 474-493) and another epitope that is yet to be determined. It is unusual to see a response by a clone, in this case HK control 215c5, to one peptide and not to the overlapping peptide since adjacent peptides overlap by 15 amino acids. A possible explanation is that the key amino acids which form the epitope peptide-HLA II complex lies completely within the 20-mer peptide (EBNA1 474-493), and adjacent peptides do not have these correct interactions. Both the clone and polyclonal culture recognise EBNA1 469-488 with a $C_{1/2}$ value of $10^{-7.5}$M. Ideally, a titration should include neighbouring peptides EBNA1 464-483 and 479-498 because these peptides share 15 amino acid sequences in common with this epitope region, but this was not possible in this case due to limited cell numbers. However, ELISpot data for both donors show that they did not respond to the EBNA1 479-498 peptide, therefore it is very likely that the optimal epitope is indeed EBNA1 474-493. We could not generate additional data using this clone because the clone would not expand further in culture.

**A** HLA types of respondents against peptide EBNA1 474-498 (peptides 48/49)

| | HLA Types | | | | | |
|---|---|---|---|---|---|---|
| Donors | DR | DR | DR | DR | DQ | DQ |
| HK Control 204 | 15 | 16 | 51 | 51 | 5 | 6 |
| HK Control 215 | 9 | 16 | 53 | 51 | 9 | 5 |
| HK Control 219 | 9 | 15 | 53 | 51 | 6 | 9 |
| HK Control 224 | 15 | 16 | 51 | 51 | 5 | 6 |
| HK Control 233 | 11 | 15 | 52b | 51 | 7 | 6 |
| HK Control 242 | 8 | 16 | 51 | 51 | 4 | 5 |
| HK Control 247 | 12 | 15 | 52a | 51 | 6 | 7 |
| HK Control 251 | 15 | 16 | 51 | 51 | 5 | 6 |
| HK Control 253 | 7 | 15 | 53 | 51 | 2 | 6 |
| HK Control 268 | 15 | 15 | 51 | 51 | 6 | 6 |
| HK Control 277 | 12 | 15 | 52a | 51 | 6 | 7 |
| HK Control 278 | 15 | 15 | 51 | 51 | 6 | 6 |
| HK Control 281 | 15 | 13 | 52b | 51 | 6 | 7 |
| HK Control 284 | 12 | 15 | 51 | 51 | 7 | 6 |
| HK Control 285 | 11 | 15 | 52b | 51 | 7 | 6 |
| HK Control 291 | 8 | 16 | 51 | 51 | 5 | 6 |

**B** Frequency of responders with the following HLA types

| HLA type | No. Respondents | Total number of respondents | Frequency (%) |
|---|---|---|---|
| DR7 | 1 | 16 | 6 |
| DR8 | 2 | 16 | 13 |
| DR9 | 2 | 16 | 13 |
| DR15 | 13 | 16 | 81 |
| DR11 | 2 | 16 | 13 |
| DR12 | 3 | 16 | 19 |
| DR13 | 1 | 16 | 6 |
| DR16 | 6 | 16 | 38 |
| DR51 | 16 | 16 | 100 |
| DR52a | 2 | 16 | 13 |
| DR52b | 3 | 16 | 19 |
| DR53 | 3 | 16 | 19 |
| DQ2 | 1 | 16 | 6 |
| DQ4 | 1 | 16 | 6 |
| DQ5 | 6 | 16 | 38 |
| DQ6 | 14 | 16 | 88 |
| DQ7 | 6 | 16 | 38 |
| DQ9 | 2 | 16 | 13 |

**C** Frequency of non-responders with the following HLA types

| HLA type | No. Non-responders | Total No. of non-responders |
|---|---|---|
| DR7 | 0 | 17 |
| DR8 | 2 | 17 |
| DR9 | 3 | 17 |
| DR15 | 2 | 17 |
| DR11 | 8 | 17 |
| DR12 | 7 | 17 |
| DR13 | 1 | 17 |
| DR16 | 1 | 17 |
| DR51 | 3 | 17 |
| DR52a | 3 | 17 |
| DR52b | 11 | 17 |
| DR53 | 4 | 17 |
| DQ2 | 2 | 17 |
| DQ4 | 0 | 17 |
| DQ5 | 6 | 17 |
| DQ6 | 3 | 17 |
| DQ7 | 14 | 17 |
| DQ9 | 3 | 17 |

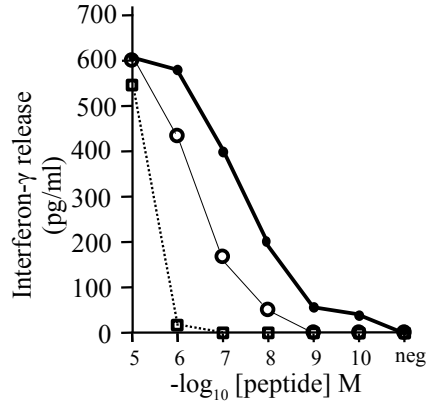
**Table 3.3.3: HLA restriction of EBNA1 474-498 (peptides 48/49) using HLA typing**

Polyclonal cultures from HK Control 215 and 233 were used in determining the HLA class II restriction element for EBNA1 474-493. Autologous LCLs were pre-loaded with 10^-7M epitope peptide for 1 hour and excess peptides were washed off. Monoclonal antibodies HLA -DR, -DQ and –DP and culture media (as no antibody control) were then added at 1μg/ml (2 μg/ml for anti-DR was used) to the individual tests for one hour before adding $5x10^5$ cells. Figure 3.3.7B shows the blocking data from the two polyclonal cultures, the axis shows the IFN-γ release expressed as pg/ml and the y-axis shows the different antibodies used and the no antibody control. There was no blocking of IFN-γ release when using anti-DQ and anti-DP monoclonal antibodies with IFN-γ release values whereas peptide recognition was completely blocked by anti-DR monoclonal antibody in both polyclonal cultures. Thereafter, these particular polyclonal cultures were lost due to bacterial infection and so the restricting allele could not be further examined using allogeneic LCLs as presenting cells. However it was clear that the epitope was likely to be presented by a DR allele shared between HK control 215 (DR 9, 16, 53, 51) and HK control 233 (DR 11, 15, 52b, 51); the only shared allele was DR51.

To examine this question of restriction another way, we re-visited the HLA typing data for the 16/33 HLA typed HK donors who responded to this epitope region in ELISpot assay. Table 3.3.3A records the HLA class II type of the 16 responders against epitope region EBNA1 474-498. Table 3.3.3B shows the frequency with which each individual allele is present among the 16 responders, and Table 3.3.3C shows the frequency with which each individual allele is present among the 17/33 HLA typed donors who did not respond to this epitope. From Table 3.3.3B, three HLA alleles are relatively common among the 16 responders, HLA DR15 (81%), DR51 (100%) and DQ6 (88%). It is therefore again likely that this epitope region is restricted through HLA DR51. It is important to note from Table 3.3.3C, however, that there were other DR51+ individuals who did not make a response against epitope region EBNA1 474-498. All of these actually made responses to other EBNA1 peptides of unknown restriction (Table 3.3.1).

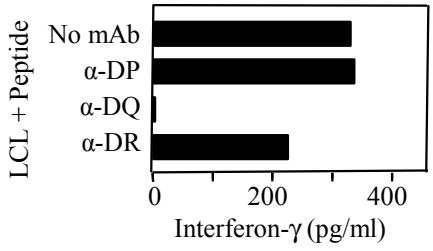
EBNA 1 479-503 HK228c81

**A**




| | |
|---|---|
| | SNPKFENIAEGLRVLLARSH |
| | ENIAEGLRVLLARSHVERTT |
| | GLRVLLARSHVERTTEEGNW |

**B**




**C**

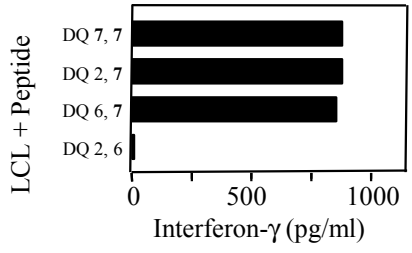


**Figure 3.3.8: Characterisation of EBNA1 479-503 using clone HK228c81**

In this assay, 500 T cells/well were cultured overnight with 50,000 autologous LCL/well pre-loaded with; (A) diluting concentrations (10-fold) of epitope region peptides or with DMSO (neg), (B) at $10^{-7}$M peptide EBNA1 484-503 and then treated with anti-DP, -DQ, -DR or culture media as no antibodies, or (C) 50,000 autologous LCL/well or allogeneic LCL/well pre-loaded with $10^{-7}$M peptide EBNA1 484-503. Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Figure 3.3.8A shows a peptide titration curve of this clones response to peptides EBNA1 474-493, 479-498, and 484-503; dose-dependant recognition was observed against all three peptides at $C_{1/2}$ values $10^{-6.5}$M (EBNA1 474-493), $10^{-7.5}$M (EBNA1 479-498) and $10^{-5.5}$M (EBNA1 484-503). Figure 3.3.8B show that this response was blocked using monoclonal antibody against HLA class II DQ and no blocking was observed with anti-DP and –DR antibodies. This epitope is restricted through the DQ7 allele (C) and not other HLA-DQ alleles (DQ2 and 6).

**A** HLA types of respondents against peptide EBNA1 479-503 (peptides 50/51)

| | HLA Types | | | | | |
|---|---|---|---|---|---|---|
| Donors | DR | DR | DR | DR | DQ | DQ |
| HK Control 201 | 11 | 13 | 52b | 52b | 7 | 7 |
| HK Control 228 | 17 | 11 | 52a | 52b | 2 | 7 |
| HK Control 230 | 11 | 14 | 52b | 52b | 7 | 5 |
| HK Control 244 | 17 | 12 | 52a | 52b | 2 | 7 |

**B** Frequency of responders with the following HLA types

| HLA type | No. Respondents | Total number of respondents | Frequency (%) |
|---|---|---|---|
| DR11 | 3 | 4 | 75 |
| DR13 | 1 | 4 | 25 |
| DR14 | 1 | 4 | 25 |
| DR17 | 1 | 4 | 25 |
| DR52a | 2 | 4 | 50 |
| DR52b | 4 | 4 | 100 |
| DQ2 | 2 | 4 | 50 |
| DQ5 | 1 | 4 | 25 |
| DQ7 | 4 | 4 | 100 |

**C** Frequency of non-responders with the following HLA types

| HLA type | No. Responders | Total number of responders |
|---|---|---|
| DR11 | 7 | 29 |
| DR13 | 1 | 29 |
| DR14 | 3 | 29 |
| DR17 | 1 | 29 |
| DR52a | 3 | 29 |
| DR52b | 10 | 29 |
| DQ2 | 1 | 29 |
| DQ5 | 11 | 29 |
| DQ7 | 16 | 29 |

**Table 3.3.4: HLA restriction of EBNA1 479-503 (peptides 50/51) using HLA typing**

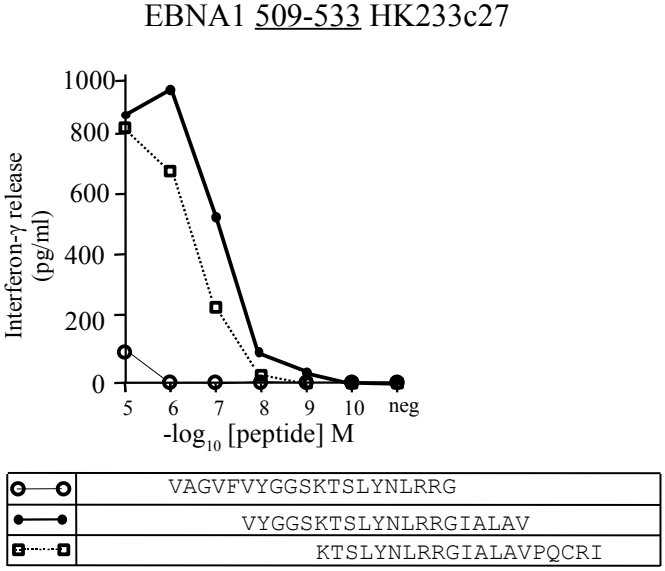
In summary, the minimal epitope for epitope region EBNA1 469-493 is EBNA1 474-493, and is likely restricted through the HLA class II DR51 allele; certainly all responding donors were DR51+, although there was a minority of DR51+ individuals in whom EBNA1 474-493 had not induced a memory response.

### 3.3.6 Analysis of responses to epitope region EBNA1 479-503

As described earlier, polyclonal cultures that were generated from HK control 201 and 228 using the epitope region peptides EBNA1 479-503 were non-specific, and therefore we could only analyse this epitope region at the clonal level. 12 clones were generated from HK Control 228, of which one (c81) was used in the initial characterisation of the epitope region. Figure 3.3.8A shows a peptide titration curve of this clones response to peptides EBNA1 474-493, 479-498, and 484-503; dose-dependant recognition was observed against all three peptides at $C_{1/2}$ values $10^{-6.5}$M (EBNA1 474-493), $10^{-7.5}$M (EBNA1 479-498) and $10^{-5.5}$M (EBNA1 484-503). The optimal peptide therefore is EBNA1 479-498. As shown in Figure 3.3.8B this response was blocked using monoclonal antibody against HLA class II DQ and no blocking was observed with anti-DP and –DR antibodies.

The HLA class II element was determined using antigen presenting cells from four different allogeneic LCLs with one or other of the relevant DQ alleles of HK control 228 (DQ2 or DQ7). These LCLs were pre-loaded with the optimal peptide, washed and incubated overnight with 500 T cells/well and supernatant harvested for detection of IFN-γ release by ELISA assay. Figure 3.3.8C shows an example of the result; DQ7-positive LCLs present the epitope peptide to the clone whereas a DQ2-positive LCL does not. This was consistant with HLA typing data. Table 3.3.4A shows the HLA class II types of the four donors who responded against this epitope region and Table 3.3.4B shows that two alleles (DR52b and DQ7) were shared by all four responders. However, note from Table 3.3.4B that the donor panel include 21 DQ7-positive donors who did not respond to this epitope region; of these 21 non-responders to EBNA1 479-498, 16 made one or more response against other epitope regions (Table 3.3.1). These findings suggest that EBNA1 epitope 479-498 is HLA DQ7-restricted but that the epitope is only immunogenic in a minority of

**A**


EBNA1 509-533 HK233c27
Interferon-γ release (pg/ml)
1000
800
600
400
200
0
5
6
7
8
9
10
neg
-log10 [peptide] M
VAGVFVYGGSKTSLYNLRRG
VYGGSKTSLYNLRRGIALAV
KTSLYNLRRGIALAVPQCRI

**B**

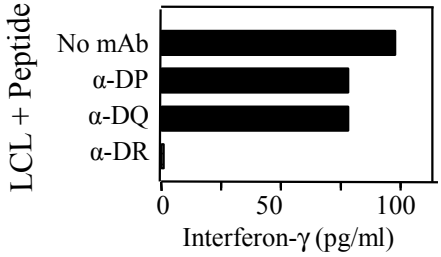

LCL + Peptide
No mAb
α-DP
α-DQ
α-DR
0
50
100
Interferon-γ (pg/ml)

**Figure 3.3.9: Characterisation of EBNA1 509-533 using clone HK233c27**

In this assay, 500 T cells/well were cultured overnight with 50,000 autologous LCL/well pre-loaded with; (A) diluting concentrations (10-fold) of epitope region peptides or with DMSO (neg) and (B) at $10^{-7}$M peptide EBNA1 514-533 and then treated with anti-DP, -DQ, -DR or culture media as no antibodies. Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Figure 3.3.9A shows the peptide titration result of peptides EBNA1 504-523, 509-528 and 514-533 against the clone. This clone responded well to two of these peptides, with a $C_{1/2}$ of $10^{-7.5}$M for EBNA1 509-528 and of $10^{-6.5}$M for EBNA1 514-533. The third peptide, EBNA1 504-523 was recognised but only at a very high concentration of $^{10-}$5M. Therefore, EBNA1 509-528 appears to be the optimal epitope. As shown in Figure 3.3.9B, recognition was blocked using monoclonal antibody against HLA class II DR alleles but was unaffected by anti-DP and anti-DQ antibodies.

**A** HLA types of respondents against peptide EBNA1 509-533 (peptides 56/57)

| | HLA Types | | | | | |
|---|---|---|---|---|---|---|
| Donors | DR | DR | DR | DR | DQ | DQ |
| HK Control 201 | 11 | 13 | 52b | 52b | 7 | 7 |
| HK Control 228 | 17 | 11 | 52a | 52b | 2 | 7 |
| HK Control 230 | 11 | 14 | 52b | 52b | 7 | 5 |
| HK Control 233 | 11 | 15 | 52b | 51 | 7 | 6 |
| HK Control 240 | 9 | 11 | 52b | 53 | 7 | 9 |
| HK Control 241 | 11 | 14 | 52b | 52b | 5 | 7 |
| HK Control 248 | 9 | 11 | 52b | 53 | 7 | 9 |
| HK Control 263 | 11 | 14 | 52b | 52b | 7 | 5 |
| HK Control 280 | 11 | 12 | 52b | 52b | 7 | 7 |
| HK Control 285 | 11 | 15 | 52b | 51 | 7 | 6 |
| HK Control 286 | 10 | 15 | 51 | 51 | 5 | 5 |

**B** Frequency of responders with the following HLA types

| HLA type | No. Respondents | Total number of respondents | Frequency (%) |
|---|---|---|---|
| DR9 | 2 | 11 | 18 |
| DR10 | 1 | 11 | 9 |
| DR11 | 10 | 11 | 91 |
| DR12 | 1 | 11 | 9 |
| DR13 | 1 | 11 | 9 |
| DR14 | 2 | 11 | 18 |
| DR15 | 3 | 11 | 27 |
| DR16 | 1 | 11 | 9 |
| DR17 | 1 | 11 | 9 |
| DR51 | 4 | 11 | 36 |
| DR52a | 1 | 11 | 9 |
| DR52b | 8 | 11 | 73 |
| DR52c | 1 | 11 | 9 |
| DR53 | 1 | 11 | 9 |
| DQ2 | 1 | 11 | 9 |
| DQ5 | 4 | 11 | 36 |
| DQ6 | 2 | 11 | 18 |
| DQ7 | 10 | 11 | 91 |
| DQ9 | 2 | 11 | 18 |

**C** Frequency of non-responders with the following HLA types

| HLA type | No. Responders | Total number of responders |
|---|---|---|
| DR9 | 3 | 22 |
| DR10 | 0 | 22 |
| DR11 | 0 | 22 |
| DR12 | 9 | 22 |
| DR13 | 1 | 22 |
| DR14 | 2 | 22 |
| DR15 | 12 | 22 |
| DR16 | 6 | 22 |
| DR17 | 1 | 22 |
| DR51 | 15 | 22 |
| DR52a | 4 | 22 |
| DR52b | 6 | 22 |
| DR52c | 2 | 22 |
| DR53 | 6 | 22 |
| DQ2 | 2 | 22 |
| DQ5 | 8 | 22 |
| DQ6 | 15 | 22 |
| DQ7 | 10 | 22 |
| DQ9 | 3 | 22 |

**Table 3.3.5: HLA restriction of EBNA1 509-533 (peptides 56/57) using HLA typing**

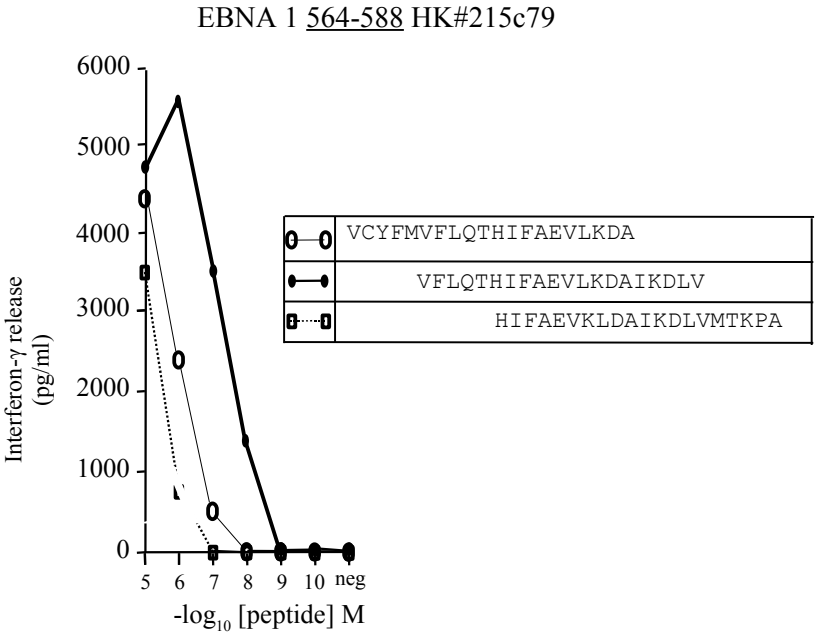
DQ7+ donors. The strength of this conclusion would have been improved by data from additional clones, but unfortunately the remaining HK control 228 clones initially identified as epitope-specific failed to grow from cryostorage.

### 3.3.7 Analysis of responses to epitope region EBNA1 509-533

Polyclonal cultures generated from HK controls 201, 228 and 233 using epitope region peptides EBNA1 509-533 were non-specific and therefore this epitope region was again characterised using just one clone, HK control 233c27. Figure 3.3.9A shows the peptide titration result of peptides EBNA1 504-523, 509-528 and 514-533 against the clone. This clone responded well to two of these peptides, with a $C_{1/2}$ of $10^{-7.5}$M for EBNA1 509-528 and of $10^{-6.5}$M for EBNA1 514-533. The third peptide, EBNA1 504-523 was recognised but only at a very high concentration of $^{10-}$5M. Therefore, EBNA1 509-528 appears to be the optimal epitope. As shown in Figure 3.3.9B, recognition was blocked using monoclonal antibody against HLA class II DR alleles but was unaffected by anti-DP and anti-DQ antibodies.

When the HLA class II types of the 11 EBNA1 509-533 responders as shown in Table 3.3.5A were analysed as in Table 3.3.5B to show the frequency of HLA class II alleles among the responding donors, there are two important points to note: there are two relatively common alleles, DR11+ (91%) and DQ7+ (91%), among responders (Table 3.3.5B) but the epitope region is restricted through a DR allele in clone HK233c27. Combining the data from the HLA class II-restriction experiments, the clone is restricted through HLA DR11. However, 10/11 responders are DR11 positive but one responder, HK control 286, is DR11 negative. It is thus likely that epitope EBNA1 509-529 is also restricted through a different HLA class II allele for HK control 286.In summary, the minimal epitope for epitope region EBNA1 509-533 is 509-528 and is possibly restricted through HLA DR11 and another HLA class II allele yet to be identified.

A


EBNA 1 564-588 HK#215c79
6000
5000
4000
3000
2000
1000
0
Interferon-γ release (pg/ml)
5 6 7 8 9 10 neg
-log10 [peptide] M
VCYFMVFLQTHIFAEVLKDA
VFLQTHIFAEVLKDAIKDLV
HIFAEVKLDAIKDLVMTKPA

B

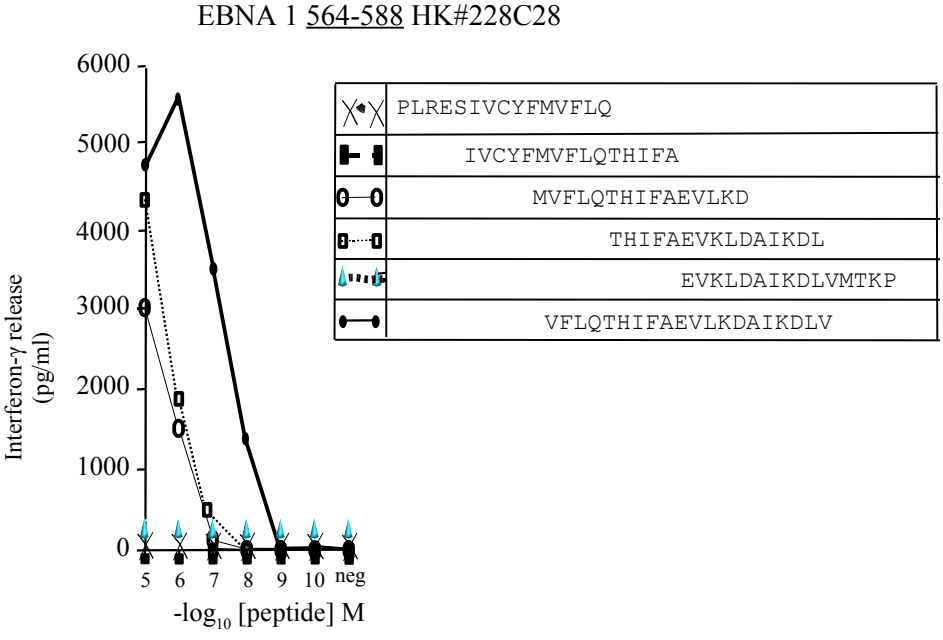

EBNA 1 564-588 HK#228C28
6000
5000
4000
3000
2000
1000
0
Interferon-γ release (pg/ml)
5 6 7 8 9 10 neg
-log10 [peptide] M
PLRESIVCYFMVFLQ
IVCYFMVFLQTHIFA
MVFLQTHIFAEVLKD
THIFAEVKLDAIKDL
EVKLDAIKDLVMTKP
VFLQTHIFAEVLKDAIKDLV

**Figure 3.3.10: Characterisation of EBNA1 564-588 using clones HK215c79 and HK228c28**

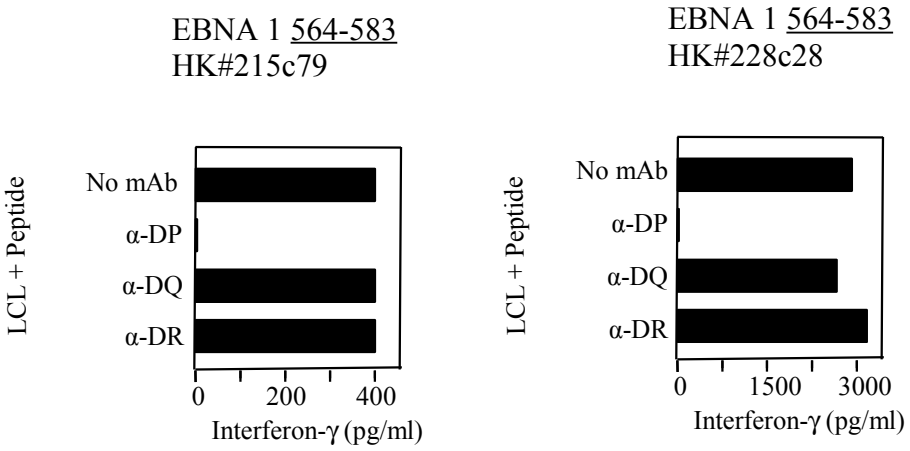
In this assay, 500 T cells/well were cultured overnight with 50,000 autologous LCL/well pre-loaded with diluting concentrations (10-fold) of epitope region peptides (20-mers and 15-mers) or with DMSO (neg). Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Figure 3.3.10A shows the titrations of EBNA1 peptides 559-578, 564-583 and 569-588 using representative clone HK control 215c79. This clone responded against the three peptides with $C_{1/2}$ values $10^{-6}$M (EBNA1 559-578) and $10^{-8}$M (EBNA1 564-583) and $>10^{-5.5}$M (EBNA1 569-588). Figure 3.3.10B shows that this clone responded to two of five 15-mer peptides (EBNA1 563-577 and 568-582) and the 20-mer peptide. However, both 15-mer peptides have a $C_{1/2}$ value at $10^{-6}$M compared to $10^{-8}$M for the 20-mer EBNA1 564-583.

### 3.3.8 Analysis of responses to epitope region EBNA1 564-588

Polyclonal cultures were generated from HK control 215 and 228 using epitope region EBNA1 564-588 peptides. Of these, HK control 228 polyclonal culture was not specific and was therefore not used in the characterisation of the epitope region. In addition the polyclonal culture from HK control 215 was lost to bacterial infection. Therefore analysis of this epitope relies on data from clones. In total, 3 HK control 215 and 23 HK control 228 clones were generated from reactivation using epitope regions peptides EBNA1 564-583 and 569-588. Figure 3.3.10A shows the titrations of EBNA1 peptides 559-578, 564-583 and 569-588 using representative clone HK control 215c79. This clone responded against the three peptides with $C_{1/2}$ values $10^{-6}$M (EBNA1 559-578) and $10^{-8}$M (EBNA1 564-583) and $>10^{-5.5}$M (EBNA1 569-588). Clearly the optimal peptide is EBNA1 564-583.

Because this epitope appeared to induce CD4+ T cell memory in 44% of the donors screened, it was of great interest to characterise it more carefully, and further work was carried out with an epitope-specific clone from the other donor HK control 228c28. Five 15-mer overlapping peptides (EBNA1 553-567, 558-572, 563-577, 568-582 and 573-587) covering the whole stretch of the three peptides from EBNA1 554-588 were made (Altabiosciences, University of Birmingham) and used in peptide titrations alongside the 20-mer EBNA1 564-583 using this clone. Figure 3.3.10B shows that this clone responded to two of five 15-mer peptides (EBNA1 563-577 and 568-582) and the 20-mer peptide. However, both 15-mer peptides have a $C_{1/2}$ value at $10^{-6}$M compared to $10^{-8}$M for the 20-mer EBNA1 564-583. Thus the optimal peptide remained as the 20-mer EBNA1 564-583. The HLA restriction element for both clones HK control 215c79 and HKcontrol 228c28 was determined using monoclonal antibody blocking assay (Figure 3.3.11A). In both cases, while no effect was observed using anti-DR and anti-DQ antibodies, there was complete blocking of IFN-γ release by anti-DP antibodies. At this point of the work, HLA DP typing was not available but subsequently four HK donor PBMC samples were HLA DP typed by the National Blood Authority (Tissue Typing Laboratory, Churchill Hospital, Oxford, United Kingdom), see Figure 3.3.11B. When these results were analysed in light of these HLA types, it became clear that presentation of this EBNA1 epitope was limited to cells

**A**


EBNA 1 564-583
HK#215c79
LCL + Peptide
No mAb
α-DP
α-DQ
α-DR
0
200
400
Interferon-γ (pg/ml)
EBNA 1 564-583
HK#228c28
LCL + Peptide
No mAb
α-DP
α-DQ
α-DR
0
1500
3000
Interferon-γ (pg/ml)

**B**

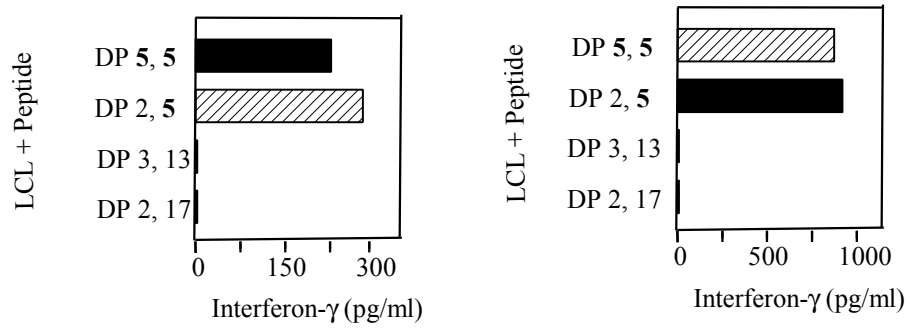
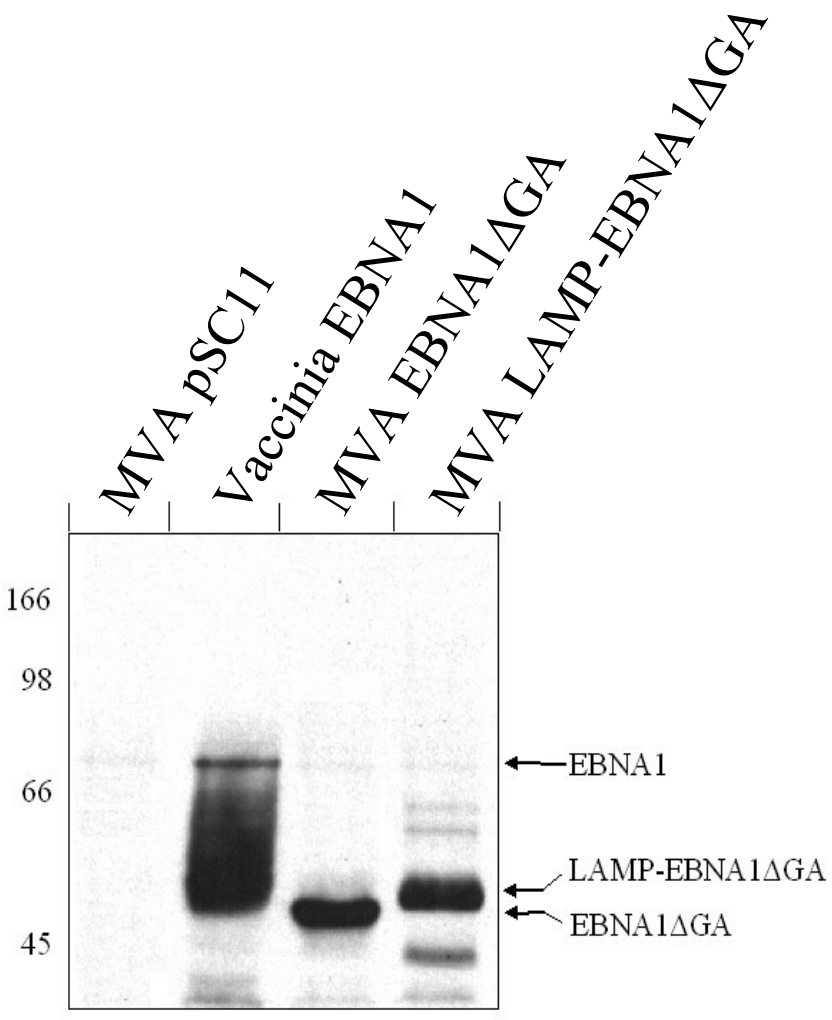

LCL + Peptide
DP **5**, **5**
DP 2, **5**
DP 3, 13
DP 2, 17
0
150
300
Interferon-γ (pg/ml)
LCL + Peptide
DP **5**, **5**
DP 2, **5**
DP 3, 13
DP 2, 17
0
500
1000
Interferon-γ (pg/ml)

**Figure 3.3.11 Determining EBNA1 564-588 restriction elements using clones HK215c79 and HK228c28**

In this assay, 500 T cells/well were cultured overnight with 50,000 autologous LCL/well pre-loaded with (A) peptide EBNA1 564-583 at $10^{-7}$M and then treated with anti-DP, -DQ, -DR or culture media as no antibodies or (B) 50,000 autologous LCL/well or allogeneic LCL/well pre-loaded with $10^{-7}$M peptide EBNA1 564-583. Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. The HLA restriction element for both clones HK215c79 and HK228c28 was determined using monoclonal antibody blocking assay (Figure 3.3.11A). In both cases, while no effect was observed using anti-DR and anti-DQ antibodies, there was complete blocking of IFN-γ release by anti-DP antibodies. Figure 3.3.11B shows that presentation of this EBNA1 epitope was limited to cells with the DP5 allele. Both donors, HK control 215c79 and HK control 228c28, were DP5-positive, and in fact HK control 215 was homozygous for this allele




western blot: anti-EBNA1 antibody

**Figure 3.3.12: Western blot of MVA protein expression**

This Figure (kindly provided by Dr. Taylor at the University of Birmingham) shows a western blot of EBNA1 expression in LCLs infected with the different viral constructs using an EBNA1 MAb for detection. Note that all LCLs show a very faint band of naturally expressed EBNA1 protein (approximately 75Kd). Cells infected with the different vaccinia viruses express the relevant EBNA1 product size; E1ΔGA (approximately 50Kd) and LAMP-E1ΔGA (approximately 55Kd). Also note cells infected with vaccinia EBNA1 (vaccinia expressing a reverse orientated construct of fully length EBNA1) shows EBNA1 of the expected size plus a range of breakdown products.

with the DP5 allele. Both donors, HK control 215c79 and HK control 228c28, were DP5-positive, and in fact HK control 215 was homozygous for this allele.

Attempts were made to test more clones cryopreserved from donors HK control 215 and 288 but unfortunately these cells again did not expand from cryostorage. Therefore in summary, the minimal epitope for the epitope region EBNA1 559-583 is 564-583 and, on the basis of clones from two independent sources, is restricted through the DP5 allele.

### 3.3.9 Functional Studies

Having determined the epitope and likely restriction element for four EBNA1 epitope regions, the next objective was to study the functional aspects of clones recognising these epitopes. At this stage only three clones HK233c27, HK228c28 and HK215c79 had maintained in culture long enough for such study. There are two questions that were of immediate interest; (i) are the clones specific to the EBNA1 antigen? and (ii) could the clones recognise naturally infected target cells?

#### 3.3.9.1 Clonal specificity for EBNA1 antigen

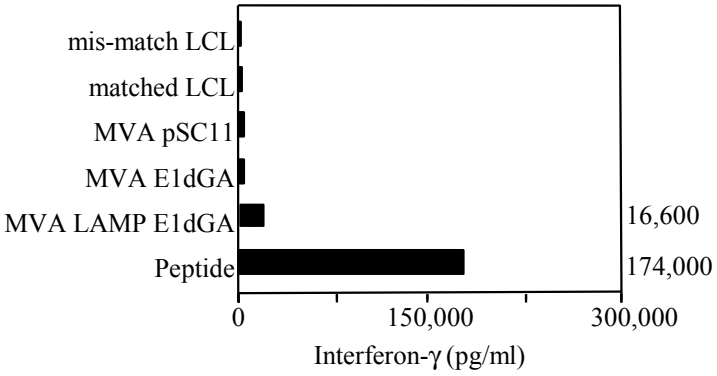
In this experiment, two modified vaccinia virus Ankara (MVA) recombinants, MVA-E1ΔGA and MVA-LAMP-E1ΔGA were used. The MVA-E1ΔGA expresses EBNA 1 with the gly-ala repeat domain removed because full-length EBNA1 vaccinia is difficult to make (Murray *et al*, 1992). The MVA-LAMP-E1ΔGA expresses gly-ala repeat domain deleted EBNA1 with a class II pathway targeting sequence (LAMP) as reported by Taylor *et al* (2004). These viruses were constructed and tested for their ability to infect LCLs as reported by Taylor *et al* (2004). Figure 3.3.12 shows a western blot of EBNA1 expression in LCLs infected with the different viral constructs using an EBNA1 MAb for detection. Note that all LCLs show a very faint band of naturally expressed EBNA1 protein (approximately 75Kd). Cells infected with the different vaccinia viruses express the relevant EBNA1 product size; E1ΔGA (approximately 50Kd) and LAMP-E1ΔGA (approximately 55Kd). Also note cells infected with vaccinia EBNA1 (vaccinia expressing

a reverse orientated construct of fully length EBNA1) shows the expression of EBNA1 of the expected size plus a range of breakdown products.

Once protein expression from these viruses was confirmed, they were used to infect selected HLA-class II matched LCLs or mis-matched LCLs for clones HK 215c79 and 228c28 to see whether the epitope EBNA1 564-583 is endogenously processed for presentation. A negative control (LCLs infected with control MVA pSC11) and a positive control (matched LCLs pre-loaded with epitope peptide) were used. The test LCLs were infected with the different viruses or loaded with epitope peptide or culture media for 1 hour. The LCLs were then washed and 500 clonal T cells were added (each assay well containing 50,000 LCLs and 500 clonal T-cells) and left to incubate overnight and assay with ELISA for IFN-γ release. Each experiment was done in triplicate wells. The results are shown in Figure 3.3.13; both clones showed recognition of LCLs infected with MVA-LAMP-E1dGA and peptide loaded LCLs but no recognition was observed against LCLs infected with MVA- E1dGA or LCL alone. It is important to note that matched LCLs infected with MVA-LAMP-E1dGA gives a lower recognition (10-20 fold difference) compared to matched LCLs preloaded with epitope peptide, this is because the epitope peptides (used at high concentrations) are fully loaded on the LCL's surface MHC class II molecules. Note that the background IFN-γ secretion is below 1000 pg/ml, therefore the response to MVA-LAMP-E1dGA infected cells are around 15-fold higher than the background.

The central point from this work is that both clones are able to detect LCLs infected with MVA-LAMP- E1dGA but not MVA- E1dGA. This clearly shows that these clones are capable of recognizing the EBNA1 antigen (if appropriately targeted into the HLA class II processing pathway) and that they are not just peptide-specific.

EBNA 1 564-583 HK#215c79




EBNA 1 564-583 HK#228c28

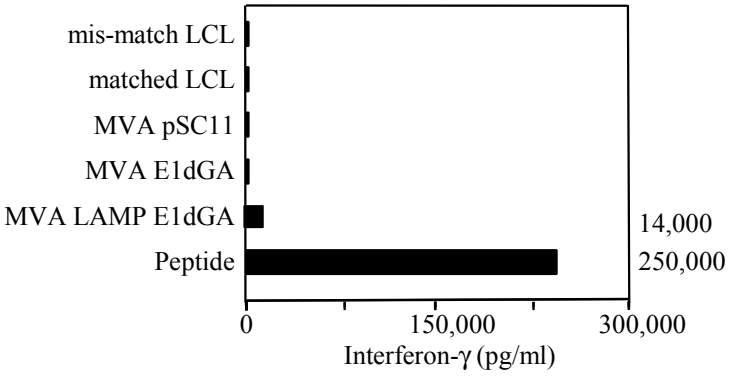


**Figure 3.3.13: EBNA1 564-583 endogenous processing and presentation**

In this assay, 500 T cells/well were cultured overnight with 50,000 autologous LCL/well pre-infected with the different MVA viruses or pre-loaded with EBNA1 564-583 peptide at $10^{-7}$M, and HLA-mismatched LCL. Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Both clones, HK215c79 (top) and HK228c28 (bottom), showed recognition of LCLs infected with MVA-LAMP-E1dGA and peptide loaded LCLs but no recognition was observed against LCLs infected with MVA- E1dGA or LCL alone. It is important to note that matched LCLs infected with MVA-LAMP-E1dGA gives a lower recognition compared to matched LCLs preloaded with epitope peptide, this is because the epitope peptides (used at high concentrations) are fully loaded on the LCL's surface MHC class II molecules.

### 3.3.9.2 Recognition of naturally infected target cells through endogenous antigen expression

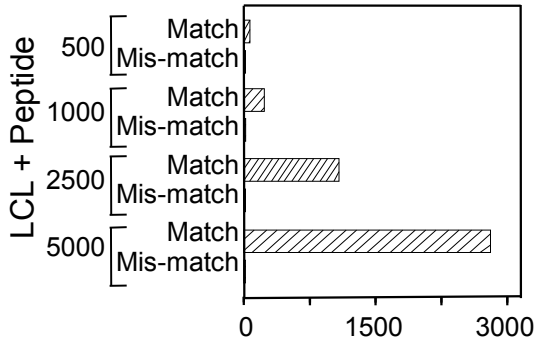
In further experiments, clones HK control 233c27 (specific for EBNA1 509-528) and 215c79 and 228c28 (specific for EBNA1 564-583) were tested for direct recognition of un-manipulated autologous LCL or allogenic mis-matched LCL. In this case, LCLs (50,000 LCL cells/well) were loaded with the epitope peptide as a positive control. And LCLs (untreated) were used at 50,000 LCL cells/well (done in triplicate wells) tested on increasing number of clonal T-cells (starting at 500, 1000, 2500 and 5000 clonal T-cells). The level of un-manipulated LCL recognition, read from a 5000 T cell input, was expressed as a percentage of that seen in the same assay against peptide-loaded LCL cells.

Figure 3.3.14A shows the data for clone HK control 215c79 against epitope EBNA1 564-583. With peptide-loaded targets, there is an increase in IFN-γ release as the number of clonal T-cells increase. There is no IFN-γ release against mis-matched LCL loaded with peptide. The same was observed with un-manipulated LCLs (Figure 3.3.14B) where an increase in T cell numbers increases recognition of matched LCLs and there is no recognition to mis-matched LCLs. This recognition of un-manipulated autologous LCL could be blocked using anti-DP blocking antibodies. Note the scales (interferon-γ (pg/ml)) between the experiment conducted with peptide and without peptide. T cell responses against LCLs loaded with peptide produces a bigger response than LCLs without peptide.

An experiment was setup to determine whether the LCL growth conditions would influence direct recognition of these LCLs by the clone HK control 215c79. Three growth densities were maintained by adjusting (where necessary) the number of cells in the culture every 3-4 days at the time of regular replacement of half the medium; condition A is maintaining LCL growth at 0.75 million cells/ml, condition B is at 1.5 million cells/ml and condition C is at 2 million cells/ml in culture for a period of 1 month prior to test. 5000 clonal T cells were use as an input since in the previous experiment described above suggests that it gives the biggest response. In the experiment, 5000 clonal T cells/well were tested on 50,000 LCL cells/well from the different growth conditions. The three

Recognition of naturally infected target cells by HK215c79

**A**




**B**

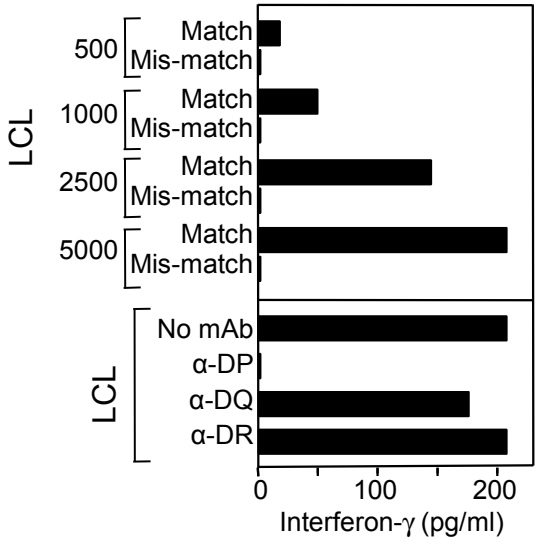


**Figure 3.3.14: Recognition of naturally infected target cells by EBNA1 564-583-specific clone HK215c79**

In this assay, increasing number of clonal T-cells (starting at 500, 1000, 2500 and 5000 clonal T-cells) were cultured overnight with 50,000 autologous LCL/well pre-loaded with EBNA1 564-583 peptide at $10^{-7}$M (A) or no peptide (B-top), or (B-bottom) 5000 clonal T cells/well were cultured overnight with 50,000 autologous LCLs treated with anti-DP, -DQ, -DR or culture media as no antibodies. Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Figure 3.3.14A shows the data for clone HK control 215c79 against epitope EBNA1 564-583. With peptide-loaded targets, there is an increase in IFN-γ release as the number of clonal T-cells increase. There is no IFN-γ release against mis-matched LCL loaded with peptide. The same was observed with un-manipulated LCLs (Figure 3.3.14B) where an increase in T cell numbers increases recognition of matched LCLs and there is no recognition to mis-matched LCLs. This recognition of un-manipulated autologous LCL could be blocked using anti-DP blocking antibodies. Note the scales (interferon-γ (pg/ml)) between the experiment conducted with peptide and without peptide, peptide coated LCLs always give a 10-fold greater response than natural targets.

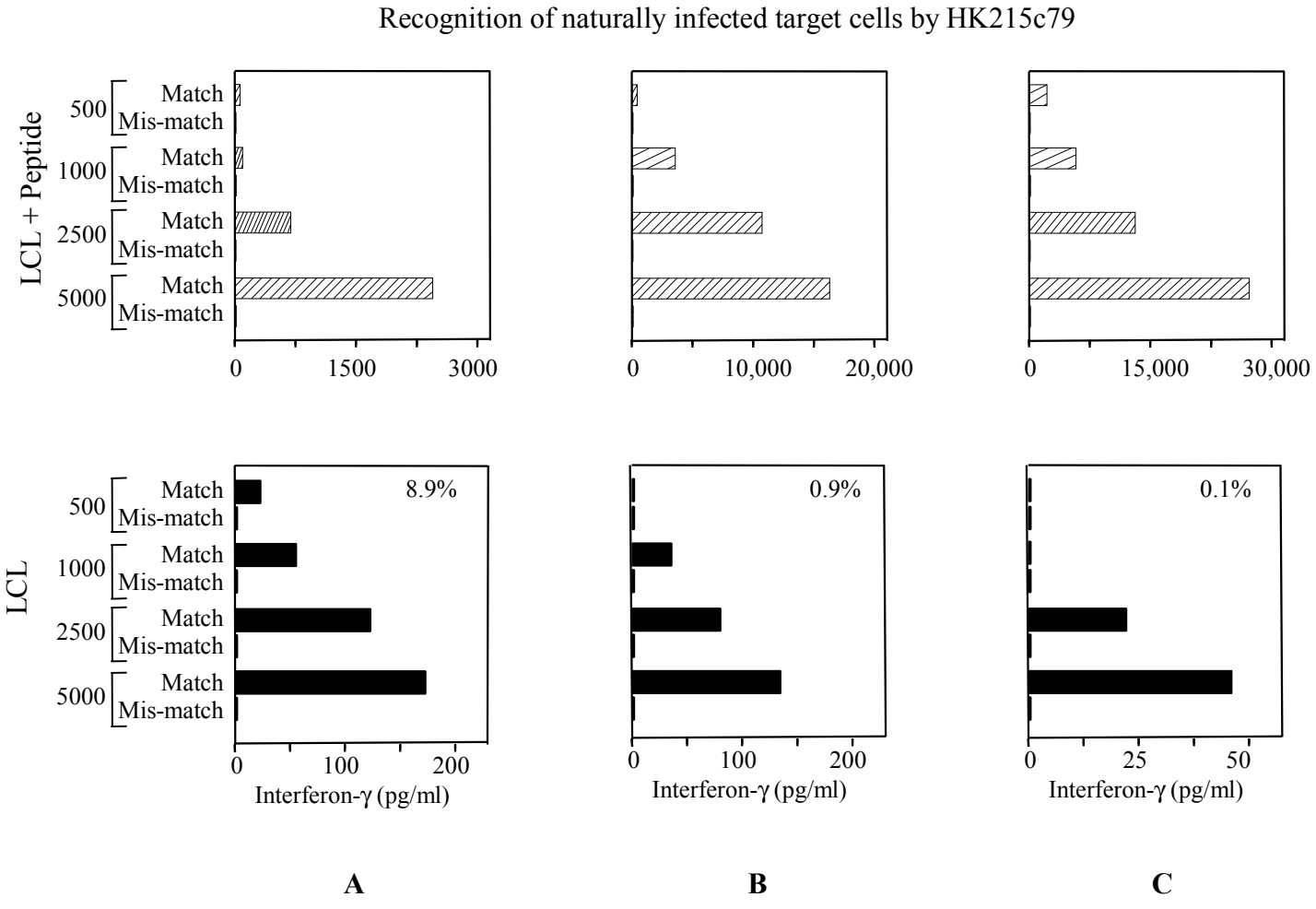

Recognition of naturally infected target cells by HK215c79
LCL + Peptide
500
Match
Mis-match
1000
Match
Mis-match
2500
Match
Mis-match
5000
Match
Mis-match
0
1500
3000
0
10,000
20,000
0
15,000
30,000
LCL
500
Match
Mis-match
1000
Match
Mis-match
2500
Match
Mis-match
5000
Match
Mis-match
8.9%
0.9%
0.1%
0
100
200
0
100
200
0
25
50
Interferon-γ (pg/ml)
Interferon-γ (pg/ml)
Interferon-γ (pg/ml)
A
B
C

**Figure 3.3.15: Recognition of naturally infected target cells, maintained at different cell density, by EBNA1 564-583-specific clone HK215c79**

In this assay, increasing number of clonal T-cells (starting at 500, 1000, 2500 and 5000 clonal T-cells) were cultured overnight with 50,000 autologous LCL/well, grown under three cell densities; (A) 0.75 million cells/ml (B) 1.5 million cells/ml and (C) 2 million cells/ml. These LCLs were either pre-loaded with EBNA1 564-583 peptide at $10^{-7}$M (top) or no peptide (bottom). Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Using clone HK control 215c79, condition A shows the lowest % LCL recognition (0.1%), with condition B at 0.9% and condition C shows the highest % LCL recognition (8.9%).

LCLs were used as targets simultaneously on the same day. Figure 3.3.15 shows the result of such experiment. Using clone HK control 215c79, condition A shows the lowest % LCL recognition (0.1%), with condition B at 0.9% and condition C shows the highest % LCL recognition (8.9%). This experiment suggests that dense LCL growth condition can increase the magnitude of LCL recognition by the T cell clones.

In the same experiment, the same target LCLs as described above, were also assayed for recognition by clone HK control 228c28. A similar pattern of results were observed. Figure 3.3.16 shows the lowest LCL recognition with condition A at 0.1%, condition B has a 0.2% LCL recognition and the highest LCL recognition at 1.8% (condition C).

Figure 3.3.17 shows the result for HK control 233c27 against epitope EBNA1 509-528. However, in this case, all three experiments were using LCL targets mentioned in condition C as described above. This is because clone HK control 233c27 was growing poorly in culture and at any one time there was insufficient T cell numbers to use multiple targets. The clone was therefore tested, on three separate occasions, using the condition that gave the highest LCL recognition. The results demonstrate that the percentage LCL recognition is repeatable, ranging from 7.9-8.6% of the optimal value seen against peptide-loaded LCL in the same assay.

### 3.3.10 Discussion

The primary objectives of this work were (i) to determine the minimal epitope within the epitope regions and its restriction element and (ii) to determine the capacity of these EBNA1-specific $CD4^+$ T cell clones to see naturally infected target cells

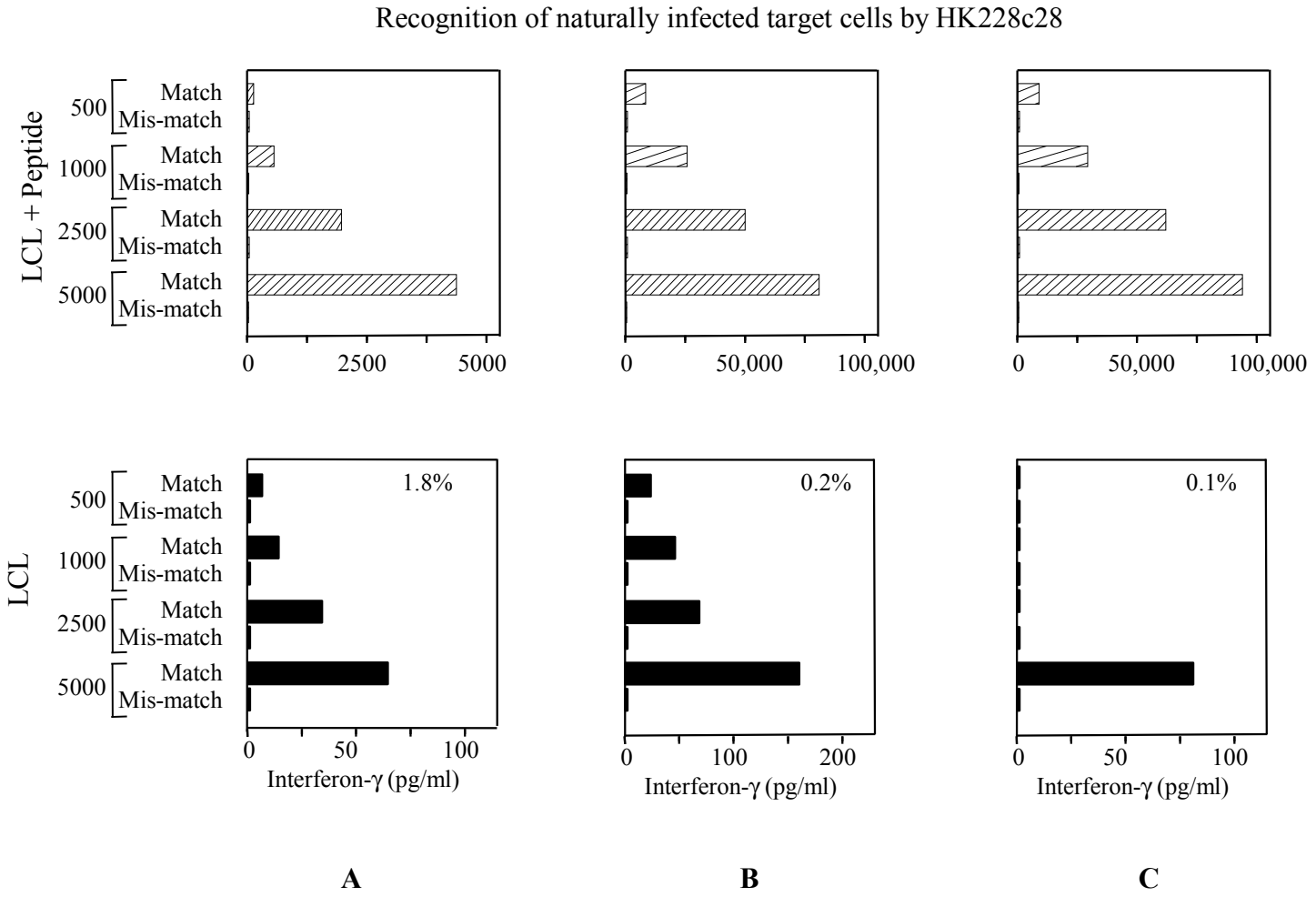

Recognition of naturally infected target cells by HK228c28
LCL + Peptide
500
1000
2500
5000
Match
Mis-match
0
2500
5000
0
50,000
100,000
0
50,000
100,000
LCL
1.8%
0.2%
0.1%
0
50
100
0
100
200
0
50
100
Interferon-γ (pg/ml)
Interferon-γ (pg/ml)
Interferon-γ (pg/ml)
A
B
C

**Figure 3.3.16: Recognition of naturally infected target cells, maintained at different cell density, by EBNA1 564-583-specific clone HK228c28**

In this assay, increasing number of clonal T-cells (starting at 500, 1000, 2500 and 5000 clonal T-cells) were cultured overnight with 50,000 autologous LCL/well, grown under three cell densities; (A) 0.75 million cells/ml (B) 1.5 million cells/ml and (C) 2 million cells/ml. These LCLs were either pre-loaded with EBNA1 564-583 peptide at $10^{-7}$M (top) or no peptide (bottom). Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Using clone HK control 228c28, shows the lowest LCL recognition with condition A at 0.1%, condition B has a 0.2% LCL recognition and the highest LCL recognition at 1.8% (condition C).

Recognition of naturally infected target cells by HK233c27

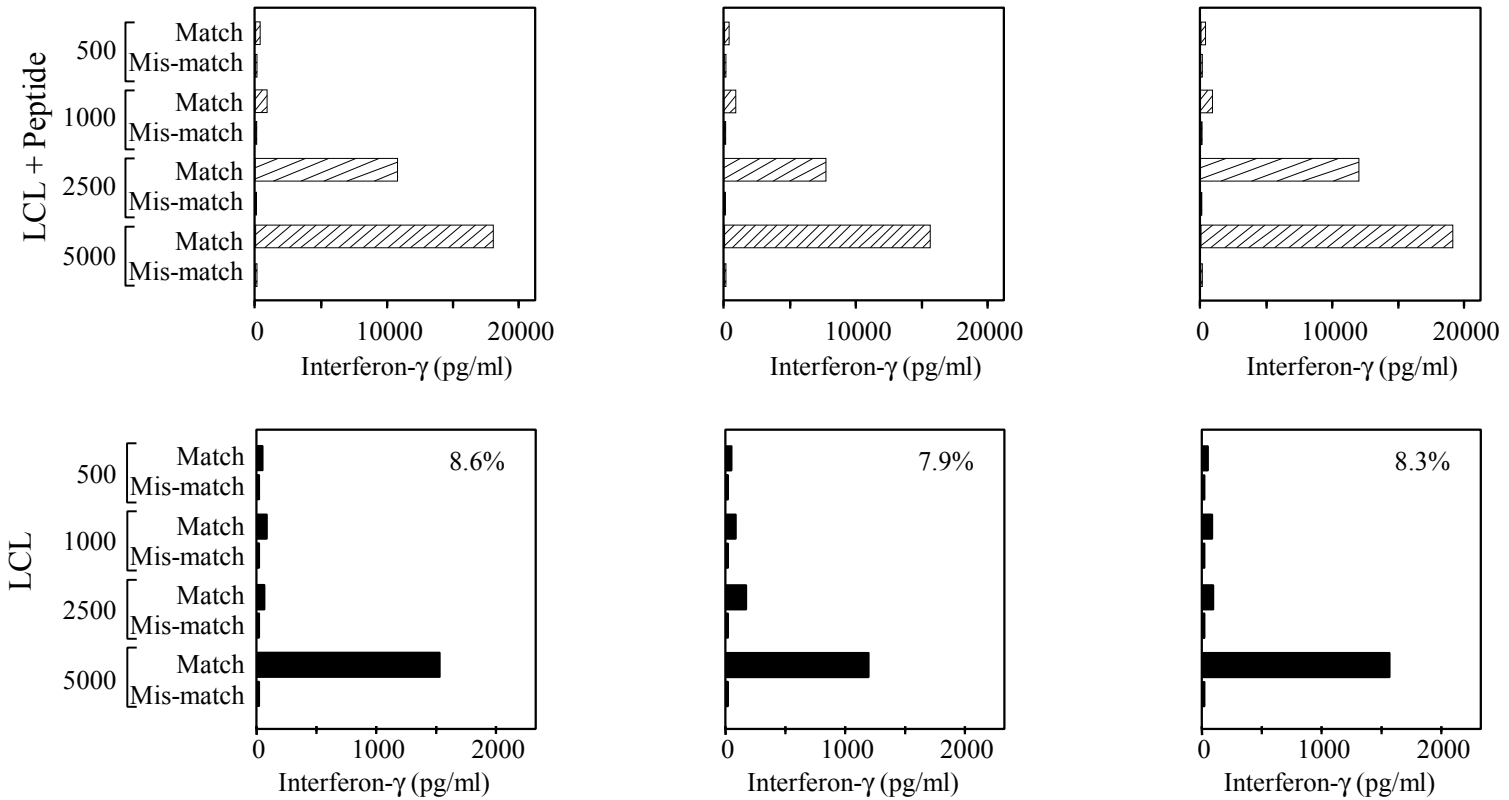


**Figure 3.3.17: Recognition of naturally infected target cells by EBNA1 509-528-specific clone HK233c27**

In this assay, increasing number of clonal T-cells (starting at 500, 1000, 2500 and 5000 clonal T-cells) were cultured overnight with 50,000 autologous LCL/well maintained in culture at 2 million cells/ml. These LCLs were either pre-loaded with EBNA1 564-583 peptide at $10^{-7}$M (top) or no peptide (bottom). Recognition was detected by IFN-γ released into the supernatant and assayed by ELISA. The results are shown as IFN-γ release expressed as pg/ml. Using clone HK control 228c28, shows the lowest LCL recognition with condition A at 0.1%, condition B has a 0.2% LCL recognition and the highest LCL recognition at 1.8% (condition C).

### 3.3.10.1 Problems with generating and maintaining clones

One of the major obstacles with this section of work was in maintaining and generating epitope-specific polyclonal cultures and clones. In theory, using peptides derived from epitope-rich region of EBNA1 should generate polyconal cell cultures that are specific to at least one or more of the epitope regions. As described earlier in section 3.3.2, various medium conditions were tested and I found that using serum free media in the reactivation of PBMCs was the optimal condition to generate epitope-specific polyclonal cell cultures. This was therefore used in the main experiments.

Of the 13 polyclonal cell cultures generated, only 3 were tested epitope-specific. It is important to note that epitope-specific clones could be generated from both epitope-specific and non-specific polyclonal cell culture. However, most of these epitope-specific clones were lost during the expansion process, after newly generated clones were transferred from their 300µl cloning wells to larger 2ml wells. This transfer of cells from a small volume environment (where cells were having sufficient cell-cell contact) to a larger volume environment (where cells may not be having sufficient cell-cell contact) may be the underlying reason why clones were lost. Possibly, if the clones were expanded in several 300µl cloning wells to reach a bigger population before transferring to the 2ml wells would have been a better option so that sufficient cell-cell contact between the clones could be maintained.

Secondly, during the expansion process, many of the clones that grew lost their peptide-specificity. Clones were re-stimulated every 2-3 weeks with γ-irradiated autologous LCLs preloaded with epitope peptides until the clones grew to a population of 1 million cells/well in 10x2ml wells. This constant bombardment of epitope peptides may have desensitized the TCR for the epitope peptide possibly causing downregulation (Taams *et al*, 1999).

Thirdly, clones that were frozen down for later experiments in cryostorage failed to recover and grow when thawed. Clones from cryostorage were thawed on day 0 and stimulated immediately with γ-irradiated autologous LCLs preloaded with epitope peptide. Maybe if

these cells were rested over a longer period of time to allow recovery before reactivation, then they would have expanded. And these cells should have been kept in 300µl wells during this period for optimal cell-cell contact before being transferred to 2ml wells so that an appropriate population could be reached to maintain the cell-cell contact needed.

**3.3.10.2 Restricting the minimal epitope and its restriction elements.**

Both polyclonal cell cultures and clones were used to determine the minimal epitope within an epitope region. Table 3.3.6 summarises the findings in this work. 4 epitope regions were minimized to individual 20-mer epitope peptide. In some cases where both polyclonal cell culture and clones were available, epitope peptide titration experiments using polyclonal cell cultures gave data consistent with data derived from clones. In other cases polyclonal cell culture and clone data could be different; for example, with the epitope region EBNA1 469-493, polyclonal cell cultures could see both peptides within this region while the clone could only see peptide EBNA1 474-493. The polyclonal data suggests the possibility that there is multiple TCR usage in the recognition of this epitope region (EBNA1 469-493). Therefore it would be important to generate different clones against this epitope region and try to correlate different fine specificities against the two different peptides with their different TCR usage.

Analyses showed that the EBNA1 epitope 519-543, seen by 44% of responders tested was restricted through the HLA DP5 allele. This allele is itself very common in Southern Chinese, seen in 48.1% of the population (Tsuji, Aizawa and Sasazuki, 1991), thereby explaining why this epitope region is frequently immunogenic. More clones need to be generated from other responding donors to determine whether it is because EBNA1 519-543 is indeed restricted through one very common HLA allele or whether it can be immunogenic in the context of other MHC class II elements. It would also be interesting to determine whether TCR usage among the response to this epitope was broader than

| Epitope | Likely restriction elements (T cell data) | HLA typing data |
|---|---|---|
| 434-453<br>439-458 | n.t | Possible DR11, DR52b and/or DQ7 |
| 474-493 | DR | Possible DR51 |
| 479-498 | DQ7 | DQ7 |
| 499-518<br>504-523 | n.t | Possible DR11, DR52b and/or DQ7 |
| 509-528 | DR | DR11 and possibly DR15 and/or other HLA alleles |
| 519-538<br>524-543 | n.t | INCONCLUSIVE |
| 549-568 | n.t | INCONCLUSIVE |
| 554-573<br>559-578 | n.t | DQ7 and possibly other HLA alleles |
| 564-583 | DP5 | DP 5 |
| 589-608<br>594-613 | n.t | INCONCLUSIVE |

**Table 3.3.6: Summary of epitope/epitope regions and their likely HLA restriction molecules**

n.t – not tested

that seen among response to other less frequent immunogenic epitopes, e.g., the DQ7 restricted epitope EBNA1 479-498.

### 3.3.10.3 Frequency of responses to EBNA1 epitopes in Chinese individuals as an indication of immunodominance

At this point immunodominance among $CD4^+$ T cell responses is revisited. As defined in Section 3.2, two factors must be considered when looking for immunodominance; the frequency of response and the response size. Immunodominance among $CD4^+$ T cell responses has to be looked at in terms of the frequency of response rather than in terms of the size of response. This is because $CD4^+$ T cell responses exist within a restricted size range and do not show consistent differences in size when comparing between epitopes. In relation to frequency of response, the DP5-restricted epitope EBNA1 564-583 is the immunodominant EBNA1 epitope seen in 44% of the healthy Chinese control screened. This immunodominance, using frequency of response among the population at large could be summarized as follow:

EBNA1 564-583>>474-493 and 519-543>554-578> 509-528>479-503>434-458 and 499-523>549-568 and 589-613

How is this different to what we see in the Caucasian study? $CD4^+$ T cell responses against EBNA1 is only partially characterised in Caucasians, but with the current information, we are seeing a different response pattern. In the Caucasian study, Leen *et al* (2001) has identified more epitopes, a total of 17 epitopes, whereas in this study only 10 epitopes has been identified even though, a considerably larger population group has been screened. The Caucasian study has identified four immunodominant epitopes (EBNA1 475-489, 485-499, 515-528, 529-543) with a frequency of response between 20-25% of the population screened. In the present study, one immunodominant DP5 restricted epitope EBNA1 564-583 has been identified with a frequency of response of 44% of the population screened, plus three relatively frequently observed epitopes EBNA1 519-543, 474-493, and 554-578 with frequency of responses matching that of the dominant epitopes identified in the

Caucasian study, of 18-22% of the population studied. Overall, fewer epitopes have been identified in this study, four epitopes (EBNA1 564-583, 519-543, 474-493, and 554-578) are seen by a larger proportion of the studied population than the epitopes identified in the Caucasian work. However, if more healthy Chinese controls were screened against the entire EBNA1 peptide panel, possibly rarer responses could be identified, such as responses to the N-terminal portion of the EBNA protein.

However, in Leen *et al* (2001) work, they have also studied $CD4^+$ T cell responses against EBNA3C, LMP1 and LMP2 and found that EBNA1 and EBNA3C is more frequently recognised than LMP1 and LMP2 (EBNA1, EBNA3C>>LMP1,LMP2). It would be interesting to carry our work further into screening for $CD4^+$ T cell responses against LMP1 and LMP2 synthetic peptides in healthy Chinese donors and to determine and compare that with the Caucasian study.

**3.3.10.4 Processing of Endogenous Expressed EBNA1**

The MVA experiments have demonstrated that over expression of EBNA1 endogenously in an overnight assay does not increase antigen presentation unless the EBNA1 is targeted (using a LAMP sequence) to the class II pathway, this has also been reported by Taylor *et al* (2004). This suggests that untargeted EBNA1 does not have direct intracellular access into the class II pathway of antigen presentation, but suggest that there may be a slower (possibly indirect) route of entry into this pathway.

Some access to the HLA class II pathway by EBNA1 has been demonstrated in the LCL recognition experiments because clones HK control 215c79 and 228c28 (to EBNA1 564-583) and 233c27 could recognise un-manipulated LCLs expressing endogenous EBNA1. LCL recognition by the clones increases when exposed to LCL cells that have been cultured at high density (2 million cells/ml) condition.

It has been suggested that EBNA1 processing by the MHC class II pathway may possibly be through autophagocytosis (Nimmerjahn *et al*, 2003; Geuze, 1998; Théry *et al*, 1999) or

other routes since the biosynthetic pathway of MHC class II intersects that of the endocytic pathway (Wolf and Ploegh, 1995; Watts, 1995). However, autophagy may seem to be a very unlikely route for the processing of EBNA1 to MHC class II molecules since overexpression of native EBNA1 by MVA vector is unable to increase $CD4^+$ T cell responses to LCLs, whereas only LAMP-target EBNA1 is able to increase $CD4^+$ T cell responses against EBNA1 epitope. However, using the same experimental model but increasing the total timeframe of infection to 42hours (including the 18 hour T cell assay) Taylor *et al* (2004) observed a slight increase in $CD4^+$ T cell responses to the LCLs above that of uninfected LCLs. This suggests that either that autophagy is taking place but it requires a longer time, or that there is another slower route of EBNA1 processing.

In another experiment with $CD4^+$ T cells specific for EBNA2 and EBNA3C epitopes, Taylor *et al* (2004) showed that co-culturing HLA matched recipient cells lacking the antigen with HLA-mismatched donor cells expressing the antigen would lead to detectable $CD4^+$ T cell recognition that increases with increasing time of co-culture. This suggested that some form of antigen transfer from one cell to another is taking place.

The present work demonstrated, at least in terms of LCL recognition, that naturally expressed EBNA1 antigen could gain access to the HLA class II pathway for processing and presentation. The conditioning of LCL growth influences the ability of the clones to recognize antigen expression, such that, LCL grown under thicker density would increase recognition by the clones. It is possible that LCL grown in high cell density are starved of nutrient thus triggering autophagy, or that there are more cells producing EBNA1 and secreting the protein into the surrounding medium for neighbouring cells to pick up and process. The mechanism of how EBNA1 could gain access to the HLA class II processing machinery was not studied. But, it would be of great interest to study the underlying mechanism, for example (1) using autophagy inhibitors to determine whether LCL recognition could be blocked, or (2) by conducting a co-culture experiment, as described by Taylor *et al* (2004), to see whether EBNA1 expressed in HLA mis-matched LCLs could be

transferred to HLA matched EBV-negative antigen presenting cells and induce recognition by the T cell clones.

### 3.3.10.5 Effector function of EBNA1 clones

It would be interesting to determine whether EBNA1 $CD4^+$ T cell clones have cytotoxic capacity as well IFN-γ release, since such cells could have the potential to eliminate EBNA1-expressing tumours. In a preliminary experiment, the cytotoxic capacity of clones HK control 215c79 and 228c28 was looked at. Appendix Figure A3.3.1 shows the result of a one-off experiment which was conducted at the end of this work. Lysis of autologous LCLs pre-loaded with epitope peptide could be seen as high as 50% in the 18 hour assay and as low as 25% in the 12 hour assay.

However, it would be important to properly conduct this experiment looking at IFN-γ release in parallel to cytotoxic capacity to determine whether killing is induced by IFN-γ release or other means. It would be interesting to conduct further experiments looking at the mechanism of killing using various inhibitors, such as cyclosporine A (CSA) inhibition of IFN-γ release, concanamycin A to block perforin-mediated killing and the blocking anti-Fas antibody 2B4 to prevent Fas-mediated lysis. Also, it would be important to determine whether these clones are capable of controlling the out-growth of LCL cells in longer term assays, since the activity may mimic tumour-control *in vivo* by the same effectors. In this type of assay, LCLs are incubated either alone or cocultured with clonal T cells (at different T cell to LCL ratios) to determine whether the clones could suppress the growth of LCLs. The mechanism of suppression, if any, could be determined using various inhibitors.

# Section 4

# Concluding Discussion

## 4.0 Concluding Discussion

In the studies presented by this thesis, it was concluded in section 3.1, that the predicted BART products and BARF1 protein are of little or no immunotherapeutic value in the treatment of NPC. This was concluded on the basis that no CD8+ and CD4+ T cell responses were detected by IFN-γ ELISpot assay. Detection of T cell responses using the ELISpot assay was best suited for this study because of its unique ability to detect T cell specificity and number (strength of the response) in PBMC samples. In the context of immunotherapy, failure to detect these responses, in healthy EBV carriers, would reflect the rarity of these responses *in vivo*, if they do exist. This may also reflect that such responses would also be rare in NPC patients. It could be argued, at least from data presented by Kienzle *et al* (1998), that BARF0-specific CD8+ T cell responses, even though are rare, could be cloned from EBV-seropositive individuals (not in EBV naïve individuals). More importantly, these clones also recognised and kill BARF0-expressing target cells. These data reflect that BARF0-specific CD8+ T cells are present *in vivo* and the BARF0 protein must also be expressed during EBV infection. There are two points that need looking into: (1) The EBV-seropositive donor in which Kienzle *et al* (1998) generated BARF0-specific CD8+ T cell clones from was also incorporated into this thesis work, UKL25. This individual, at the time of this thesis work, was EBV-seronegative as indicated from the VCA staining study. Furthermore, CD8+ T cell responses against the BARF0 peptides were not detectable by IFN-γ ELISpot assay, and the 9-mer HLA-A2 peptide was also incorporated into this study, but no response was detected against this peptide. A possible explanation is that Kienzle *et al* (1998) cloned a naïve CD8+ T cell by chance from the T cell repertoire. However, Kienzle *et al* (1998) also report that they found BARF0-specific CD8+ T cell response in another EBV-seropositive donor and this response could not be detected in 2 EBV seronegative donors. Considering UKL25 is EBV seronegative, of the four donors tested, 3 were EBV-seronegative and 1 was EBV-seropositive. More importantly, as indicated by the study in this thesis, if responses to BARF0 or the other BART products and BARF1 exist, it could not be detected by IFN-γ assay. (2) There is still yet no substantial evidence which suggest the BART products and BARF1 protein are expressed at the protein level.

Failure to detect T cell responses against the predicted BART products and the BARF1 protein resulted in a decision to shift the direction of this thesis work looking for CD4+ T cell responses to EBNA1, a protein that is uniformly expressed in NPC tumours. CD4+ T cell responses to EBNA1 have been studied in Caucasian people, however, little is known about the EBNA1-CD4+ T cell epitopes presented by the HLA alleles in Chinese people, the population in which NPC is very common. In this section of work, section 3.2, CD4+ T cell responses were detected in 78 healthy Chinese EBV carriers by IFN-γ ELISpot assay. Analysis of these CD4+ T cell responses lead to the mapping of 10 epitope regions, all of which clustered at the C-terminal half of the protein. One particular epitope region stood out from the rest, epitope region EBNA1 564-588, to which almost half of the donors tested had detectable CD4+ T cell response to this region. We speculated that this epitope region is presented through an HLA allele that is possibly common to Chinese people, but further tests are required to confirm this. Comparison of the Chinese work with the Caucasian study identified 6 epitope regions that are unique to Chinese responses and 4 epitope regions which overlap responses that have already been identified in the Caucasian study. This section of work demonstrated that HLA polymorphism between the Chinese and Caucasian population would contribute to the differences in the presentation of EBNA1 epitopes to CD4+ T cells. This is because CD4+ T cell epitopes identified in the Chinese study are different, at least 6 of the epitopes are unique to Chinese, from those identified in the Caucasian study. It could be argued that the differences in the amino acid composition in Chinese EBNA1 and Caucasian EBNA1 may contribute to this factor. However, we also determined that Chinese CD4+ T cells could also recognise Caucasian EBNA1 peptides. This strongly suggested that the amino acid composition, in this case, does not contribute to the identification of 6 unique Chinese epitopes. More importantly, all CD4+ T cell responses are possibly of the Th1 phenotype since the ELISpot assay was set to measure IFN-γ secretion by responding CD4+ T cells. Other studies have shown that Th1 cells are important in the control of EBV infection and could recognise and suppress LCL growth. In this study, the phenotype of these EBNA1-specific CD4+ T cells were not determined and further studies are required to determine the phenotype of these IFN-γ secreting cells and their possible role in EBV infection. However, if these Chinese EBNA1-specific Th1 cells

do recognise and control LCL growth *in vitro*, then it would be a good indication that they could also recognise and control tumour cells *in vivo*.

Therefore, in section 3.3 of this work, the EBNA1-specific CD4+ T cells were cloned to determine their function roles in EBV infection. Generating CD4+ T cell clones for the epitope region EBNA1 559-583 was of great interest because around 50% of Chinese donors tested gave a response to this region of peptides. Two clones, HK215c79 and HK228c28, from two separate responders, HK control 215 and 228, were generated. Both clones were specific to a 20-mer peptide presented through an HLA-DP5 allele, an allele which is common in Chinese, >50% of Chinese people were reported to carry this allele (Tsuji *et al*, 1993). The frequency of the HLA-DP5 allele was reflected in the ELISpot data, of which, around 50% of Chinese donors tested had a response to the epitope region. In addition to recognition of the peptide EBNA1 564-583, these clones were demonstrated to also recognise the EBNA1 protein and also recognition of naturally infected HLA-DP5+ target cells. More importantly, preliminary CTL data suggest both clones also kill naturally infected target cells. Furthermore, another clone was described, generated from HK control 233, HK233c27, recognises an HLA-DR restricted epitope EBNA1 509-528 (the exact HLA-DR restriction element is yet to be determined). Preliminary data suggest this clone also recognises naturally infected autologous target cells. These data strongly suggest that CD4+ T cells generate from different EBNA1 epitopes could recognise EBV infected cells exist in the T cell repertoire *in vivo*, and they can kill EBV infected cells. In the context of immunotherapy to treat NPC, such data suggest adoptive T cell therapy of tumour recognising and tumour killing EBNA1-specific CD4+ T cells is a possible strategy towards treating the NPC. Or, the EBNA1 protein could be made into a vaccine, either as a polyepitope vaccine as in the experiments described by Duraiswamy *et al* (2004) to express only the epitopes which induce EBNA1-specific CD4+ T cells that recognise and kill tumour cells, or as an EBNA1-LMP1 chimeric vaccine to boost both CD4+ and CD8+ T cell responses against the tumour (Taylor *et al*, 2004). The HLA-DP5 restricted epitope is of great importance because of the high frequency of the HLA-DP 5 allele in the Chinese population would suggest >50% of NPC patients would also have this allele. However,

further studies looking at CD4+ T cell responses against EBNA1 in NPC patients are need to determine if this speculation is also true for patients with NPC.

The future direction of this work would be to generate more EBNA1-specific CD4+ T cell clones and characterise these clones for recognition and killing of naturally infected target cells. So far, much of the focus has been centred on CD4+ T cells as effectors, however, the role of CD4+ T cells as 'helper cells' to maintain an effective CD8+ T cell response to EBV infection is as important. Therefore, phenotype studies would be as important in the characterisation of EBNA1-specific CD4+ T cells, such as their cytokine secretion profile. In addition to studying the role of EBNA1-specific CD4+ T cells in the control of EBV infection, it would be important to study whether EBNA1 would induce Treg cells. Treg cells can suppress the activation and proliferation of CD4+ and CD8+ T cells and is important in controlling autoreactive T cells. *In vitro* studies have shown that EBNA1 can induce IFN-γ secreting CD4+ T cells with Treg phenotype CD25+Foxp3+GITR+ (Voo *et al*, 2005). More importantly, Lau *et* al (2005) demonstrated that there is an increase in Treg cells in the peripheral blood and TILs of NPC patients, however, in this study the authors did not determine whether these Treg cells were induced by EBNA1. Inducing Treg response would be a problem in immunotherapy since these cells would inhibit T cell control of EBV infection *in vivo*. This would not be a problem for adoptive T cell therapy since T cells that are used in adoptive T cell therapy are expanded *ex vivo* and selectively chosen for their antitumour function. However, when designing a therapeutic vaccine to boost T cell responses *in vivo*, it would be important not to incorporate epitopes that induce Treg cells.

Furthermore, it would be interesting to study CD4+ T cell responses to LMP1 and LMP2 in healthy Chinese EBV carriers, since LMP1 and LMP2 are two other proteins that are expressed NPC tumours. Eventually, this work would extend further into determining CD4+ T cell responses against EBNA1, LMP1 and LMP2 in the peripheral blood and TILs of NPC patients. This would provide an overview of the EBV-immune status of NPC patients to help identify responses that are 'missing' so that 'missing' responses could be

put back into the patients. Or NPC patients may have ‘inappropriate responses’ such as Treg responses, in this case, Treg cells could be depleted and effector and ‘helper’ cells could be boosted. These are several of the scenarios that future work would be looking into.

# Section 5

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# Section 6

# Appendix

| Derived sequence | Peptide coordinate | B95.8 Peptide sequence | Denoted peptide reference to text |
|---|---|---|---|
| **A73**‡ | 1-20† | MSMPPKGFLKKEMKPETRLL‡‡ | A1†† |
| **A73** | 6-25 | KGFLKKEMKPETRLLNKPPT | A2 |
| **A73** | 11-30 | KEMKPETRLLNKPPTVLTRP | A3 |
| **A73** | 16-35 | ETRLLNKPPTVLTRPAMFCA | A4 |
| **A73** | 21-40 | NKPPTVLTRPAMFCAWKLYS | A5 |
| **A73** | 26-45 | VLTRPAMFCAWKLYSRKMPS | A6 |
| **A73** | 31-50 | AMFCAWKLYSRKMPSRSKTL | A7 |
| **A73** | 36-55 | WKLYSRKMPSRSKTLEARCS | A8 |
| **A73** | 41-60 | RKMPSRSKTLEARCSSRPPC | A9 |
| **A73** | 46-65 | RSKTLEARCSSRPPCDSPAC | A10 |
| **A73** | 51-70 | EARCSSRPPCDSPACQTRDT | A11 |
| **A73** | 56-75 | SRPPCDSPACQTRDTGCPRR | A12 |
| **A73** | 61-80 | DSPACQTRDTGCPRRSGTGR | A13 |
| **A73** | 66-85 | QTRDTGCPRRSGTGRRGWRA | A14 |
| **A73** | 71-90 | GCPRRSGTGRRGWRARRLGK | A15 |
| **A73** | 76-95 | SGTGRRGWRARRLGKESWFA | A16 |
| **A73** | 81-100 | RGWRARRLGKESWFADAWRM | A17 |
| **A73** | 86-105 | RRLGKESWFADAWRMARYWG | A18 |
| **A73** | 91-110 | ESWFADAWRMARYWGCAVKA | A19 |
| **A73** | 96-115 | DAWRMARYWGCAVKAAAQSA | A20 |
| **A73** | 101-120 | ARYWGCAVKAAAQSAFSAST | A21 |
| **A73** | 106-126 | CAVKAAAQSAFSASTASPEEL | A22 |

**Table A3.1.1: 22 overlapping peptides covering A73**

‡The peptide sequence is derived from this predicted polypeptide product

†Peptide coordinate in relation to the full length product

‡‡The amino acid sequence of the 20-mer peptide

††The denotation used in the main text to refer to this 20-mer peptide

| Derived sequence | Peptide coordinate | B95.8 Peptide sequence | Denoted peptide reference to text |
|---|---|---|---|
| **BARFO** | 1-20 | MAATLPLPRCTDSMAARVPI | RK1 |
| **BARFO** | 6-25 | PLPRCTDSMAARVPIEELRE | RK2 |
| **BARFO** | 11-30 | TDSMAARVPIEELRELRHLR | RK3 |
| **BARFO** | 16-35 | ARVPIEELRELRHLRGHCRE | RK4 |
| **BARFO** | 21-40 | EELRELRHLRGHCREDVVGV | RK5 |
| **BARFO** | 26-45 | LRHLRGHCREDVVGVQRSGR | RK6 |
| **BARFO** | 31-50 | GHCREDVVGVQRSGRPLCLR | RK7 |
| **BARFO** | 36-55 | DVVGVQRSGRPLCLRPPRAR | RK8 |
| **BARFO** | 41-60 | QRSGRPLCLRPPRARDRALL | RK9 |
| **BARFO** | 46-65 | PLCLRPPRARDRALLWAARP | RK10 |
| **BARFO** | 51-70 | PPRARDRALLWAARPRLLLS | RK11 |
| **BARFO** | 56-75 | DRALLWAARPRLLLSLQQVP | RK12 |
| **BARFO** | 59-67 | LLWAARPRL* | RK13 |
| **BARFO** | 66-85 | RLLLSLQQVPEPRLADFILK | RK14 |
| **BARFO** | 71-90 | LQQVPEPRLADFILKQSRDR | RK15 |
| **BARFO** | 76-95 | EPRLADFILKQSRDRLRIHR | RK16 |
| **BARFO** | 81-100 | DFILKQSRDRLRIHRHRQVV | RK17 |
| **BARFO** | 86-105 | QSRDRLRIHRHRQVVTGDVG | RK18 |
| **BARFO** | 91-110 | LRIHRHRQVVTGDVGPLCRG | RK19 |
| **BARFO** | 96-115 | HRQVVTGDVGPLCRGRVAVV | RK20 |
| **BARFO** | 101-120 | TGDVGPLCRGRVAVVGQNHQ | RK21 |
| **BARFO** | 106-125 | PLCRGRVAVVGQNHQLAHTA | RK22 |
| **BARFO** | 111-130 | RVAVVGQNHQLAHTAPAGHR | RK23 |
| **BARFO** | 116-135 | GQNHQLAHTAPAGHRGDVEA | RK24 |
| **BARFO** | 121-140 | LAHTAPAGHRGDVEARVWDG | RK25 |
| **BARFO** | 126-145 | PAGHRGDVEARVWDGTYAPK | RK26 |
| **BARFO** | 131-150 | GDVEARVWDGTYAPKAAQQI | RK27 |
| **BARFO** | 136-155 | RVWDGTYAPKAAQQIQGPFQ | RK28 |
| **BARFO** | 141-160 | TYAPKAAQQIQGPFQALQPH | RK29 |
| **BARFO** | 146-165 | AAQQIQGPFQALQPHGVRHA | RK30 |
| **BARFO** | 151-170 | QGPFQALQPHGVRHAIKHAI | RK31 |
| **BARFO** | 156-174 | ALQPHGVRHAIKHAIDSLH | RK32 |

**Table A3.1.2: 32 overlapping peptides covering BARF0**

*Previously published 9-mer epitope (Kienzle *et al* , 1998)

| Derived sequence | Peptide coordinate | B95.8 Peptide sequence | Denoted peptide reference to text |
|---|---|---|---|
| **RPMS1** | 1-20 | MAGARRRARCPASAGCAYSA | R1 |
| **RPMS1** | 6-25 | RRARCPASAGCAYSARPPPL | R2 |
| **RPMS1** | 11-30 | PASAGCAYSARPPPLSTRGR | R3 |
| **RPMS1** | 16-35 | CAYSARPPPLSTRGRRISAG | R4 |
| **RPMS1** | 21-40 | RPPPLSTRGRRISAGSGQPR | R5 |
| **RPMS1** | 26-45 | STRGRRISAGSGQPRWWPWG | R6 |
| **RPMS1** | 31-50 | RISAGSGQPRWWPWGSPPPP | R7 |
| **RPMS1** | 36-55 | SGQPRWWPWGSPPPPDTRYR | R8 |
| **RPMS1** | 41-60 | WWPWGSPPPPDTRYRRPGPG | R9 |
| **RPMS1** | 46-65 | SPPPPDTRYRRPGPGRRARS | R10 |
| **RPMS1** | 51-70 | DTRYRRPGPGRRARSCLHAG | R11 |
| **RPMS1** | 56-75 | RPGPGRRARSCLHAGPRGRP | R12 |
| **RPMS1** | 61-80 | RRARSCLHAGPRGRPPHSRT | R13 |
| **RPMS1** | 66-85 | CLHAGPRGRPPHSRTRARRT | R14 |
| **RPMS1** | 71-90 | PRGRPPHSRTRARRTSPGAG | R15 |
| **RPMS1** | 76-95 | PHSRTRARRTSPGAGGGGWR | R16 |
| **RPMS1** | 81-100 | RARRTSPGAGGGGWRGGSCT | R17 |
| **RPMS1** | 86-103 | SPGAGGGGWRGGSCTSQR | R18 |

**Table A3.1.3: 18 overlapping peptides covering RPMS1**

| Derived sequence | Peptide coordinate | B95.8 Peptide sequence | Denoted peptide reference to text |
|---|---|---|---|
| **BARF1** | 1-20 | MARFIAQLLLLASCVAAGQA | B1 |
| **BARF1** | 6-52 | AQLLLLASCVAAGQAVTAFL | B2 |
| **BARF1** | 11-30 | LASCVAAGQAVTAFLGERVT | B3 |
| **BARF1** | 16-35 | AAGQAVTAFLGERVTLTSYW | B4 |
| **BARF1** | 21-40 | VTAFLGERVTLTSYWRRVSL | B5 |
| **BARF1** | 26-45 | GERVTLTSYWRRVSLGPEIV | B6 |
| **BARF1** | 31-50 | LTSYWRRVSLGPEIVSWFKL | B7 |
| **BARF1** | 36-55 | RRVSLGPEIVSWFKLGPGEE | B8 |
| **BARF1** | 41-60 | GPEIVSWFKLGPGEEQVLIG | B9 |
| **BARF1** | 46-65 | SWFKLGPGEEQVLIGRMHHD | B10 |
| **BARF1** | 51-70 | GPGEEQVLIGRMHHDVIFIE | B11 |
| **BARF1** | 56-75 | QVLIGRMHHDVIFIEWPFRG | B12 |
| **BARF1** | 61-80 | RMHHDVIFIEWPFRGFFDIH | B13 |
| **BARF1** | 66-85 | VIFIEWPFRGFFDIHRSANT | B14 |
| **BARF1** | 71-90 | WPFRGFFDIHRSANTFFLVV | B15 |
| **BARF1** | 76-95 | FFDIHRSANTFFLVVTAANI | B16 |
| **BARF1** | 81-100 | RSANTFFLVVTAANISHDGN | B17 |
| **BARF1** | 86-105 | FFLVVTAANISHDGNYLCRM | B18 |
| **BARF1** | 91-110 | TAANISHDGNYLCRMKLGET | B19 |
| **BARF1** | 96-115 | SHDGNYLCRMKLGETEVTKQ | B20 |
| **BARF1** | 101-120 | YLCRMKLGETEVTKQEHLSV | B21 |
| **BARF1** | 106-126 | KLGETEVTKQEHLSVVKPLT | B22 |
| **BARF1** | 111-130 | EVTKQEHLSVVKPLTLSVHS | B23 |
| **BARF1** | 116-135 | EHLSVVKPLTLSVHSERSQF | B24 |
| **BARF1** | 121-140 | VKPLTLSVHSERSQFPDFSV | B25 |
| **BARF1** | 126-145 | LSVHSERSQFPDFSVLTVTC | B26 |
| **BARF1** | 131-150 | ERSQFPDFSVLTVTCTVNAF | B27 |
| **BARF1** | 136-155 | PDFSVLTVTCTVNAFPHPHV | B28 |
| **BARF1** | 141-160 | LTVTCTVNAFPHPHVQWLMP | B29 |
| **BARF1** | 146-165 | TVNAFPHPHVQWLMPEGVEP | B30 |
| **BARF1** | 151-170 | PHPHVQWLMPEGVEPAPTAA | B31 |
| **BARF1** | 156-175 | QWLMPEGVEPAPTAANGGVM | B32 |
| **BARF1** | 161-180 | EGVEPAPTAANGGVMKEKDG | B33 |
| **BARF1** | 166-185 | APTAANGGVMKEKDGSLSVA | B34 |
| **BARF1** | 171-190 | NGGVMKEKDGSLSVAVDLSL | B35 |
| **BARF1** | 176-195 | KEKDGSLSVAVDLSLPKPWH | B36 |
| **BARF1** | 181-200 | SLSVAVDLSLPKPWHLPVTC | B37 |
| **BARF1** | 186-205 | VDLSLPKPWHLPVTCVGKND | B38 |
| **BARF1** | 191-210 | PKPWHLPVTCVGKNDKEEAH | B39 |
| **BARF1** | 196-215 | LPVTCVGKNDKEEAHGVYVS | B40 |
| **BARF1** | 201-220 | VGKNDKEEAHGVYVSGYLSQ | B41 |

**Table A3.1.4: 42 overlapping peptides covering BARF1**

| Known EBV Epitopes | Peptide coordinate | Epitope sequence | CD4/CD8 | HLA restriction molecule |
|---|---|---|---|---|
| EBNA1 | 475-489 | NPKFENIAEGLRALL | CD4 | †DR 11 |
| EBNA1 | 515-528 | TSLYNLRRGTALAI | CD4 | †DR 1 |
| EBNA1 | 529-543 | PQCRLTPLSRLPFGM | CD4 | *DR 13<br>*DQ 3, 5, 6 |
| EBNA1 | 563-577 | MVFLQTHIFAEVLKD | CD4 | †DR 15 |
| EBNA 3C | 386-400 | SDDELPYIDPNMEPV | CD4 | *DR 1 |
| EBNA 3C | 916-930 | PSMPFASDYSQGAFT | CD4 | *DR 4 |
| EBNA 3C | 961-986 | AQEILSDNSEISVFPK | CD4 | *DR 4<br>*DQ 2, 3, 5 |
| LMP1 | 212-226 | SGHESDSNSNEGRHH | CD4 | *DQ 2 |
| EBNA 3A | 158-166 | QAKWRLQTL | CD8 | †B8 |
| EBNA 3B | 416-424 | IVTDFSVIK | CD8 | †A1101 |
| LMP2 | 329-337 | LLWTLVVLL | CD8 | †A0201 |
| LMP2 | 419-427 | TYGPVFMCL | CD8 | †A2402 |

**Table A3.1.5: Known $CD4^+/CD8^+$ EBV epitopes within the control peptide pool**

†Known HLA class II restriction allele
*Possible HLA class II restriction alleles

```
B95.8     165504 ATGGCCAGGT TCATCGCTCA GCTCCTCCTG TTGGCCTCCT GTGTGGCCGC CGGCCAGGCT GTCACCGCTT TCTTGGGTGA 165583
CKL       165504 ATGGCCAGGT TCATCGCTCA GCTCCTCCTG TTGGCCTCCT GCGTGGCCGC CGGCCAGGCT GTCACCGCTT TCTTGGGTGA 165583
C5        165504 ATGGCCAGGT TCATCGCTCA GCTCCTCCTG TTGGCCTCCT GCGTGGCCGC CGGCCAGGCT GTCACCGCTT TCTTGGGTGA 165583
Ag876     165504 ATGGCCAGGT TCATCGCTCA GCTCCTCCTG TTGGCCTCCT GTGTGGCCGC CGGCCAGGCT GTCACCGCTT TCTTGGGTGA 165583

B95.8     165584 GCGAGTCACC CTGACCTCCT ACTGGAGGAG GGTGAGCCTC GGTCCAGAGA TTGAGGTCAG CTGGTTTAAA CTGGGCCCAG 165663
CKL       165584 GCGAGTCACC CTGACCTCCT ACTGGAGGAG GGTGAGCCTC GGTCCAGAGA TTGAGGTCAG CTGGTTTAAA CTGGGCCCAG 165663
C5        165584 GCGAGTCACC CTGACCTCCT ACTGGAGGAG GGTGAGCCTC GGTCCAGAGA TTGAGGTCAG CTGGTTTAAA CTGGGCCCAG 165663
Ag876     165584 GCGAGTCACC CTGACCTCCT ACTGGAGGAG GGTGAGCCTC GGTCCAGAGA TTGAGGTCAG CTGGTTTAAA CTGGGCCCAG 165663

B95.8     165664 GAGAGGAGCA GGTGCTTATT GGGCGCATGC ACCACGATGT CATCTTTATA GAGTGGCCTT TCAGGGGCTT CTTTGATATC 165743
CKL       165664 GAGAGGAGCA GGTGCTTATT GGGCGCATGC ACCACGATGT CATCTTTATA GAGTGGCCTT TCAGGGGCTT CTTTGATATC 165743
C5        165664 GAGAGGAGCA GGTGCTTATT GGGCGCATGC ACCACGATGT CATCTTTATA GAGTGGCCTT TCAGGGGCTT CTTTGATATC 165743
Ag876     165664 GAGAGGAGCA GGTGCTTATT GGGCGCATGC ACCACGATGT CATCTTTATA GAGTGGCCTT TCAGGGGCTT CTTTGATATC 165743

B95.8     165744 CACAGAAGTG CCAACACCTT CTTTTTAGTA GTCACCGCTG CCAACATCTC CCATGACGGC AACTACCTGT GCCGCATGAA 165823
CKL       165744 CACAGAAGTG CCAACACCTT CTTTTTAGTA GTCACCGCTG CCAACATCTC CCATGACGGC AACTACCTGT GCCGCATGAA 165823
C5        165744 CACAGAAGTG CCAACACCTT CTTTTTAGTA GTCACCGCTG CCAACATCTC CCATGACGGC AACTACCTGT GCCGCATGAA 165823
Ag876     165744 CACAGAAGTG CCAACACCTT CTTTTTAGTA GTCACCGCTG CCAACATCTC CCATGACGGC AACTACCTGT GCCGCATGAA 165823

B95.8     165824 ACTGGGCGAG ACCGAGGTCA CCAAGCAGGA ACACCTGAGC GTGGTGAAGC CTCTAACGCT GTCTGTCCAC TCCGAAAGGT 165903
CKL       165824 ACTGGGCGAG ACCGAGGTCA CCAAGCAGGA ACACCTGAGC GTGGTGAAGC CTCTAACGCT GTCTGTCCAC TCCGAAAGGT 165903
C5        165824 ACTGGGCGAG ACCGAGGTCA CCAAGCAGGA ACACCTGAGC GTGGTGAAGC CTCTAACGCT GTCTGTCCAC TCCGAAAGGT 165903
Ag876     165824 ACTGGGCGAG ACCGAGGTCA CCAAGCAGGA ACACCTGAGC GTGGTGAAGC CTCTAACGCT GTCTGTCCAC TCCGAAAGGT 165903

B95.8     165904 CTCAGTTCCC AGACTTCTCT GTCCTTACTG TGACATGCAC CGTGAATGCA TTTCCCCATC CCCACGTCCA GTGGCTCATG 165983
CKL       165904 CTCAGTTCCC AGACTTCTCT GTCCTTACTG TGACATGCAC CGTGAATGCA TTTCCCCATC CCCACGTCCA GTGGCTCATG 165983
C5        165904 CTCAGTTCCC AGACTTCTCT GTCCTTACTG TGACATGCAC CGTGAATGCA TTTCCCCATC CCCACGTCCA GTGGCTCATG 165983
Ag876     165904 CTCAGTTCCC AGACTTCTCT GTCCTTACTG TGACATGCAC CGTGAATGCA TTTCCCCATC CCCACGTCCA GTGGCTCATG 165983

B95.8     165984 CCCGAGGGCG TGGAGCCCGC ACCAACTGCG GCAAATGGCG GTGTTATGAA GGAAAAGGAT GGGAGCCTCT CTGTTGCTGT 166063
CKL       165984 CCCGAGGGCG TGGAGCCCGC ACCAACTGCG GCAAATGGCG GTGTTATGAA GGAAAAGGAT GGGAGCCTCT CTGTTGCTGT 166063
C5        165984 CCCGAGGGCG TGGAGCCCGC ACCAACTGCG GCAAATGGCG GTGTTATGAA GGAAAAGGAT GGGAGCCTCT CTGTTGCTGT 166063
Ag876     165984 CCCGAGGGCG TGGAGCCCGC ACCAACTGCG GCAAATGGCG GTGTTATGAA GGAAAAGGAT GGGAGCCTCT CTGTTGCTGT 166063

B95.8     166064 TGACCTGTCA CTTCCCAAGC CCTGGCACCT GCCAGTGACC TGCGTTGGGA AAAATGACAA GGAGGAAGCC CACGGGGTTT 166143
CKL       166064 TGACCTGTCA CTTCCCAAGC CCTGGCACCT GCCAGTGACC TGCGTTGGGA AAAATGACAA GGAGGAAGCC CACGGGGTTT 166143
C5        166064 TGACCTGTCA CTTCCCAAGC CCTGGCACCT GCCAGTGACC TGCGTTGGGA AAAATGACAA GGAGGAAGCC CACGGGGTTT 166143
Ag876     166064 TGACCTGTCA CTTCCCAAGC CCTGGCACCT GCCAGTGACC TGCGTTGGGA AAAATGACAA GGAGGAAGCC CACGGGGTTT 166143

B95.8     166144 ATGTTTCTGG ATACTTGTCG 166164
CKL       166144 ATGTTTCTGG ATACTTGTCG 166164
C5        166144 ATGTTTCTGG ATACTTGTCG 166164
Ag876     166144 ATGTTTCTGG ATACTTGTCG 166164
```

**Figure A3.1.1: Nucleotide sequence of BARF1 from four EBV strains**

This Figure shows the nucleotide sequence alignment of BARF1 from four EBV strains; (reading from top to bottom) B95.8, CKL, C5 and Ag876. The B95.8 EBV strain is the Caucasian type I virus, CKL is the type I Chinese EBV isolate from Chinese NPC patient, C5 is the type I EBV isolate from a healthy Chinese donor as reported by Midgley *et al* (2003), and Ag876 is a African type II virus. The numbers situated to the sides of the nucleotide sequence are the nucleotide coordinates in relation to the entire EBV B95.8 strain genome. The B95.8 nucleotide sequence is underline, to which it would serve as a reference point for comparison with the other EBV strain sequence. The letters which appear in red represent a difference in the nucleotide when comparison with the B95.8 sequence. Note there is a single nucleotide difference, at position 165545, in the BARF1 sequence of CKL and C5 EBV strain. This change in nucleotide sequence does not affect the amino acid sequence of the protein. Therefore, the amino acid sequence of BARF1 is fully conserved in the four EBV strains studied.

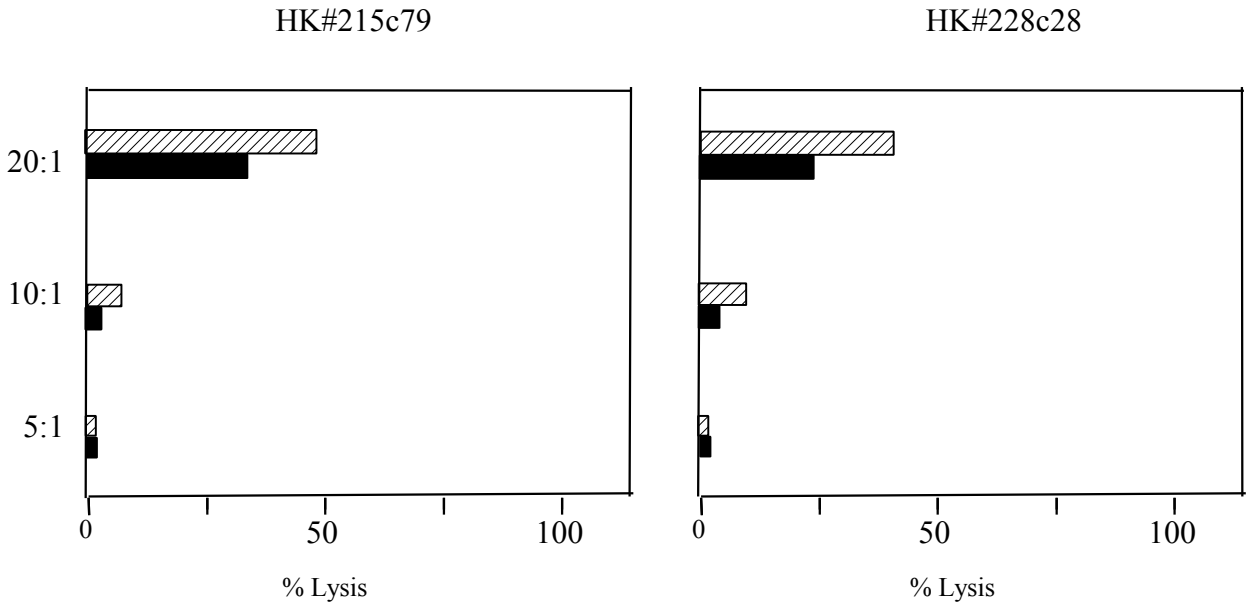
| | Peptide sequence | Coordinates | Virus type | Peptide no. |
|---|---|---|---|---|
| **EBNA1** | *MSDEGPGTGPGNGLGQKEDS | 1-20 | #Chinese | **1 |
| **EBNA1** | PGTGPGNGLG**Q**K**E**D**S**SGPEG | 6-25 | Chinese | 2 |
| **EBNA1** | GNGLG**Q**K**E**D**S**SGPEGSGGSG | 11-30 | Chinese | 3 |
| **EBNA1** | **Q**K**E**D**S**SGPEGSGGSGPQRRG | 16-35 | Chinese | 4 |
| **EBNA1** | SGPEGSGGSGPQRRGGDNHG | 21-40 | B95-8 | 5 |
| **EBNA1** | SGGSGPQRRGGDNHGRGRGR | 26-45 | B95-8 | 6 |
| **EBNA1** | PQRRGGDNHGRGRGRGRGRG | 31-50 | B95-8 | 7 |
| **EBNA1** | GDNHGRGRGRGRGGGRPG | 36-55 | B95-8 | 8 |
| **EBNA1** | RGRGRGRGRGGGRPGAPGGS | 41-60 | B95-8 | 9 |
| **EBNA1** | GRGRGGGRPGAPGGSGSGPR | 46-65 | B95-8 | 10 |
| **EBNA1** | GGRPGAPGGSGSGPRHRDGV | 51-70 | B95-8 | 11 |
| **EBNA1** | APGGSGSGPRHRDGVRRPQK | 56-75 | B95-8 | 12 |
| **EBNA1** | GSGPRHRDGVRRPQKRPSCI | 61-80 | B95-8 | 13 |
| **EBNA1** | HRDGVRRPQKRPSCIGCKGT | 66-85 | B95-8 | 14 |
| **EBNA1** | RRPQKRPSCIGCKGTHGGTG | 71-90 | B95-8 | 15 |
| **EBNA1** | RPSCIGCKGTHGGTGAGGGG | 76-90/324-328 | B95-8 | 16 |
| **EBNA1** | GCKGTHGGTGAGGGGRGRGG | 81-95/324-333 | B95-8 | 17 |
| **EBNA1** | HGGTGAGGGGRGRGGSGGRG | 86-100/338 | B95-8 | 18 |
| **EBNA1** | AGGGGRGRGGSGGRGRGGSG | 324-343 | B95-8 | 19 |
| **EBNA1** | RGRGGSGGRGRGGSGGRGRG | 329-348 | B95-8 | 20 |
| **EBNA1** | SGGRGRGGSGGRGRGGSGGR | 334-353 | B95-8 | 21 |
| **EBNA1** | RGGSGGRGRGGSGGRRGRGR | 339-358 | B95-8 | 22 |
| **EBNA1** | ERARGGRGRGGSGGRRGRGR | 344-363 | B95-8 | 23 |
| **EBNA1** | GSGGRRGRGRERARG**R**SRER | 349-368 | Chinese | 24 |
| **EBNA1** | RGRGRERARG**R**SRERARGRG | 354-373 | Chinese | 25 |
| **EBNA1** | ERARG**R**SRERARGRGRGRGE | 359-378 | Chinese | 26 |
| **EBNA1** | **R**SRERARGRGRGRGEKRPRS | 364-383 | Chinese | 27 |
| **EBNA1** | ARGRGRGRGEKRPRSPSSQS | 359-388 | B95-8 | 28 |
| **EBNA1** | RGRGEKRPRSPSSQSSSSGS | 374-393 | B95-8 | 29 |
| **EBNA1** | KRPRSPSSQSSSSGSPPRRP | 379-398 | B95-8 | 30 |
| **EBNA1** | PSSQSSSSGSPPRRPPPGRR | 384-403 | B95-8 | 31 |
| **EBNA1** | SSSGSPPRRPPPGRRPFFHP | 389-408 | B95-8 | 32 |
| **EBNA1** | PPRRPPPGRRPFFHPVG**D**AD | 394-413 | Chinese | 33 |
| **EBNA1** | PPGRRPFFHPVG**D**ADYFEY**L** | 399-418 | Chinese | 34 |
| **EBNA1** | PFFHPVG**D**ADYFEY**L**QEGGP | 404-423 | Chinese | 35 |
| **EBNA1** | VG**D**ADYFEY**L**QEGGPDGEPD | 409-428 | Chinese | 36 |
| **EBNA1** | YFEY**L**QEGGPDGEPDVPPGA | 414-433 | Chinese | 37 |
| **EBNA1** | QEGGPDGEPDVPPGAIEQGP | 419-438 | Chinese | 38 |
| **EBNA1** | DGEPDVPPGAIEQGP**T**DDPG | 424-443 | Chinese | 39 |
| **EBNA1** | VPPGAIEQGP**T**DDPGEGPST | 429-448 | Chinese | 40 |
| **EBNA1** | IEQGP**T**DDPGEGPSTGPRGQ | 434-453 | Chinese | 41 |
| **EBNA1** | **T**DDPGEGPSTGPRGQGDGGR | 439-458 | Chinese | 42 |
| **EBNA1** | EGPSTGPRGQGDGGRRKKGG | 444-463 | B95-8 | 43 |
| **EBNA1** | GPRGQGDGGRRKKGGWFGKH | 449-468 | B95-8 | 44 |
| **EBNA1** | GDGGRRKKGGWFGKHRGQGG | 454-473 | B95-8 | 45 |
| **EBNA1** | RKKGGWFGKHRGQGGSNPKF | 459-478 | B95-8 | 46 |
| **EBNA1** | WFGKHRGQGGSNPKFENIAE | 464-483 | B95-8 | 47 |
| **EBNA1** | RGQGGSNPKFENIAEGLR**V**L | 469-488 | Chinese | 48 |
| **EBNA1** | SNPKFENIAEGLR**V**LLARSH | 474-493 | Chinese | 49 |
| **EBNA1** | ENIAEGLR**V**LLARSHVERTT | 479-498 | Chinese | 50 |
| **EBNA1** | GLR**V**LLARSHVERTT**E**EG**N**W | 484-503 | Chinese | 51 |
| **EBNA1** | LARSHVERTT**E**EG**N**WVAGVF | 489-508 | Chinese | 52 |
| **EBNA1** | VERTT**E**EG**N**WVAGVFVYGGS | 494-513 | Chinese | 53 |
| **EBNA1** | **E**EG**N**WVAGVFVYGGSKTSLY | 499-518 | Chinese | 54 |
| **EBNA1** | VAGVFVYGGSKTSLYNLRRG | 504-523 | B95-8 | 55 |
| **EBNA1** | VYGGSKTSLYNLRRG**I**ALA**V** | 509-528 | Chinese | 56 |
| **EBNA1** | KTSLYNLRRG**I**ALA**V**PQCR**I** | 514-533 | Chinese | 57 |
| **EBNA1** | NLRRG**I**ALA**V**PQCR**I**TPLSR | 519-538 | Chinese | 58 |
| **EBNA1** | **I**ALA**V**PQCR**I**TPLSRLPFGM | 524-543 | Chinese | 59 |
| **EBNA1** | PQCR**I**TPLSRLPFGMAPGPG | 529-548 | Chinese | 60 |
| **EBNA1** | TPLSRLPFGMAPGPGPQPGP | 534-553 | B95-8 | 61 |
| **EBNA1** | LPFGMAPGPGPQPGPLRESI | 539-558 | B95-8 | 62 |
| **EBNA1** | APGPGPQPGPLRESIVCYFM | 544-563 | B95-8 | 63 |
| **EBNA1** | PQPGPLRESIVCYFMVFLQT | 549-568 | B95-8 | 64 |
| **EBNA1** | LRESIVCYFMVFLQTHIFAE | 554-573 | B95-8 | 65 |
| **EBNA1** | VCYFMVFLQTHIFAEVLKDA | 559-578 | B95-8 | 66 |
| **EBNA1** | VFLQTHIFAEVLKDAIKDLV | 564-583 | B95-8 | 67 |
| **EBNA1** | HIFAEVLKDAIKDLVMTKPA | 569-588 | B95-8 | 68 |
| **EBNA1** | VLKDAIKDLVMTKPAPTCNI | 574-593 | B95-8 | 69 |
| **EBNA1** | IKDLVMTKPAPTCNI**K**VTVC | 579-598 | Chinese | 70 |
| **EBNA1** | MTKPAPTCNI**K**VTVCSFDDG | 584-603 | Chinese | 71 |
| **EBNA1** | PTCNI**K**VTVCSFDDGVDLPP | 589-608 | Chinese | 72 |
| **EBNA1** | **K**VTVCSFDDGVDLPPWFPPM | 594-613 | Chinese | 73 |
| **EBNA1** | SFDDGVDLPPWFPPMVEGAA | 599-618 | B95-8 | 74 |
| **EBNA1** | VDLPPWFPPMVEGAAAEGDD | 604-623 | B95-8 | 75 |
| **EBNA1** | WFPPMVEGAAAEGDDGDDGD | 609-628 | B95-8 | 76 |
| **EBNA1** | VEGAAAEGDDGDDGDEGGDG | 614-633 | B95-8 | 77 |
| **EBNA1** | AEGDDGDDGDEGGDGDEGEE | 619-638 | B95-8 | 78 |
| **EBNA1** | GDDGDEGGDGDEGEEEGQE | 624-641 | B95-8 | 79 |
| **EBNA1** | AGAGGGAGGAGAGGGAGGAG | Gly/Ala repeat | B95-8 | 80 |
| **EBNA1** | GGGAGAGGAGGAGGAGAGGAGAGA | Gly/Ala repeat | B95-8 | 81 |

**Table A3.2.1: EBNA1 20-mer peptide panel**

# EBNA1 sequence unique to the Chinese CKL EBV strain

* In bold are the amino acid substitution

** Peptide numbers that are being referred to then in main text




**A** **B**

**18hour assay**

**12hour assay**

**Figure A3.3.1: Killing of peptide-loaded target cells by EBNA1 564-583-specific clones HK215c79 and HK228c28**

Autologous LCLs and HLA-mismatched LCLs were preloaded with EBNA1 564-583 peptide at $10^{-7}$M and $^{51}$Cr. Clonal T cells from HK215c79 (A) and HK228c28 (B) were co-cultured with these LCLs at different clonal T cell to LCL rations (20:1, 10:1 and 5:1), or LCLs cultured with SDS, or LCLs alone for 12 hours (bold bar) and 18 hours (striped bar). Lysis is represented as a (%) calculated using the equation shown in section 2.2.11 where; LCLs cultured in SDS is the maximum $^{51}$Cr release, LCLs in culture media is the spontaneous $^{51}$Cr release, and LCLs co-cultured with clonal T cells assess for cytotoxicity. In the 18 hour assay, both clones were calculated to have 40-50% lysis of autologous LCLs pre-loaded with epitope peptide at a 20:1 clonal T cell to LCL ratio, and lysis decreases with decreasing clonal T cell to LCL ratio. In the 12 hour assay, lysis of autologous LCLs were lower than seen in the 18 hour assay, at 25-30% lysis in the 20:1 clonal T cell to LCL ratio, and lysis also decreases with decreasing clonal T cell to LCL ratio. Note that no lysis was observed on HLA-mismatched LCLs (data not shown).

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# CD4$^+$ T-Cell Responses to Epstein-Barr Virus Nuclear Antigen EBNA1 in Chinese Populations Are Highly Focused on Novel C-Terminal Domain-Derived Epitopes

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**Epstein-Barr virus nuclear antigen EBNA1, the one viral protein uniformly expressed in nasopharyngeal carcinoma (NPC), represents a prime target for T-cell-based immunotherapy. However, little is known about the EBNA1 epitopes, particularly CD4 epitopes, presented by HLA alleles in Chinese people, the group at highest risk for NPC. We analyzed the CD4$^+$ T-cell responses to EBNA1 in 78 healthy Chinese donors and found marked focusing on a small number of epitopes in the EBNA1 C-terminal region, including a DP5-restricted epitope that was recognized by almost half of the donors tested and elicited responses able to recognize EBNA1-expressing, DP5-positive target cells.**

Undifferentiated nasopharyngeal carcinoma (NPC), an unusually prevalent tumor in Southeast Asian and particularly Chinese populations, is consistently Epstein-Barr virus (EBV) positive and expresses a subset of EBV latent proteins. These include the nuclear antigen EBNA1, the latent membrane protein LMP2 in most cases, and in some cases LMP1 (12). A detailed knowledge of both CD4$^+$ and CD8$^+$ T-cell responses to these antigens is needed if one is to exploit such responses for immunotherapeutic use (1, 7, 15). While there has been extensive work on CD8$^+$ T-cell responses in that regard (4), CD4-based studies have focused almost exclusively on Caucasian donors (3, 6, 8, 10, 11, 14, 20) and little is known about responses restricted through the different array of HLA II alleles found in the Chinese population. Here we focus on CD4 responses to EBNA1, the only viral protein known to be expressed in all NPC tumors and one which proves to be a rich source of CD4 epitopes.

**ELISPOT assay mapping of CD4 epitope regions.** Eighty-one peptides (20-mers overlapping by 15 residues) were synthesized according to the EBNA1 sequence common to 27/31 Chinese EBV strains, of which the NPC 15 (CKL) virus strain is the prototype (9). On the basis of published protocols (6), these peptides were tested individually in enzyme-linked immunospot (ELISPOT) assays of gamma interferon (IFN-γ) release with peripheral blood mononuclear cells (PBMCs, CD8$^+$ T cell depleted) obtained with informed consent from healthy, EBV-seropositive Chinese donors resident in Hong Kong, an area with a high incidence of NPC. Figure 1 shows representative results from four responsive donors, HK 201, HK 215, HK 263, and HK 280, to illustrate the low backgrounds (0 to 10 spots/well) usually seen and the clear focusing of responses mostly on adjacent sets of two or three overlapping peptides in specific regions of the molecule. Overall, 50/78 donors tested in repeat assays showed reproducible responses to particular peptides.

On this basis, we identified 10 distinct epitope regions in the Chinese EBNA1 sequence. These are located on a linear map of the EBNA1 protein (Fig. 2, right) where the filled horizontal bars represent the percentages of donors responding to each epitope region. Alongside (Fig. 2, left) are shown the corresponding data from 37 healthy seropositive Caucasian donors tested against the standard B95.8 EBNA1 peptide panel (6; unpublished data). In both cases, CD4 epitopes are concentrated within the C-terminal half of the molecule. However, compared to those of Caucasians, the EBNA1 responses of Chinese donors are focused on a smaller number of epitope regions, with correspondingly higher percentages of donors responding to individual regions. In particular, EBNA1 peptides 67 and 68 were recognized by 47% (37/78) of the Chinese donors tested, a far higher frequency than that seen for any Caucasian donor response.

**Epitope definition by CD4$^+$ T-cell cloning.** Four epitope regions, represented by Chinese EBNA1 peptides 48 and 49, 50 and 51, 56 and 57, and 67 and 68, were selected for detailed analysis. On the basis of previously published protocols (8), PBMCs from selected donors were stimulated in vitro with relevant peptide pairs and cloned on day 7 at limiting dilution (0.3 cell/well) on autologous CKL virus-transformed B-lymphoblastoid cell line (LCL) feeders, and clones reactive to the stimulating peptide pair were identified by specific IFN-γ release in an enzyme-linked immunosorbent assay. Such clones were assayed on the autologous LCL loaded with serial dilutions of individual peptides from the epitope region in order to identify the optimal 20-mer epitope. Representative results are

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† C.W.T. and X.L. contributed equally to this work.

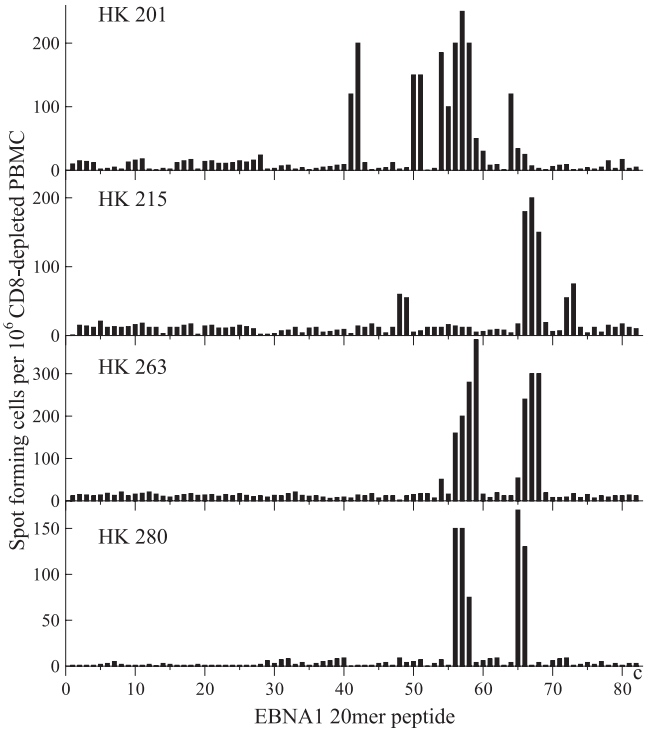


FIG. 1. Results of IFN-γ ELISPOT assays using CD8-depleted PBMCs from healthy Chinese donors exposed to individual CKL strain EBNA1 peptides (1 to 81) or assayed in the absence of peptide as a control (c). Results from four different donors are shown as numbers of spot-forming cells per $10^6$ CD8-depleted PBMCs tested. Note that responses occasionally mapped to a single peptide (e.g., HK 201, peptide 64) but usually mapped to two or three adjacent peptides (e.g., HK 201, peptides 41 and 42 and peptides 50 and 51; HK 215, peptides 48 and 49 and peptides 66, 67, and 68). Where there was recognition of four or five adjacent peptides, cloning frequently revealed coresident responses to two separate epitopes.

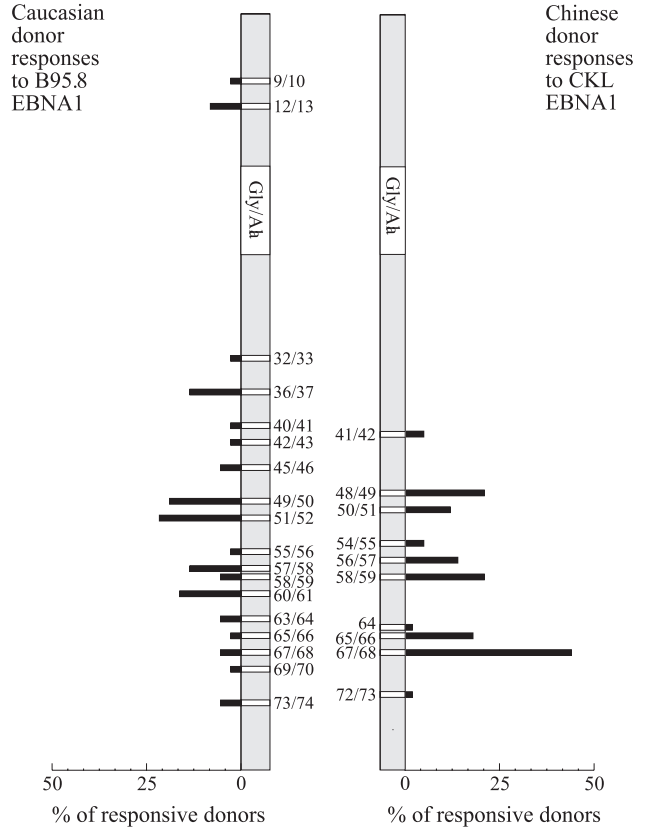


FIG. 2. Locations of CD4 epitope regions, identified by peptide number, on a linear map of the EBNA1 protein (with the position of the internal glycine-alanine repeat domain also shown). (Right) Summary of data from 78 healthy Chinese donors screened by using the CKL strain EBNA1 peptide panel. (Left) Summary of data from 37 healthy Caucasian donors from the United Kingdom who were screened by using the B95.8 strain EBNA1 peptide panel. In each case, the filled bars indicate the percentages of donors tested who responded to these particular epitope regions.

shown in Fig. 3A. Clones from donor HK 215 against the peptide 48 and 49 region recognized peptide 49 but, surprisingly, not peptide 48 even though both peptides had elicited equally strong responses in the original ELISPOT assays. Clones against the other epitope regions showed recognition of two or more adjacent peptides but consistently identified one of those peptides most efficiently, i.e., peptide 50 for clones from donor HK 228, peptide 56 for clones from donor HK 233, and peptide 67 for clones from two donors, HK 215 and HK 228 (data not shown). Note also that these EBNA1 epitope-specific clones all showed functional avidities (defined as the minimum peptide concentration required for 50% monoclonal IFN-γ release) within the 10- to 100-nM range, similar to those seen for most EBNA1-specific reactivities isolated from Caucasian donors (8). Moreover, they were also confirmed as antigen specific since they recognized autologous target cells expressing a class II-targeted EBNA1 protein expressed from a recombinant vaccinia virus vector (17; data not shown).

**HLA class II restriction of epitope-specific responses.** Figure 3B shows the restriction data obtained from these clones in two types of assay. As previously described (8), these used (i) individual HLA-DP-, -DQ-, or -DR-specific monoclonal antibodies (MAbs) to block the recognition of autologous peptide-loaded cells and (ii) allogeneic target cells of known HLA type as peptide-presenting cells. Both the peptide 49-specific and peptide 56-specific clones proved to be HLA-DR restricted by blocking, but the precise alleles could not be identified by using the allogeneic targets available at that time. We therefore HLA typed 47 donors from the original panel; this revealed strong correlations between HLA-DR51 positivity and responsiveness to peptide 49 and between HLA-DR11 positivity and responsiveness to peptide 56, suggesting that these were the likely restricting alleles. With regard to peptide 50, the MAb blocking and allogeneic LCL assays suggested that this was HLA-DQ7 restricted and, indeed, all five donors showing this response in ELISPOT assays were DQ7 positive; however, another 15 DQ7-positive individuals showed no response, suggesting that this is not a consistently immunodominant epitope. Most importantly, the peptide 67 response proved to be HLA-DP5 restricted. This immediately suggested why it was seen so frequently in the ELISPOT assays, since HLA-DP5 is carried by >50% of Chinese people (19) and, indeed, all responders to this allele who were HLA typed proved to be DP5 positive.

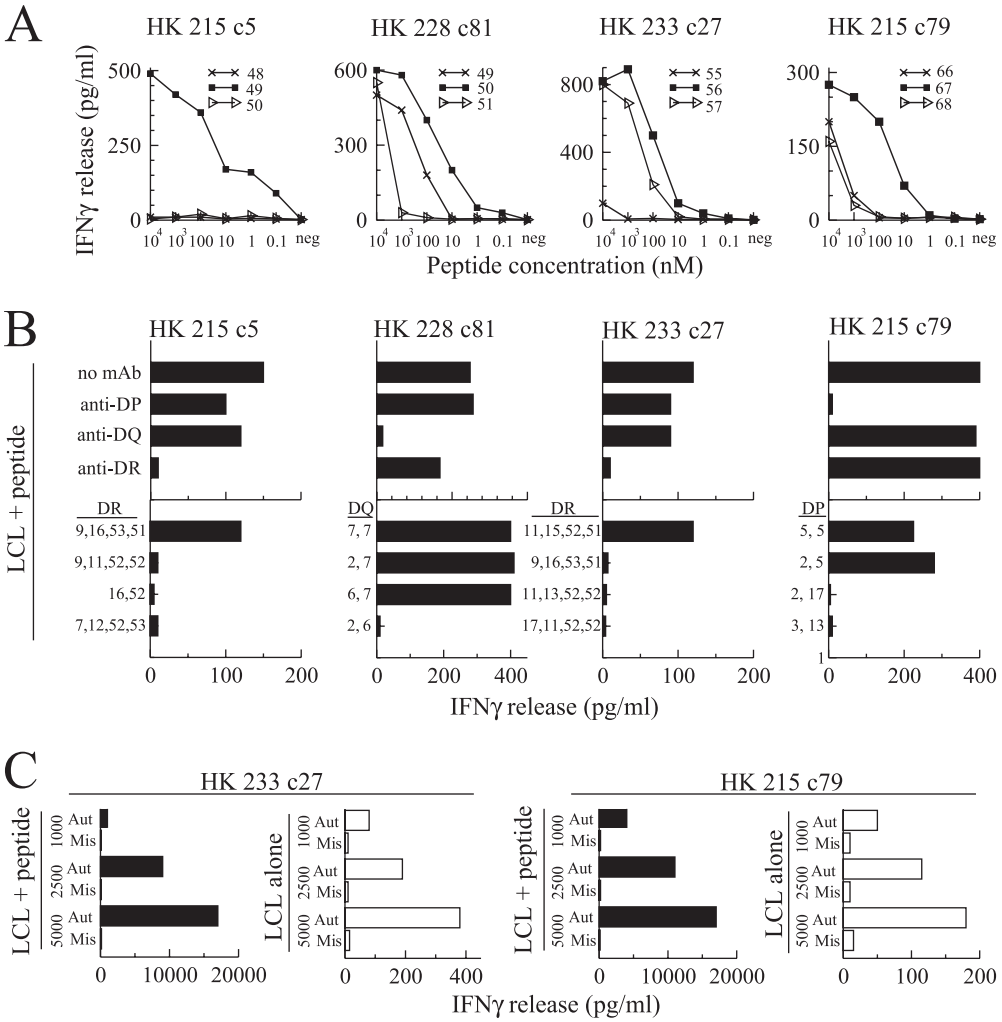


FIG. 3. Functional analysis of CD4$^+$ T-cell clones established from selected healthy Chinese donors by stimulating PBMCs with CKL strain EBNA1 peptide pair 48 and 49, 49 and 50, 56 and 57, or 67 and 68. (A) Identifying the optimal 20-mer epitope. Clones were stimulated with the autologous LCL preloaded with limiting concentrations of individual peptides from the epitope region; recognition was assayed as IFN-γ release by enzyme-linked immunosorbent assay. neg, negative. (B) Identifying HLA class II restriction. (Top) Clones were stimulated with the autologous LCL preloaded with a just-optimal concentration of the epitope peptide in the presence of MAbs to HLA-DP, -DQ, or -DR. (Bottom) Clones were stimulated with the autologous LCL or with an HLA-typed allogeneic LCL preloaded with a concentration of peptide similar to that above; relevant HLA class II alleles shared between the LCL and donor T cells are shown. Results are expressed as in panel A. (C) Recognizing unmanipulated LCL. Clones seeded at different cell numbers were stimulated with a standard number of cells of an autologous LCL (Aut) or an allogeneic HLA-mismatched LCL (Mis), either alone or after loading with an optimal concentration of epitope peptide, and recognition was assayed as above.

TABLE 1. Summary of EBNA1 epitope responses

| EBNA1 peptide no. | Amino acid coordinates | Epitope sequence[a] | No. of responders[b] | Restriction element | % LCL recognition[e] |
|---|---|---|---|---|---|
| 41/42 | 434–458 | IEQGPTDDPGEGPSTGPRGQGDGGR | 2/43 | ND[c] | ND |
| 49 | 474–493 | SNPKFENIAEGLRVLLARSH | 16/78 | DR51 | <0.2 |
| 50 | 479–498 | ENIAEGLRVLLARSHVERTT | 5/43 | DQ7 | <0.2 |
| 54/55 | 499–523 | EEGNWVAGVFVYGGSKTSLYNLRRG | 4/78 | ND | ND |
| 56 | 509–528 | VYGGSKTSLYNLRRGIALAV | 11/78 | DR11[d] | 2 |
| 58/59 | 519–543 | NLRRGIALAVPQCRITPLSRLPFGM | 16/78 | ND | ND |
| 64 | 549–568 | PQPGPLRESIVCYFMVFLQT | 1/43 | ND | ND |
| 65/66 | 554–578 | LRESIVCYFMVFLQTHIFAEVLKDA | 14/78 | ND | ND |
| 67 | 564–583 | VFLQTHIFAEVLKDAIKDL | 34/78 | DP5 | 1 |
| 72/73 | 589–613 | PTCNIKVTVCSFDDGVDLPPWFPPM | 1/43 | ND | ND |

[a] Amino acid changes in CKL strain EBNA1 relative to B95.8 EBNA1 are underlined.
[b] Note that of the 78 donors used, 43 were tested on all 81 EBNA1 peptides and a further 35 were tested on a limited panel of peptides that did not include peptide 41, 42, 50, 64, 72, or 73.
[c] ND, not determined.
[d] DR-restricted responses where the restricting allele is inferred to be DR51 and DR11 from the HLA type of epitope-responsive donors.
[e] IFN-γ levels from CD4$^+$ T cells challenged with the autologous LCL are expressed as a percentage of the levels seen on challenge with the same LCL optimally loaded with epitope peptide.

**CD4$^+$ T-cell recognition of latently infected cells.** Interest in CD4$^+$ T-cell responses to EBV latent proteins reflects not just their likely importance in the maintenance of effective CD8$^+$ T-cell surveillance (2, 13, 16) but also their possible role as direct effectors capable of recognizing latently infected cells that, like NPC tumors (5, 18), express HLA class II molecules. To investigate this latter point, we used published protocols (8) to assay the EBNA1-specific clones for recognition of autologous LCL targets. Specific recognition could not be detected with clones to peptide 49 or 50 (data not shown). However, clones to peptides 56 and 67 clearly did recognize the autologous LCL, with recognition titrating against T-cell input; for these clones, levels of IFN-γ produced in response to the autologous LCL (Fig. 3C, open bars, LCL alone) were around 2% and 1%, respectively, of those produced in response to the same target cells optimally loaded with epitope peptide (Fig. 3C, filled bars, LCL + peptide).

Table 1 summarizes the essential data from the present study. We conclude that most healthy Chinese donors possess a strong CD4$^+$ T-cell memory for EBNA1; that responses are focused on a small number of C-terminal EBNA1 epitopes, including a DP5-restricted epitope seen by almost 50% of the donors tested; and that some of the above responses (including that to the DP5 epitope) have the potential to directly recognize a latently infected HLA II-positive LCL. These findings will help in both the design and testing of immunotherapeutic strategies targeting NPC, whether based on vaccines to stimulate the patient's own responses (7, 17) or on the adoptive transfer of in vitro-expanded T-cell populations (1, 15).

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# $CD4^+$ T-Cell Responses to Epstein-Barr Virus (EBV) Latent-Cycle Antigens and the Recognition of EBV-Transformed Lymphoblastoid Cell Lines

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**There is considerable interest in the potential of Epstein-Barr virus (EBV) latent antigen-specific $CD4^+$ T cells to act as direct effectors controlling EBV-induced B lymphoproliferations. Such activity would require direct $CD4^+$ T-cell recognition of latently infected cells through epitopes derived from endogenously expressed viral proteins and presented on the target cell surface in association with HLA class II molecules. It is therefore important to know how often these conditions are met. Here we provide $CD4^+$ epitope maps for four EBV nuclear antigens, EBNA1, -2, -3A, and -3C, and establish $CD4^+$ T-cell clones against 12 representative epitopes. For each epitope we identify the relevant HLA class II restricting allele and determine the efficiency with which epitope-specific effectors recognize the autologous EBV-transformed B-lymphoblastoid cell line (LCL). The level of recognition measured by gamma interferon release was consistent among clones to the same epitope but varied between epitopes, with values ranging from 0 to 35% of the maximum seen against the epitope peptide-loaded LCL. These epitope-specific differences, also apparent in short-term cytotoxicity and longer-term outgrowth assays on LCL targets, did not relate to the identity of the source antigen and could not be explained by the different functional avidities of the $CD4^+$ clones; rather, they appeared to reflect different levels of epitope display at the LCL surface. Thus, while $CD4^+$ T-cell responses are detectable against many epitopes in EBV latent proteins, only a minority of these responses are likely to have therapeutic potential as effectors directly recognizing latently infected target cells.**

Epstein-Barr virus (EBV), a herpesvirus with B-cell growth transforming ability and lymphomagenic potential, provides one of the most instructive systems in which to study T-cell responses to viral infection in humans (11, 25). Primary infection is usually asymptomatic but in some individuals can present as infectious mononucleosis, a self-limiting lymphoproliferative disease where the symptoms are coincident with the appearance of a large reactive T-cell response. Following primary infection, the virus is carried for life as a latent infection of the circulating memory B-cell pool (1), with low-level reactivation from latency into virus productive (lytic) infection at oropharyngeal sites. Immune T-cell responses clearly play some role in the maintenance of this virus-host balance since T-cell-immunocompromised individuals show increased virus replication in the oropharynx (4) and an increased risk of EBV-driven B-lymphoproliferative disease (22). One of the outstanding issues to resolve in this respect is the relative contribution made by $CD4^+$ and $CD8^+$ T-cell responses to this host control.

The HLA class I-restricted CD8 response has attracted the most attention, for two reasons. Firstly, primary T-cell responses seen in the blood of infectious mononucleosis patients are largely of $CD8^+$ T-cell origin; indeed, many of these in vivo-primed reactivities have been mapped to epitopes drawn from EBV lytic- and latent-cycle antigens (28, 29). Secondly, memory T-cell responses reactivated from immune donors by in vitro stimulation with the virus-transformed B-lymphoblastoid cell line (LCL) are likewise dominated by $CD8^+$ effectors. Many such effectors recognize epitopes drawn from the latent-cycle proteins that are expressed in all LCLs, namely, nuclear antigens EBNA1, -2, -3A, -3B, -3C, and -LP and latent membrane proteins LMP1 and -2, with responses to EBNA3A-, -3B-, and -3C-derived epitopes frequently in the majority (8, 18). Such CD8 responses to EBV latent antigens are of particular interest because of their ability to recognize and kill virus-transformed B cells in vitro and their therapeutic potential against EBV-driven B-lymphoproliferative disease in vivo (24).

The growing interest in HLA class II-restricted $CD4^+$ T-cell responses to the virus reflects not only an appreciation of the general role that $CD4^+$ T cells are thought to play in the maintenance of effective CD8 immunity (7, 26, 30) but also the fact that EBV infects target cells in which the HLA class II pathway of antigen presentation is active (33). This raises the possibility that virus-specific $CD4^+$ T cells are able to recognize infected cells directly and, if they are, could act (like $CD8^+$ T cells) as effectors in their own right. Certainly there are examples where indicator antigens have been expressed endogenously within LCLs and appear to have gained direct intracellular entry into the HLA class II processing pathway (2, 20, 23, 34), in some way bypassing the usual means of HLA class II presentation involving uptake as exogenously acquired antigen (33). The first $CD4^+$ T-cell clones to EBV latent pro-

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teins, specific for an EBNA1-derived and an EBNA2-derived epitope, respectively, were identified as rare components of LCL-reactivated memory T-cell preparations (9, 10). Of these, only the EBNA2-specific clone appeared to be capable of recognizing LCLs directly in cytotoxicity assays (10). Since that time, $CD4^+$ recall responses to more latent-cycle epitopes have been generated by a variety of protocols, many involving in vitro stimulation with dendritic cells either overexpressing antigen from vaccinia virus vectors or preloaded with exogenous antigen or with epitope peptides (13, 17, 21, 27, 32). Although these clones display specificity for antigen in protein loading assays, there are differing reports of their ability to recognize LCLs naturally expressing cognate antigen from the resident EBV genome. It is not clear whether these divergent results reflect technical differences in the way in which the clones are generated in vitro or genuine differences in the way in which either individual antigens or individual epitopes are processed and presented. The present study (i) provides detailed CD4 epitope maps for four EBV latent-cycle antigens, EBNA1, -2, -3A, and -3C; (ii) establishes $CD4^+$ T-cell clones to 12 selected epitopes from these antigens; and (iii) characterizes these clones in terms of their HLA class II restriction, their functional avidity in peptide titration assays, and their ability to recognize autologous LCL targets in gamma interferon (IFN-γ) release, cytotoxicity, and outgrowth assays.

## MATERIALS AND METHODS

**Cell preparations and cell lines.** Peripheral blood mononuclear cells (PBMCs) were separated from healthy, EBV-immune donors by Ficoll-Hypaque centrifugation into RPMI 1640 medium (Invitrogen) supplemented with 2 mM glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 5% autologous serum. Where specified, PBMCs were depleted of $CD8^+$ T cells with CD8 Dynabeads (Dynal) in accordance with the manufacturer's recommended protocol. Dendritic cells were prepared as previously described (13), by 6-day culture of adherent PBMCs in the above medium supplemented with granulocyte-macrophage colony-stimulating factor and interleukin-4, and then matured for 24 h in 50 ng of tumor necrosis factor alpha per ml. EBV-transformed LCLs were prepared with prototype 1 strain B95.8 or prototype 2 strain Ag876. All LCLs were cultured in medium (as described above) containing 10% fetal calf serum, and all assays involved B95.8 virus-transformed LCLs unless otherwise stated.

**Synthetic peptides and protein preparations.** Epitope peptides were synthesized by 9-fluorenylmethoxycarbonyl chemistry (Alta Bioscience, University of Birmingham) and dissolved in dimethyl sulfoxide (DMSO), and their concentrations were determined by biuret assay. The preparation and purification of baculovirus-expressed EBNA1 protein have been described elsewhere (13). EBNA2 protein and appropriate control preparations made in baculovirus expression systems were kindly provided by Friedrich Grässer, Homburg-Saar, Germany (6). EBNA3A and EBNA3C protein preparations and controls were likewise made from appropriate baculovirus vectors by infection of insect cells and purification of nuclear proteins.

**ELISPOT assays.** CD8-depleted PBMC preparations were tested in ELISPOT assays of IFN-γ release as previously described (13), with pools of overlapping peptides (three or four peptides per pool) from the antigen sequence of interest, followed by assays on individual peptides within positive pools. The EBNA1 and EBNA2 panels were 20-mer peptides (overlapping by 15 residues), and the EBNA3A and -3C panels were 15-mer peptides (overlapping by 10 residues), all based on the B95.8 EBV strain sequence.

**In vitro reactivation protocols.** $CD4^+$ T-cell clones were generated from cultures of CD8-depleted PBMCs 7 days after in vitro stimulation either with 5 μM epitope peptide directly loaded onto the cells for 1 h or with autologous dendritic cells loaded either with peptide or with protein (13) or (as for $CD8^+$ T-cell activation) with gamma-irradiated autologous LCLs (18). On day 7, cells were cloned by limiting dilution at 3 cells per well on autologous gamma-irradiated LCLs ($10^4$/well) loaded with the relevant peptide at 5 μM and allogeneic gamma-irradiated, phytohemagglutinin-treated PBMCs ($10^5$/well) in interleukin-2-supplemented medium with 5% autologous serum as previously described (12). Growing microcultures were screened for peptide reactivity by IFN-γ enzyme-linked immunosorbent assay (ELISA), and selected clones were expanded as described above with fetal calf serum-supplemented medium.

**ELISAs of IFN-γ release and MAb blocking.** Cloned T cells were incubated in U-bottom or V-bottom microtest plate wells with standard numbers of autologous, HLA-matched, or HLA-mismatched LCLs that were either unmanipulated or prepulsed for 1 h with 5 μM peptide (or an equivalent concentration of DMSO solvent as a control) or preexposed in serum-free medium for 2 h to specific EBV antigen preparations (or to control antigen preparations) and then washed. The supernatant medium harvested after 18 h was assayed for IFN-γ by ELISA (Endogen) in accordance with the manufacturer's recommended protocol. In blocking assays, LCLs were preincubated with monoclonal antibodies (MAbs) specific for HLA-DR (L243; ATCC clone HB-55), HLA-DQ (SPV-L3; Serotec), and HLA-DP (B7.21; kindly provided by A. M. de Jong, Leiden University, Leiden, The Netherlands) for 1 h before addition of T-cells to the assay.

**Chromium release assays.** $CD4^+$ T-cell clones were tested for killing of target cells at known effector/target ratios in 5- and 18-h chromium release assays, and results were expressed as percent specific lysis of the target line. Targets were HLA-matched or HLA-mismatched LCLs preexposed for 1 h to 5 μM epitope peptide or to an equivalent concentration of DMSO solvent as a control.

**Outgrowth assays.** Target LCLs (HLA matched and HLA mismatched, either unmanipulated or prepulsed with 5 μM epitope peptide and then washed) were serially diluted into replicate U-bottom microtest plate wells at $10^4$ to 300 per well, and T cells were added to half of the replicates at $10^4$/well. Plates were incubated at 37°C in 5% $CO_2$ with weekly refeeding, and LCL outgrowth was scored after 4 weeks. Results are expressed as the minimum seeding of LCLs required for successful outgrowth.

## RESULTS

**CD4 epitope mapping of EBV latent-cycle antigens.** In initial epitope mapping experiments, we increased the number of EBV-seropositive donors screened for $CD4^+$ T-cell reactivity to EBNA1 and EBNA3C peptide panels (13) and extended the analysis to new peptide panels covering the primary sequences of EBNA2 and EBNA3A. The results of these assays are summarized in Fig. 1, showing all of the individual peptides against which $CD4^+$ T-cell memory was detected; numbers refer to the coordinate of the first amino acid in the peptide sequence. For each peptide, the histogram indicates the overall percentage of seropositive donors who made a detectable response. These results, based on the screening of 23 to 32 donors per peptide panel, confirm that a 200-amino-acid stretch in the C-terminal half of EBNA1 is a rich source of CD4 epitopes, with 75% of the donor cells reactive to 1 or more of the 17 epitopes in this area. In addition 70% of the donor cells also responded to 1 or more of 11 epitopes in the much larger EBNA3C protein. Of note are individual epitopes, for example, EBNA1 515 (TSL) and EBNA3C 386 (SDD), that were recognized by about 30% of the donors tested. Although almost as large as EBNA3C, the 944-amino-acid EBNA3A protein proved to be much less immunogenic to the $CD4^+$ T-cell response, with only three epitope peptides detected and <25% of the donors reactive to any one of these epitopes. Screening of EBNA2, a protein with a unique sequence similar in size to that of EBNA1, revealed only six epitopes; however, one of these (EBNA2 276, PRS) was recognized by 40% of the donors tested, and this was largely responsible for the overall frequency of EBNA2-reactive donors reaching 65%.

Throughout these ELISPOT assays, we consistently found that the size of CD4 epitope-specific memory populations in peripheral blood lay between the detection threshold of 30 spot-forming cells (SFC) and a maximum value of 350 SFC per $10^6$ CD8-depleted PBMCs. This range is 10-fold smaller than

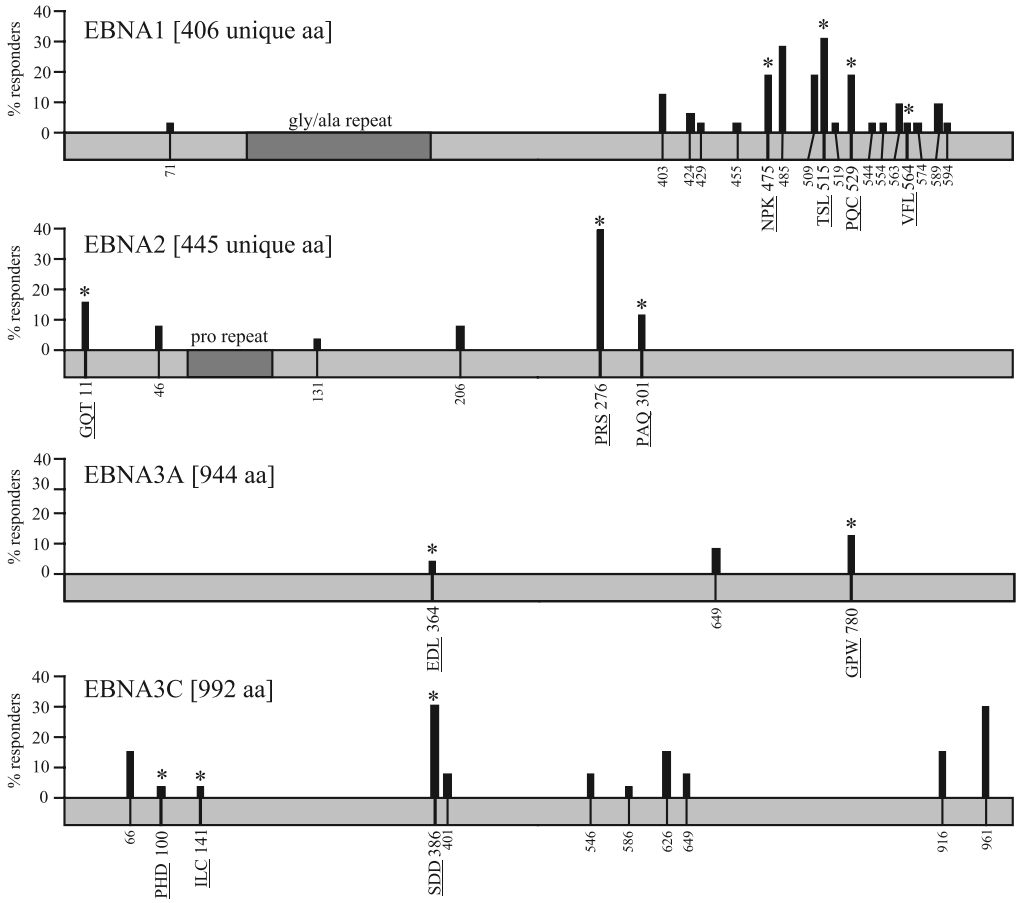


FIG. 1. Mapping of CD4$^+$ epitopes within the primary sequences of EBNA1, -2, -3A, and -3C. The position of each epitope is identified by a number that refers to the coordinate of the first amino acid in the protein as encoded by EBV strain B95.8. The bar above each epitope position indicates the percentage of all EBV-seropositive donors tested who responded to the relevant epitope peptide in ELISPOT assays. Epitopes against which CD4$^+$ T-cell clones were later derived are also identified by a three-letter code corresponding to the first three amino acids of the epitope sequence and by an asterisk above the bar. For each protein, the number of unique amino acids (aa) is shown; this excludes the 233-amino-acid glycine-alanine repeat domain (residues 93 to 325) for EBNA1 and the 42-amino-acid polyproline repeat domain (residues 59 to 100) for EBNA2.

the equivalent range of EBV latent epitope-specific CD8$^+$ T-cell memory, which, for immunodominant CD8 epitopes, can reach up to 3,000 SFC/10$^6$ PBMCs (5, 31). Furthermore, the size of the CD4 response to any particular epitope peptide varied between individual donors across the full range, again in contrast to certain immunodominant CD8 epitopes, which in donors with the appropriate HLA type consistently produce large responses in the ELISPOT assay (5, 31; data not shown).

**Antigen specificity of CD4$^+$ T-cell clones generated by epitope-peptide stimulation.** On the basis of the above assays, we selected 12 epitopes (4 from EBNA1, 3 from EBNA2, 2 from EBNA3A, and 3 from EBNA3C) against which to generate CD4$^+$ T-cell clones. These epitopes included some that were recognized by a large proportion of donors, for example, EBNA1 515 (TSL), EBNA2 276 (PRS), and EBNA3C 386 (SDD), and others that represented relatively rare responses.

Using donors with detectable CD4$^+$ T-cell memory to the individual peptides in ELISPOT assays, we generated CD4$^+$ T-cell clones by limiting-dilution cloning of PBMCs 7 days after peptide stimulation in vitro. All proliferating cultures were first screened for peptide reactivity in ELISAs of IFN-γ release, and peptide-specific clones were checked for antigen specificity by testing on autologous antigen-presenting cells that had been loaded with an exogenous supply of the relevant EBV protein preparation. Figure 2 shows representative results from these antigen specificity assays, with clones raised against the EBNA1 515 (TSL), EBNA2 276 (PRS), EBNA3A 780 (GPW), and EBNA3C 386 (SDD) epitopes. In each case, we were able to confirm that the clones recognized not only the epitope peptide but also processed antigen, whereas there was no significant response to control epitope or antigen preparations. Specific recognition of antigen-loaded cells clearly required processing and could not be ascribed to contamination of the protein preparation with preformed peptide, since prefixing the presenting cells eliminated the response to antigen but not that to exogenously loaded peptide (data not shown). Figure 2 also shows representative MAb blocking assays in which peptide-loaded autologous cells were used as targets in the presence of high concentrations of HLA-DP-, -DQ-, and -DR-specific MAbs. The results show that the TSL-, PRS- and GPW-specific clones were all restricted through an HLA-DR allele and the SDD-specific clones were all restricted through

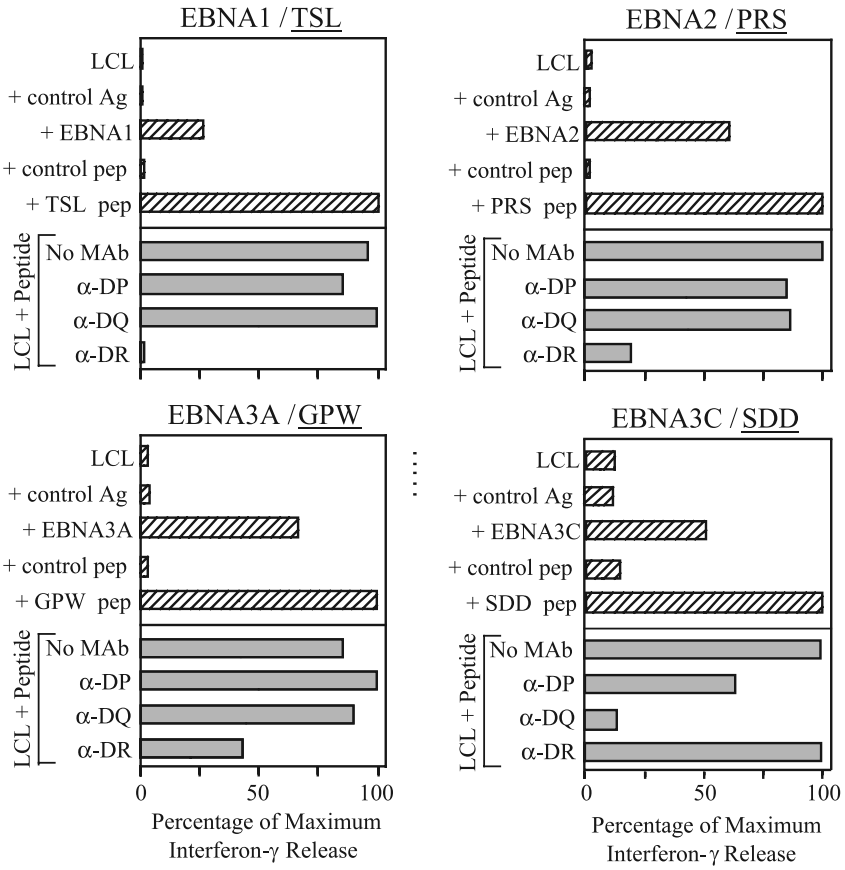


FIG. 2. Antigen (Ag) specificity of CD4+ T-cell clones raised against the TSL (EBNA1 515), PRS (EBNA2 276), GPW (EBNA3A 780), and SDD (EBNA3C 386) epitopes. (Top panels) Clones (500 T cells per well) were stimulated overnight with autologous LCLs that were either unmanipulated, prepulsed for 1 h with 5 μM epitope peptide (pep; or an equivalent concentration of control peptide), or preexposed in serum-free medium for 2 h to specific EBV antigen preparations (or to a control antigen) and then washed before the assay. Responses are expressed as percentages of the maximum IFN-γ induced by peptide-loaded target cells in each case. (Bottom panels) The results shown in the bottom panels are from a separate experiment in which autologous LCLs were preexposed for 1 h to epitope peptide at a concentration mediating half-maximal responses, washed, and then incubated for a further hour in the presence of MAb to HLA-DP, HLA-DQ, or HLA-DR or in medium as a control (No MAb) before the addition of 500 T cells to the cell suspension. Results are expressed as described above. Note that assays conducted with the PRS-specific clones were carried out with LCLs transformed with the type 2 Ag876 EBV strain, in which the PRS epitope sequence is mutated (10), thereby reducing background LCL recognition to zero.

an HLA-DQ allele. In each case, the relevant allele could be determined by screening partially HLA-matched target cells for the ability to present the peptide. This identified the restricting alleles as HLA-DR103 for TSL, HLA-DR52 for PRS, HLA-DR1 for GPW, and HLA-DQ5 for SDD (data not shown). In this way, restriction was mapped to defined HLA-DR or HLA-DQ alleles for 10 of the 12 epitopes being studied and to HLA-DP alleles for the other 2 epitopes (see Table 1).

**LCL recognition by CD4+ T-cell clones: IFN-γ release assays.** For the 12 sets of clones reactive to individual epitopes, we then conducted a series of IFN-γ release assays (i) to determine each clone's functional avidity, defined in peptide titration assays as that peptide concentration mediating 50% maximal recognition, and (ii) to determine the efficiency with which each clone recognized the unmanipulated LCL, expressing this as a percentage of the maximal response seen on the same targets loaded with an optimal concentration of epitope peptide. All of the clones generated against the same epitope-HLA allele combination were functionally similar to one another, and so the data for individual epitopes are illustrated with a representative clone in each case.

Figure 3 presents the results for three CD4 epitopes in EBNA1, PQC (529) restricted through HLA-DR14, VFL (564) restricted through an HLA-DP allele, and TSL (515) restricted through HLA-DR103. Peptide titrations (top panels) determined the functional avidities as 15 nM for PQC-specific clones, 30 nM for VFL-specific clones, and 90 nM for TSL-specific clones. The same clones were then assayed at a range of T-cell inputs (500 to 5,000 per well) against the autologous LCL and an HLA-mismatched LCL (seeded at $5 \times 10^4$ per well) where the LCL targets had either been preexposed to the cognate peptide at a 5 μM concentration and then washed (middle panels) or left untreated (bottom panels). The PQC-specific clone showed strong recognition of the peptide-loaded autologous LCL, with levels of IFN-γ release increasing with increased T-cell input, and not of peptide-loaded control targets. However, this clone, and several other PQC-specific clones, showed no detectable recognition of the autologous LCL without added peptide. This was also true of clones di-

TABLE 1. Summary of epitope-specific CD4$^+$ T-cell clones

| Protein and epitope | Epitope coordinates | HLA restriction | Functional avidity[a] (concn [nM]) | % Recognition of LCL[b] |
|---|---|---|---|---|
| EBNA1 | | | | |
| PQC | 529–543 | DR14 | 15 | 0 |
| NPK | 475–494 | DP | 50 | 0 |
| VFL | 564–583 | DP | 30 | 0.2 |
| TSL | 515–529 | DR103 | 90 | 3 |
| EBNA2 | | | | |
| PAQ | 301–320 | DR17 | 100 | 3 |
| GQT | 11–30 | DR4 | 80 | 15 |
| PRS | 276–295 | DR52a | 6 | 1 |
| PRS | 276–295 | DR52c | 7 | 1 |
| PRS | 276–295 | DR7 | 30 | 15 |
| PRS | 276–295 | DR52b | 3 | 35 |
| EBNA3A | | | | |
| GPW | 780–799 | DR1 | 30 | 1 |
| EDL | 364–383 | DR15 | 10 | 4 |
| EBNA3C | | | | |
| ILC | 141–155 | DR13 | 50 | 0 |
| PHD | 100–119 | DR16 | 100 | 3 |
| SDD | 386–400 | DQ5 | 30 | 7 |

[a] Functional avidity is defined as the concentration of epitope peptide mediating 50% maximal IFN-γ release in peptide titration assays. Values shown are the means of results of assays on several epitope-specific clones (except for PHD and DR52c PRS, where only one clone was available).

[b] Recognition of the unmanipulated autologous LCL, as measured by IFN-γ release, is expressed as a percentage of the IFN-γ release seen in the same assay against the same LCL optimally loaded with epitope peptide. The value for each epitope is the mean from assays on several epitope-specific clones (except for PHD and DR52c PRS, where only one clone was available).

rected against a different EBNA1 epitope, NPK (475) (data not shown). By contrast, clones specific for the VFL epitope again responded well to peptide-loaded autologous targets but in this case also showed low-level IFN-γ release in response to the autologous LCL itself. This recognition was reproducible, titrated against the input T-cell number, and also was blocked specifically by the same anti-HLA-DP MAb as had blocked the recognition of exogenously loaded peptide in the earlier assays. However, comparing the observed levels of release of IFN-γ against peptide-loaded and unloaded targets in the same assay gave an efficiency of recognition of the unmanipulated LCL of only 0.2%. Other VFL-specific clones gave similarly low values. Interestingly, CD4 clones to the third EBNA1 epitope, TSL, were also capable of recognizing the untreated autologous LCL, but in this case at higher levels representing around 3% of that seen on peptide-loaded targets. Recognition again titrated against the input T-cell number and was blocked with the appropriate antibody, in this case the anti-DR MAb.

The latter result was of particular interest since different groups have reported either no LCL recognition by TSL-specific CD4$^+$ T-cell clones (9) or recognition that appeared to be strong, albeit never directly compared with that seen on peptide-loaded cells (17). To determine whether such differences might have arisen wholly or in part through the use of different in vitro reactivation protocols, we established TSL-specific CD4$^+$ T-cell clones by standard peptide stimulation and compared these to clones generated from the same donor by stimulation with EBNA1 protein-loaded dendritic cells. We found that the two sets of clones were very similar in the above types of assay (data not shown), and indeed their functional avidities were similar to those reported by others for TSL-specific clones reactivated by autologous LCL stimulation (9).

Figure 4 shows corresponding data for CD4$^+$ T-cell clones raised against EBNA2 epitope PAQ (301), EBNA3A epitope GPW (780), and EBNA3C epitope SDD (386). These showed functional avidities of 100, 30, and 30 nM, respectively, a range not dissimilar from that seen with the EBNA1 epitope-specific clones. Again, all three epitope-specific reactivities in Fig. 4 showed strong recognition of peptide-loaded autologous (but not HLA-mismatched) LCL targets and also recognized the unmanipulated autologous LCL at levels representing 3% (for PAQ), 1% (for GPW), and 7% (for SDD) of the optimal levels seen on peptide-loaded targets. These values were reproducibly observed both on repeat testing of the particular clones illustrated and on testing of other clones generated against the same epitopes. In this case, we also compared SDD-specific clones generated by standard peptide stimulation against clones produced by autologous LCL stimulation and found that the two sets were again functionally very similar (data not shown).

Similar experiments were conducted on a further five epitopes from the EBNA2, EBNA3A, and EBNA3C proteins, and we again observed that each individual epitope was associated with its own characteristic level of LCL recognition (for a summary, see Table 1). Of particular interest was EBNA2 epitope PRS (276) because it had been recognized by 40% of the seropositive donors tested in ELISPOT assays (Fig. 1) and in an earlier study (21) appeared to be presented in several different HLA class II contexts. On the basis of our ELISPOT screening, we selected four PRS-responsive donors with disparate HLA class II types and from each generated PRS-specific clones that, when tested in MAb blocking assays, were all found to be restricted through an HLA-DR allele. Since all individuals possess two DRB1 alleles and, in some cases, also one or two additional DR alleles (designated DR51, -52a, -52b, -52c, and -53), we screened each set of clones on a large panel of fully DR-typed target cells loaded with the PRS peptide. Figure 5 shows data from a representative range of targets sufficient to map the HLA restricting allele in each case. We found that PRS-specific clones from the four different donors used four different alleles, DR52a, DR52b, DR52c, and DR7.

The four different sets of clones were then tested for functional avidity and for autologous LCL recognition. As shown in Fig. 6, all clones restricted through DR52 alleles had unusually high avidities in peptide titration assays, with 50% end points at 6 nM (DR52a), 3 nM (DR52b), and 7 nM (DR52c), whereas the value of 30 nM for DR7-restricted clones was in the range seen earlier for clones restricted through other DRB1 alleles. Interestingly, all four sets of clones showed detectable recognition of the unmanipulated autologous LCL but at widely different efficiencies. These ranged from 1% of that seen on peptide-loaded targets for the DR52a- and DR52c-restricted clones to 15% for the DR7-restricted clones and to as high as 35% for DR52b-restricted clones. To check the reproducibility of the latter result, we generated PRS-specific clones from a second DR52b-positive donor by standard peptide stimulation and from the same donor by two other in vitro reactivation protocols, namely, stimulation with peptide-loaded dendritic

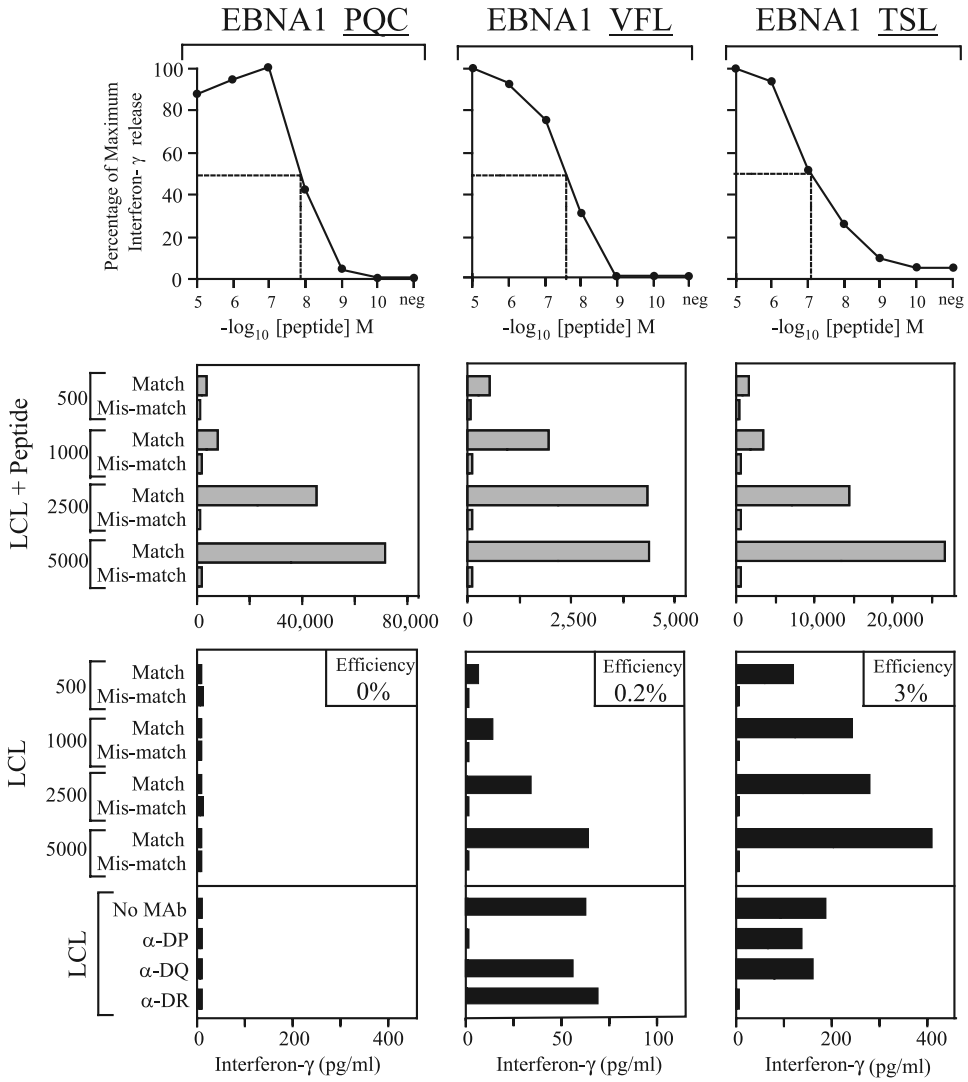


FIG. 3. Functional analysis of CD4+ T-cell clones against the PQC (EBNA1 529), VFL (EBNA1 564), and TSL (EBNA1 515) epitopes. (Top panels) Clones (100 T cells per well) were stimulated overnight with autologous LCLs ($5 \times 10^4$ per well) either unmanipulated (neg) or loaded with epitope peptide at $10^{-5}$ to $10^{-10}$ M concentrations. Responses were assayed by IFN-γ release and expressed as a percentage of the maximum peptide-induced response. (Middle panels) Clones (500 to 5,000 T cells per well) were stimulated as described above with the autologous LCLs (match) or with HLA class II-mismatched LCLs ($5 \times 10^4$ per well), both previously exposed to 5 μM epitope peptide and then washed before the assay. Responses are expressed as IFN-γ release in picograms per milliliter. (Bottom panels, upper section) Clones were tested at the same time as above, on the same autologous and HLA class II-mismatched LCLs but with no exogenous peptide treatment. Responses are expressed as IFN-γ release in picograms per milliliter. The efficiency with which each clone is able to recognize unmanipulated autologous LCL targets is expressed as a percentage of the maximal response seen on the same targets loaded with peptide (box at upper right of each graph). (Bottom panels, lower section) The results shown in the lower section of the bottom panels are from a separate experiment in which the responses of 500 T cells to the autologous LCL (non-peptide loaded) were assayed either alone (no MAb) or in the presence of MAbs to HLA-DP, HLA-DQ, or HLA-DR as described above.

cells and stimulation with the LCL alone. All epitope-specific DR52b-restricted clones, regardless of the stimulation protocol or individual donor, gave a similar pattern of results.

Table 1 summarizes the data obtained with CD4+ T-cell clones to all 12 of the epitopes studied, including the PRS epitope in its four different HLA contexts. Epitopes are grouped in accordance with their source antigen and, within each group, ordered by the efficiency of LCL recognition shown by epitope-specific clones; the functional avidity of these clones is shown alongside.

**LCL recognition by CD4+ T-cell clones: cytotoxicity and LCL outgrowth assays.** The ability of EBV latent-epitope-specific CD4+ T cells to recognize naturally infected B-cell targets implies that such T cells might have a direct effector role in the control of EBV infection. For that reason, we examined whether the CD4+ T-cell recognition of LCLs observed by IFN-γ release was also detectable with other functional readouts, namely, short-term cytotoxicity and longer-term LCL outgrowth assays. This work was conducted with CD4+ T-cell clones to five selected epitopes; these were (in

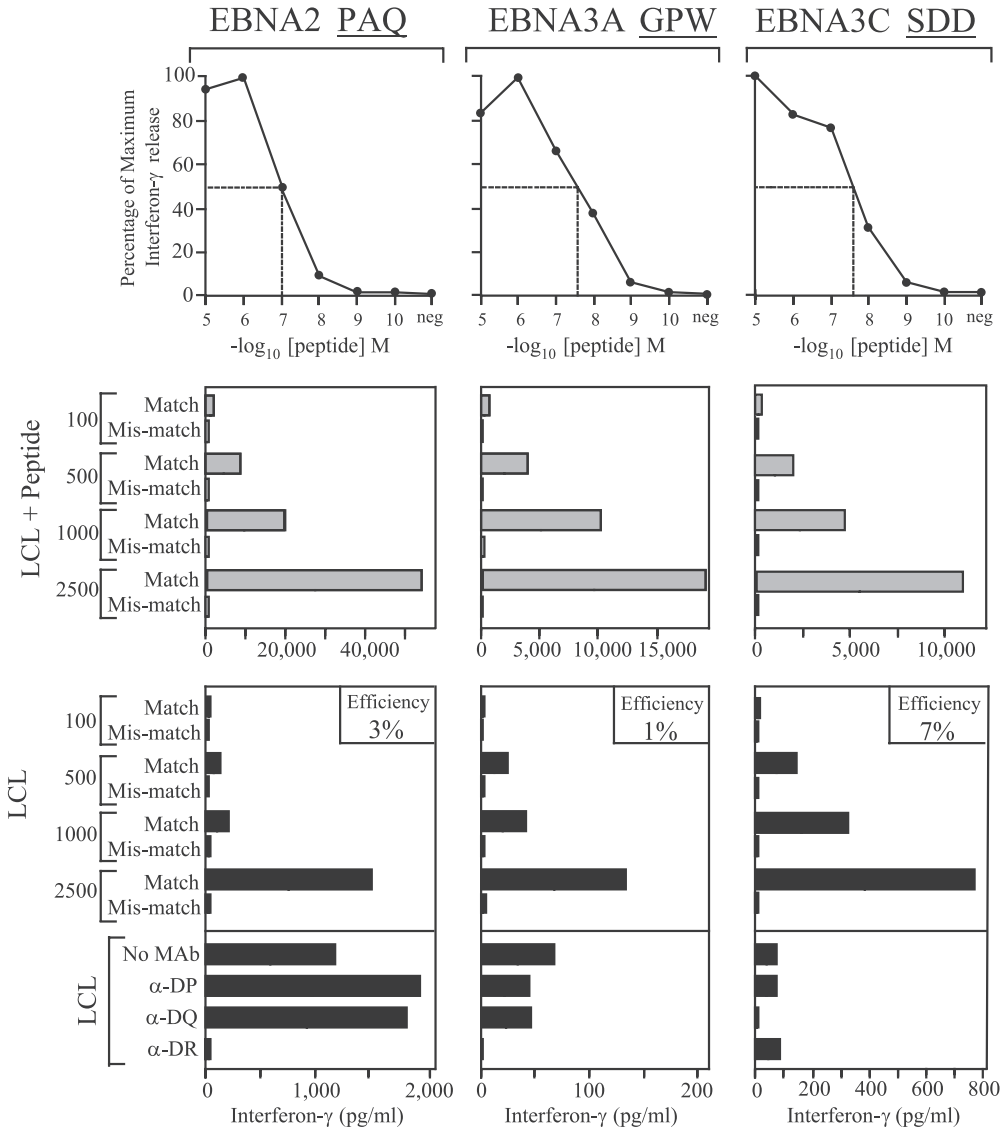


FIG. 4. Functional analysis of $CD4^+$ T-cell clones against the PAQ (EBNA2, 301), GPW (EBNA3A, 780), and SDD (EBNA3C, 386) epitopes. The experimental design and expression of results are the same as in Fig. 3, except that the range of T-cell numbers used in LCL stimulation experiments extended from 100 to 2,500 per well.

order of increasing efficiency of LCL recognition by IFN-γ release) clones specific for EBNA1 epitopes PQC and TSL, EBNA2 epitope PAQ, EBNA3C epitope SDD, and DR52b-restricted EBNA2 epitope PRS.

Figure 7 presents chromium release assay data showing the levels of killing observed against HLA class II-matched and mismatched target LCLs, each tested with and without preloading with the relevant epitope peptide. Because pilot experiments had shown that killing was not always apparent within the conventional 5-h assay period, we measured specific lysis after both 5 and 18 h. All of the clones showed killing of the HLA-matched peptide-loaded LCL, apparent within 5 h and stronger by 18 h, and no killing of the mismatched peptide-loaded target. However, the clones differed in the ability to kill the unmanipulated HLA-matched LCL. The PQC (data not shown)- and TSL-specific $CD4^+$ effectors did not lyse these targets significantly, even within 18 h. The PAQ-specific clone gave marginal killing after 18 h only, while the SDD-specific and PRS-specific clones both gave a hint of killing within 5 h and clearly detectable lysis at the later time.

We then set up outgrowth assays in which replicate cultures of HLA-matched and mismatched LCLs, either untreated or preloaded with the relevant epitope peptide, were seeded into U-bottom microtest plate wells at doubling dilutions of $10^4$ to 300 cells per well; to some wells at each seeding, a standard number of $CD4^+$ T cells ($10^4$ cells per well) were then added from the same clones as tested above. The cultures were maintained in standard cell culture medium for 3 to 4 weeks and examined for successful LCL outgrowth. Although these experiments could be accurately scored by microscopic inspection of the cultures, in several cases we confirmed by CD19 staining that successful outgrowth involved the LCL and not

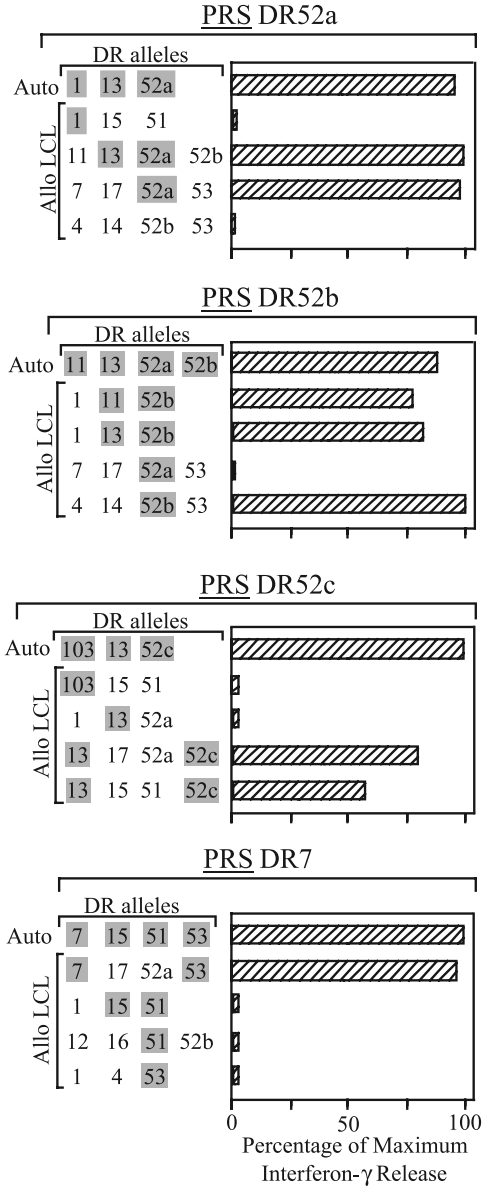


FIG. 5. Analysis of HLA class II allele restriction of CD4+ T-cell clones specific for the PRS (EBNA2, 276) epitope. Clones were established from four donors whose HLA-DR types are in each case identified (Auto). These clones were then tested as described in the legend to Fig. 3 (middle panels) against peptide-loaded cells of the autologous LCL and of allogeneic LCLs of known HLA-DR type (Allo LCL). Alleles matched with the autologous cells are identified by shading. Results are expressed as a percentage of the maximum IFN-γ release observed in the assay.

surviving T cells. Figure 8 expresses the results of these assays as the minimum number of each LCL required for successful outgrowth under the various conditions. The results are consistent with the earlier experiments in that clear evidence of LCL growth inhibition was limited to T-cell–LCL combinations with the higher levels of LCL recognition as determined by IFN-γ release. Thus, PQC (data not shown)-, TSL-, and PAQ-specific CD4+ T-cell clones, which had shown levels of unmanipulated LCL recognition of 0 to 3% of that seen against peptide-loaded targets, markedly inhibited outgrowth of HLA-matched LCLs if they had been peptide loaded but had no effect on the corresponding nonloaded cells; as a specificity control, these same clones had little if any effect on HLA-mismatched LCLs with or without peptide loading. By contrast, clones specific for the SDD and PRS epitopes, which had shown stronger recognition of autologous LCL in IFN-γ assays, clearly were able to inhibit the outgrowth of HLA-matched (but not mismatched) LCLs even without peptide loading.

## DISCUSSION

The extent to which EBV latent-specific CD4+ T cells are able to recognize naturally infected LCL targets is an important in vitro indicator of their likely potential as direct effectors controlling EBV-driven lymphoproliferations in vivo. Most in vitro studies to date have focused on CD4+ T-cell responses to just one of the available latent proteins, EBNA1 (9, 13, 17, 27, 32) and, even when studying responses to the same epitope (9, 17), have reported discordant results with respect to LCL recognition. To address this issue in a more systematic way, the present work set out to identify CD4 epitopes in a broader range of latent-cycle antigens, EBNA1, -2, -3A, and -3C, and then to generate CD4+ T-cell clones to a representative panel of epitopes drawn from these four proteins.

Screening with peptide panels showed that three antigens, EBNA1, -2, and -3C, are each recognized by a majority (65 to 75%) of EBV-seropositive donors, whereas only a small number (<25%) respond to EBNA3A. Interestingly certain epitopes, for example, TSL in EBNA1, PRS in EBNA2, and SDD in EBNA3C, were recognized by 30 to 40% of the donors tested. Such a high frequency is explained in the case of the PRS epitope by its capacity to elicit responses in the context of several different HLA alleles (Fig. 5) (21). The same also appears to be true of the TSL epitope because, although we established epitope-specific clones that were DR103-restricted, not all responders to TSL in ELISPOT screening assays express this allele (13; data not shown). However, this was not the case for the SDD epitope; this was restricted to HLA-DQ5, a high-incidence allele in Caucasian populations (16), and all responders to this epitope indeed proved to be HLA-DQ5 positive. It is important to note that, while a high percentage of immune donors might respond to certain epitopes, these are not necessarily immunodominant responses in terms of absolute size. Thus, both here and in an earlier study (13), we found that responses to all CD4 epitopes fell within a rather narrow size range and the strongest responses did not consistently map to a particular set of epitopes or to epitopes from a particular antigen. This contrasts with the CD8 response to EBV latent-cycle antigens, which is not only much larger than the CD4 response but also tends to focus preferentially on immunodominant epitopes from the EBNA3A, -3B, and -3C proteins (11, 25).

Having established CD4+ T-cell clones to 12 representative epitopes and confirmed their specificity for the relevant EBV target antigen in protein loading assays, we determined their functional avidity by peptide titration. There were two important findings in this regard. Firstly, all clones produced by peptide stimulation of PBMCs and specific for the same

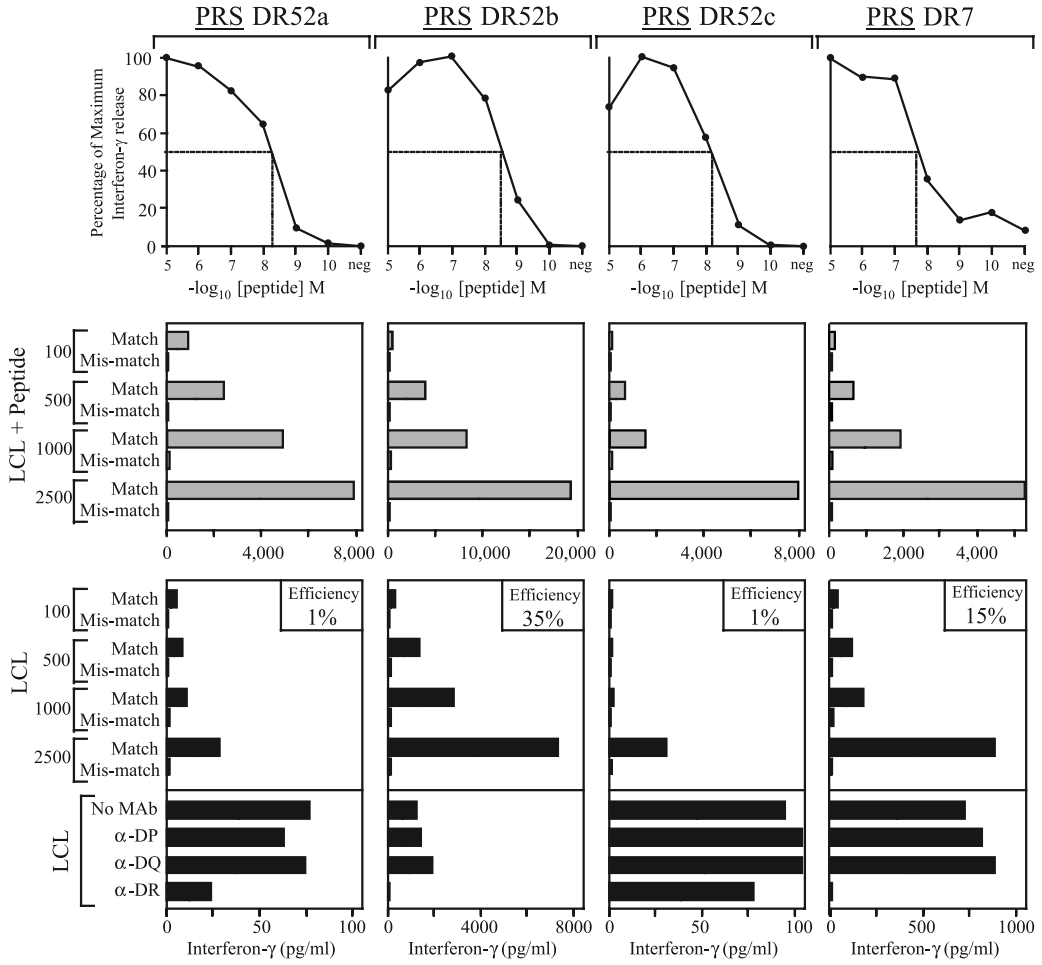


FIG. 6. Functional analysis of $CD4^+$ T-cell clones specific for the PRS (EBNA2, 276) epitope derived from different EBV-seropositive donors and restricted through HLA-DR52a, -DR52b, -DR52c, and -DR7, respectively. The experimental design and expression of results are the same as in Fig. 4. Note that peptide titration assays involving the DR52b-restricted PRS clones were conducted with the Ag876 virus-transformed LCL as in Fig. 2.

epitope-HLA allele combination, whether from the same donor or different donors, gave similar peptide titration curves. Secondly, in several cases we compared epitope-specific $CD4^+$ T-cell clones that had been generated from individual donors either by conventional peptide stimulation, by peptide- or antigen-loaded dendritic cells, or by LCL stimulation and, whenever we tested them, found no significant differences in functional avidity. We infer that stimulation protocols are not major sources of artifacts in these experiments and that the $CD4^+$ T-cell clones being used are genuinely representative of the epitope-specific memory populations present in our EBV-immune donors.

We then turned to the question of LCL recognition. To allow comparisons to be made between individual clones to a single epitope and between clones specific for different epitopes, in each case we expressed the level of recognition of the unmanipulated LCL as a percentage of that seen in parallel against the same LCL preloaded with an optimal concentration of epitope peptide. The overall findings, summarized in Table 1, allow a number of conclusions to be drawn. Firstly, the level of LCL recognition is consistent among clones with the same epitope specificity but differs markedly, from 0 to 35% of optimal peptide-loaded values, between clones with different specificities. Secondly, these interepitope differences are not obviously related to the antigenic source of the epitope. Thus, levels of LCL recognition ranged from 0 to 3% of peptide-loaded values for EBNA1 epitopes, from 1 to 35% for EBNA2 epitopes, from 1 to 4% for EBNA3A epitopes, and from 0 to 7% for EBNA3C epitopes. Thirdly, the differences in LCL recognition cannot solely be explained by differences in the functional avidities of the $CD4^+$ T-cell clones. For example, clones to the PQC epitope in EBNA1 had an avidity of 15 nM and showed no LCL recognition whereas clones to the TSL epitope also in EBNA1 recognized the LCL at an efficiency of 3% yet required a sixfold higher peptide concentration (90 nM) for half-maximal IFN-γ release in peptide titration assays. Likewise, among EBNA2-specific clones, those against the GQT epitope showed a 15% efficiency of LCL recognition yet were 10-fold less avid in peptide titration assays than DR52a-restricted, PRS-specific clones, which show much less efficient (1%) LCL recognition. We conclude that, as for $CD8^+$ T-cell clones in this viral system (3, 14, 15), the observed level of LCL

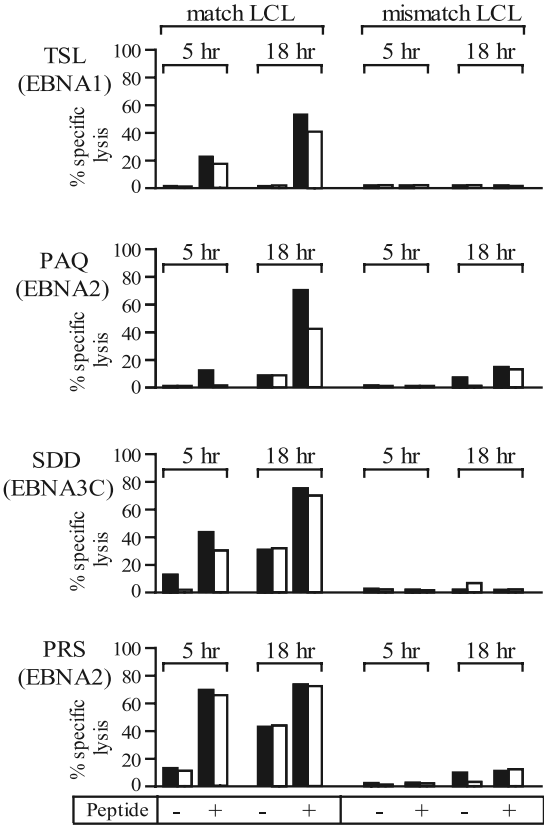


FIG. 7. Killing of LCL targets by CD4+ T-cell clones against the TSL (EBNA1 515), PAQ (EBNA2 301), SDD (EBNA3C 386), and PRS (EBNA2 276) epitopes. Five- and 18-h chromium release assays were conducted with HLA class II-matched and mismatched LCL targets either unmanipulated or previously exposed to 5 μM epitope peptide and then washed before the assay. Results are expressed as percent specific chromium release from target cells at effector/target ratios of 5:1 (■) and 2.5:1 (□).

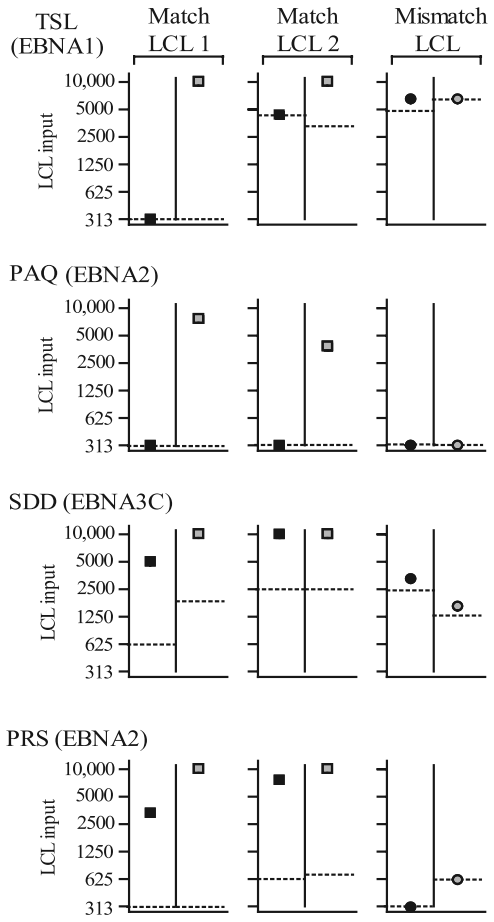


FIG. 8. Inhibition of LCL outgrowth by the epitope-specific CD4+ T-cell clones used in Fig. 7. For each clone, two HLA class II-matched LCLs and one mismatched LCL were seeded at doubling dilutions of $10^4$ to 300 cells per well either alone or with the addition of $10^4$ CD4+ T cells. The LCLs were either unmanipulated or previously exposed to 5 μM epitope peptide and then washed before seeding. Results are expressed as the minimum LCL seeding required for successful outgrowth in each case. For each clone, the results from LCL–T-cell cocultures are shown for the unmanipulated LCL (■) and in the adjacent column for the peptide-loaded LCL (▨). These values are in each case compared with the corresponding results for outgrowth of the unmanipulated LCL or of the peptide-loaded LCL cultured in the absence of T cells (dotted lines).

recognition will be a function both of the inherent avidity of the CD4+ T-cell clones and of the degree of representation of the epitope-HLA complex on the LCL surface.

The example of the PRS epitope is particularly interesting in this regard. It was already clear from the literature that this epitope can elicit responses in the context of several different HLA class II alleles. Thus, Khanna et al. reported strong killing of the autologous LCL by a PRS-specific clone reactivated by LCL stimulation and restricted through an HLA-DQ allele (10). Subsequently, Omiya and colleagues generated PRS-specific clones by peptide stimulation from several different donors and mapped their restriction to five different HLA alleles. Interestingly, all clones recognized the autologous LCL by IFN-γ release, although at levels that were never compared to the maximal peptide-induced response, whereas only clones restricted through an unidentified DR52 allele killed LCL targets in cytotoxicity assays (21). Our work extends this analysis by accurately quantitating LCL recognition by PRS-specific clones restricted through four different HLA class II alleles. Recognition ranged from 1% efficiency for DR52a- and DR52c-restricted clones through 15% efficiency for DR7-restricted clones to 35% efficiency for DR52b-restricted clones. Again, these differences cannot be explained by differences in functional avidity but instead reflect how the level of representation of the PRS epitope on the LCL surface is critically influenced by the identity of the restricting allele.

A similar phenomenon may underlie the apparent discrepancy in the literature with respect to CD4+ T-cell clones specific for the TSL epitope in EBNA1. Khanna et al. reported no LCL recognition in cytotoxicity assays by a DR1-restricted TSL clone (9). This is reminiscent of the DR103-restricted clones described in the present work, which again did not kill the LCL but showed low-level (3%) efficiency of recognition by IFN-γ release. In contrast, the TSL-specific clones with strong LCL killing described by Munz and colleagues (17) may be restricted through a different HLA class II allele that mediates more efficient epitope presentation at the LCL surface. With

promiscuous epitopes such as PRS and TSL, one can begin to look for correlations between the level of epitope presentation on infected cells and immunogenicity of the epitope in vivo. Clearly in the case of PRS, even low-level presentation in the context of the HLA-DR52a or -52c allele can elicit a response; however, we found that only a small number of individuals with the DR52a or DR52c allele screened actually made a detectable response to the peptide in ELISPOT assays. By contrast, most individuals positive for HLA-DR7 or -DR52b, an allele that mediates more efficient presentation of the epitope, did have detectable PRS-specific memory (data not shown). These questions will be better addressed once the restricting alleles for other apparently promiscuous EBV epitopes have been identified.

One of our main motivations for this work was to address the potential importance of EBV latent epitope-specific $CD4^+$ T cells as direct effectors capable of recognizing and eliminating EBV-driven lymphoproliferations in vivo. Our final series of experiments, measuring both short-term cytotoxicity and long-term inhibition of target cell outgrowth in vitro, show firstly that all of the epitope-specific $CD4^+$ T-cell clones tested were cytotoxic on epitope-loaded LCL targets and could inhibit their outgrowth in cocultivation assays. However, parallel assays on unmanipulated LCL targets split the clones into two groups. Clones with an efficiency of LCL recognition at or below 3% in IFN-γ assays showed no detectable killing in 18-h assays and no detectable inhibition of outgrowth against the unmanipulated LCL, whereas clones with efficiencies of 7% or greater were active in both situations. These results, and those of other studies with $CD4^+$ T-cell clones against as yet undefined targets on the LCL surface (5), are all consistent with the view that inhibition of LCL outgrowth correlates strongly with cytotoxic activity. The present work confirms that $CD4^+$ T cells specific for some EBV latent-cycle epitopes can prevent LCL outgrowth in vitro (17, 19, 21) and therefore, like their $CD8^+$ T-cell counterparts, could act directly against EBV-driven lymphoproliferative lesions in vivo (24). However, our data suggest that this is only true of $CD4^+$ T cells against a minority of epitopes, namely, those epitopes represented on the surface of latently infected cells above a critical threshold. This is not to imply that T cells specific for other latent-cycle epitopes do not play an important role in vivo; for example, they may act to help $CD8^+$ responses but such helper activity is more likely to be induced by specific recognition of antigen exogenously acquired and presented by dendritic cells rather than of antigen endogenously expressed by infected cells themselves. From a therapeutic standpoint, identifying the subset of epitope-HLA combinations that can mediate direct T-cell recognition and determining the route whereby these epitopes access the HLA class II pathway in infected cells represent important priorities for future work.

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# Amino acid changes in functional domains of latent membrane protein 1 of Epstein–Barr virus in nasopharyngeal carcinoma of southern China and Taiwan: prevalence of an HLA A2-restricted 'epitope-loss variant'

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Full-length sequences of the Epstein–Barr virus (EBV) gene for latent membrane protein (LMP)-1 from 22 nasopharyngeal carcinoma (NPC) biopsy specimens and 18 non-neoplastic counterparts (NPI) were determined. Relative to the B95-8 strain, the amino acid sequences of the toxic-signal and transformation domains were changed variably in NPC and NPI specimens; in contrast, no change was observed in the NF-$\kappa$B (nuclear factor $\kappa$B) activation domain. HLA typing revealed that 47 % of NPC and 31 % of NPI specimens were HLA A2-positive. A major A2-restricted epitope within LMP-1 (residues 125–133) was analysed. At residue 126, a change of L→F was detected in 91 % (20/22) of NPC and 67 % (12/18) of NPI specimens. In addition, a deletion at residue 126 was detected in one NPC sample from Taiwan. At residue 129, a change of M→I was observed in all samples, regardless of whether they were NPC or NPI. The changes in this peptide between NPC and NPI specimens, including mutation and deletion, are statistically significant ($P < 0{\cdot}05$). A recent report indicated that this variant sequence is recognized poorly by epitope-specific T cells. Genotyping results indicated that 96 % of NPC and 67 % of NPI samples carried a type A virus. By scanning the entire sequence of LMP-1, eight distinct patterns were identified. Detailed examination of these patterns revealed that type A strains are more prevalent in NPC than in NPI specimens and are marked by the loss of an *Xho*I site, the presence of a 30 bp deletion and the presence of a mutated, A2-restricted, T cell target epitope sequence. These results suggest that an EBV strain carrying an HLA A2-restricted 'epitope-loss variant' of LMP-1 is prevalent in NPC in southern China and Taiwan.

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