TARGETING EBV ASSOCIATED MALIGNANCIES USING HLA-A2/EBNA-1 SPECIFIC MONOCLONAL ANTIBODIES

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SUMMARY

Pathogen infected cells express antigens that are recognised by cytotoxic T lymphocytes (CTLs) as short peptides complexed with surface MHC class I molecules. These MHC/peptide complexes provide a specific and unique target for immune based therapeutic approaches for targeting cells expressing viral antigens or tumour specific epitopes. Hence an antibody that mimics the specificity of cytotoxic T lymphocytes and recognises MHC/peptide complexes on the surface of tumour cells, represents an attractive new targeting strategy.

We have developed a T cell receptor-like antibody (TCR-like antibody) which targets EBV nuclear antigen 1 (EBNA-1) in the context of HLA-A201 using conventional hybridoma techniques with a high degree of specificity. EBNA-1, a latent protein of Epstein-Barr Virus (EBV), is a DNA-binding protein that is essential for the replication and maintenance of the EBV genome and is expressed in all EBV-infected cells. EBV is a gamma herpesvirus found in all human populations and is associated with malignancies such as Burkitt's lymphoma (BL), Hodgkin's lymphoma (HL) and nasopharyngeal carcinoma (NPC).

In our study, we detail the generation and characterisation of this monoclonal antibody. We demonstrate that this monoclonal antibody exhibit a specific binding pattern and binds with high affinity to HLA-A201/EBNA-1 complexes on the surface of EBV transformed B-lymphoblastoid cell lines (BLCLs), tumour cell lines and NPC biopsies. In addition, this monoclonal antibody is able to detect constitutive levels of HLA-A201/EBNA-1 complexes and as such represents a novel reagent for EBV research and for targeting EBV-associated malignant cells.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percentage
µg	Microgram
µl	Microlitre
µM	Micromolar
µm	Micrometre
APCs	Antigen presenting cells
APS	Ammonium persulphate
BCR	B cell receptor
BL	Burkitt's lymphoma
BLCLs	B-lymphoblastoid cell lines
BSA	Bovine serum albumin
cm^2	Square centimetre
CR2	Complement receptor 2
CTLs	Cytotoxic T lymphocytes
CV	Column volume
DCs	Dendritic cells
DEAE	Diethylaminoethyl
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EBNA	EBV nuclear antigen
EBV	Epstein-Barr Virus
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
FBS	Foetal bovine serum
g	Gram/gravitational force
Gly-Gly-Ala	Glycine-glycine-alanine
HAT	Hypoxanthine-aminopterin-thymidine

HD	Hodgkin's disease
HL	Hodgkin's lymphoma
HLA	Human leucocyte antigen
Hr/hrs	Hour/Hours
HRP	Horseradish peroxidase
HT	Hypoxanthine-thymidine
IFN-γ	Interferon gamma
IM	Infectious mononucleosis
IPTG	Isopropyl β-D-I-thiogalactopyranoside
Kbp	Kilobase pair
kDa	Kilo dalton
L	Litre
Lat	Latency
LA	Luria-Bertani Agar
LB	Luria-Bertani Broth
LCLs	Lymphoblastoid cell lines
LCV	*Lymphocryptovirus*
LK	LB with kanamycin
LMP	Latent membrane protein
M	Molar
mA	Milliampere
mg	Milligram
MHC	Major histocompatibility complex
mins	Minutes
ml	Millilitre
mM	Millimolar
NaEDTA	Sodium ethylenediaminetetraacetate
ng	Nanogram
NPC	Nasopharyngeal carcinoma
^{o}C	Degree Celsius
OD	Optical density
OriP	Origin of replication

PBS	Phosphate buffered saline
PEG	Polyethylene glycol
Pen/strep	Penicillin/streptomycin
PFA	Paraformaldehyde
PMSF	Phenylmethylsulphonylfluoride
PVDF	Polyvinylidene Difluoride
R10	RPMI-1640 with 10% FBS
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RSC	Reed-Sternberg cell
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
s	Seconds
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAP	Transporter associated with antigen processing
TCR-like antibodies	T cell receptor-like antibodies
TEMED	N,N,N',N' – tetramethyl ethylenediamine
TfBI	Transformation buffer I
TfBII	Transformation buffer II
TM	Transmembrane region
TMB	3,3´,5,5´-tetramethylbenzidine
TR	Terminal repeat
V	Voltage
XLA	X-linked agammaglobulinaemia
β_2m	Beta-2-microglobulin
κ	Kappa

LITERATURE REVIEW

Chapter 1

1.1 T Cell Receptor-Like Antibodies (TCR-Like Antibodies)

The advent of major histocompatibility complex (MHC) tetramer technology has enabled the detection and analysis of rare populations of antigen-specific T cells. However, the ability to visualise cells expressing tumour antigens remains suboptimal due to the lack of appropriate reagents that enable the detection of intracellular antigens on live or fresh cells. It is well established that tumour cells express antigens that are recognised by cytotoxic T lymphocytes (CTLs) derived from cancer patients (Renkvist *et al*., 2001; Rosenberg 2001). Antigens are expressed as short peptides in complex with MHC class I molecules on the tumour cell surface. Such tumour-specific MHC/peptide complexes on the surface of tumour cells provide a specific and unique target for immune based therapeutic approaches.

Whilst soluble T cell receptors are able to target these tumour-specific MHC/peptide complexes, they have low affinity for their ligands and are unstable as recombinant-engineered molecules, thus making them potentially unreliable for immunotherapy (Davis *et al*., 1998). An alternative strategy is to engineer a high affinity antibody that can mimic the specificity of the T cell and specifically recognise MHC/peptide complexes on the surface of the tumour cells. These antibodies are termed T cell receptor like antibodies (TCR-like antibodies). Hence, this project involves the generation of a TCR-like antibody targeting a peptide from an Epstein-Barr virus tumour antigen as a model.

1.2 Epstein-Barr Virus

Epstein-Barr virus (EBV) is a gamma herpesvirus of the genus *Lymphocryptovirus* (LCV). Its genome consists of a linear double-stranded DNA that is approximately 184 kilobase pairs (kbp) in length (Kieff and Rickinson, 2001). It was discovered in 1964 by Epstein, Achong and Barr who

identified the herpesvirus by electron microscopy in a cell line from Burkitt's lymphoma (BL), a childhood tumour that is common in Africa (Epstein *et al*., 1965). This virus was shown to be unable to replicate in cultured cells unlike the known human herpesvirus and was not reactive with antibodies of other known human herpesviruses. Hence EBV became the first human tumour virus (Kieff and Rickinson, 2001).

EBV can be classified as either Type I or Type II based on the differences in the EBV nuclear antigens EBNA-2, EBNA-3A, EBNA-3B and EBNA-3C genes (Sample *et al*., 1990). Type I is the predominant form of EBV found in most areas and it has been found in diseases such as nasopharyngeal carcinoma (Chen *et al*., 1992) and head and neck carcinomas (Shu *et al*., 1992). Type II is found mainly in Burkitt's lymphomas and has been isolated in areas such as Africa (Kenya) and New Guinea (Young *et al*., 1987). In addition, Type I EBV transforms and immortalises peripheral blood lymphocytes into B-lymphoblastoid cell lines (BLCLs) more efficiently than Type II EBV (Shu *et al*., 1992).

Epstein Barr virus infection occurs worldwide and the majority of the population are infected with EBV in some stage of their lives. In the United States, as many as 95% of adults between 35 and 40 years old have been infected with EBV which is spread by oral transmission via the saliva of an infected individual. Most primary infections occur during childhood and are asymptomatic. However, if the infection is delayed until adolescence or adulthood, it can result in infectious mononucleosis (IM) (Crawford, 2001). Infectious mononucleosis is associated with the hyperactivation of $CD8^+$ T-cell response and the symptoms of IM include fever, sore throat, swollen lymph glands and severe fatigue. Occasionally, the spleen or liver may swell and on rare occasions, IM may cause heart disorders and affect the central nervous system. However, it is

almost never fatal and patients usually recover within one to two months. Despite this, EBV remains dormant or latent in the infected individual for life (CDC, 2008).

1.3 Routes Of Infection

In normal healthy carriers, EBV infection may infect both epithelial and/or B cells. In the former, the infection is predominantly replicative while in the latter, EBV infection is predominantly latent and has the potential to induce transformation. From studies carried out on patients with IM, high titres of infectious EBV were found in the saliva. This suggests that EBV can reproduce productively in the oral cavity or throat of the patient. Despite these findings, the exact location of productive infection is still under debate with some favouring the oropharyngeal epithelial cell infection while others supporting the direct infection of local mucosal B lymphocytes (Crawford, 2001; Young and Rickinson, 2004).

Evidence supporting epithelial cell infection is seen in cases where EBV is detected in oral hairy leukoplakia. This is a benign lesion of the oral epithelia caused by opportunistic infection of EBV in immuno-compromised patients (De Souza *et al*., 1989). The virus is also detected in pre-invasive undifferentiated nasopharyngeal carcinoma (Pathmanathan *et al*., 1995) and dysplastic gastric epithelium (Gulley *et al*., 1996). Hence EBV could be an early trigger for tumourgenesis in these diseases.

However, EBV has not been detected in normal epithelial cells in tonsillar epithelium of IM patients (Niedobitek *et al*., 2000). Moreover, it is difficult to obtain convincing results of primary infection of epithelial cells since they lack complement receptor 2 (CR2) or CD21, an EBV B cell receptor (Young *et al*., 1989).

Conversely, there is strong evidence to suggest that primary infection involves B cells. A study was carried out on a group of patients with a rare genetic defect in Bruton's thymidine kinase gene (X-linked agammaglobulinaemia, XLA) who lack the mature CR2-expressing B cells. They were found to be free of EBV infection which suggests that without B cells, they are unable to support EBV infection (Crawford, 2001). In addition, sections taken from tonsils removed in acute IM showed the presence of latently and lytically infected B cells while no evidence of epithelial cell infection was detected (Anagnostopolous *et al*., 1995). Thus to date, there is more evidence that favours the direct EBV infection of B cells.

Epstein-Barr virus preferentially infects B cells through the adsorption of EBV outer envelope glycoprotein gp350/220 to complement receptor CR2/CD21 (receptor for complement component C3d) on the cell surface (Nemerow *et al*., 1987). In addition, the viral glycoprotein gp25 (gL) and gp42/38 complexed with viral gp85 (gH) also interacts with human leucocyte antigen (HLA) class II which is a co-receptor for virus entry into B cells (Knox and Young, 1995). The aggregation and cross-linking of CD21 probably results in CD21 mediated tyrosine kinase signal transduction thus activating B cells from their resting state (Masucci *et al*., 1987; Martin *et al*., 1994).

Resting B cells are transformed into permanent, latently infected lymphoblastoid cell lines (LCLs) *in vitro*. Every cell of the LCLs carries multiple extrachromosomal copies of the viral episome and constitutively expresses a set of viral gene products known as the latent proteins (Young and Rickinson, 2004). Occasionally, EBV infects other cells such as epithelial cells. However, infection of these cells is not as efficient and occurs through a separate pathway that is still poorly defined (Borza and Hutt-Fletcher, 2002).

1.4 EBV Latent Gene Products

The latent gene products consist of six EBV nuclear antigens (EBNAs 1, 2, 3A, 3B, 3C and LP), three latent membrane proteins (LMPs 1, 2A and 2B) and transcripts from the *Bam*HI A region (BART transcripts). In addition, LCLs consistently express abundant amounts of small, non-polyadenylated (and therefore non-coding) RNAs, (EBERs 1 and 2) whose functions are still unclear (Kieff and Rickinson, 2001). These latent gene products activate resting B cells to enter into the cell cycle, maintain continuous proliferation and prevent cells from undergoing apoptosis (Tsurumi *et al*., 2005). Figure 1.1 shows the linearised *Bam*HI restriction map of the EBV genome. The *Bam*HI fragments are named according to size with A being the largest fragment. Lowercase letters indicates the smallest fragments. The region labelled N_{het} is a heterogenous region due to the variable number of terminal repeats in different viral isolates and clones of EBV-infected cells (Young and Rickinson, 2004; Murray and Young, 2001).

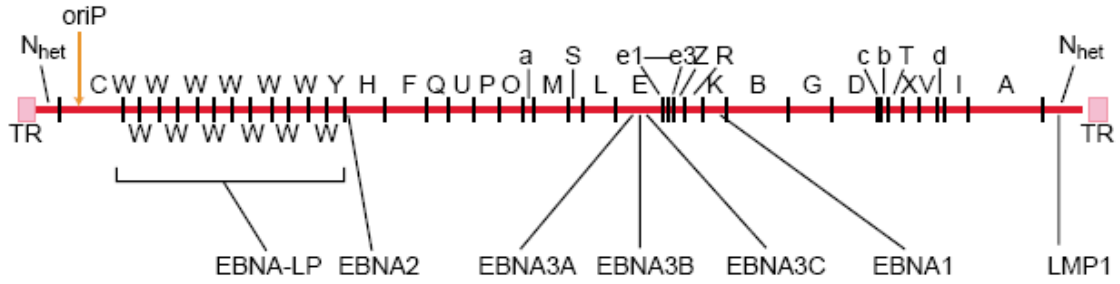


Figure 1.1 Open Reading Frames For EBV Latent Proteins
Location of open reading frames for EBV latent proteins on the *Bam*HI restriction-endonuclease map of prototype B95.8 genome.
(Murray and Young, 2001)

This pattern of latent EBV gene expression is referred to as 'Latency III' (Lat III) (Young and Murray, 2003). Besides this, two other forms of latency patterns are observed. In 'Latency I' (Lat I), which is characteristic of Burkitt's lymphoma (BL), only EBNA-1, the EBERs and *Bam*HI transcripts are detected. In 'Latency II' (Lat II), EBNA-1, the EBERs, *Bam*HI transcripts, LMP-1 and

LMP-2 are expressed and this pattern is observed in EBV-associated Hodgkin's disease (HD) and undifferentiated nasopharyngeal carcinoma (UNPC) (Murray and Young, 2001).

The location and transcription of these latent proteins are illustrated in Figure 1.2. The origin of replication (OriP) is shown in orange. The large green solid arrows represent the coding exons for each of the latent proteins (EBNAs 1, 2, 3A, 3B, 3C, LP and LMPs 1, 2A and 2B) and the direction in which they are transcribed. EBNA-LP is transcribed from various repetitive exons while LMP-2A and LMP-2B are made up of multiple exons located on either side of the terminal repeat (TR) region which is formed during the circularisation of linear DNA to produce the episome. The blue arrows at the top show the highly transcribed RNAs - EBERs 1 and 2. The outer long green arrow shows EBV transcription during Lat III, in which all the EBNAs are transcribed from either the Cp or Wp promoter. The various EBNAs are encoded by individual mRNAs generated by differential splicing of the same long primary transcript. The inner shorter red arrow represents the transcription of EBNA-1 during Lat I and II where the promoter originates at Qp. The blue arrows show the location of BARF0 and BARF1. Although *Bam*HI transcripts are detected during latent infection, no protein has been definitely identified (Young and Rickinson, 2004).

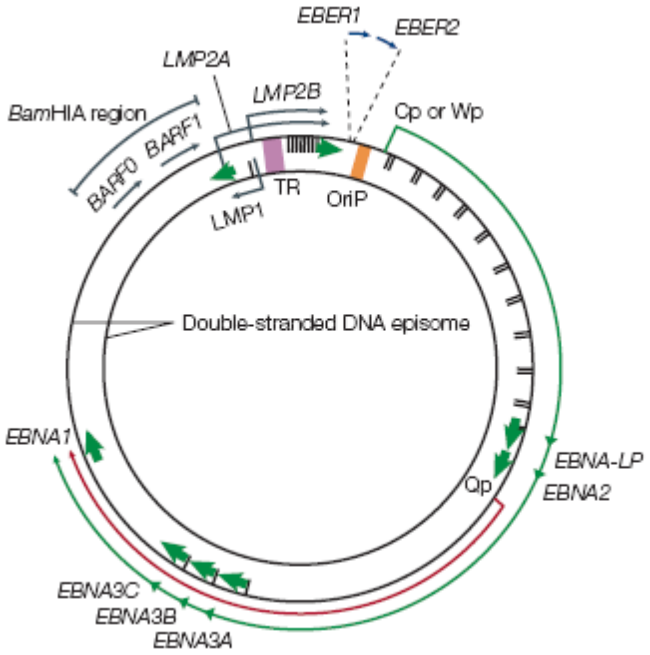


Figure 1.2 The Epstein-Barr Virus Genome
A diagram showing the locations and transcription of latent proteins on the double stranded DNA episome.
(Young and Rickinson, 2004)

Table 1.1 below provides a summary of the various EBV genes, their locations in the cell and the latency types in which they are expressed.

EBV-encoded genes	Location	Latency Type
EBNA-1	Nucleus	I, II, III
EBNA-2	Nucleus	III
EBNA-3	Nucleus	III
LMP-1	Membrane	II, III
LMP-2	Membrane	II, III
EBER-1 and EBER-2	Nucleus	I, II, III

Table 1.1 Locations And Latency Types Of EBV-Encoded Genes

1.5 Replication Cycle

1.5.1 Lytic Cycle

Although most of the LCLs are in the latent state, a small percentage of cells proceed to the lytic cycle. This switch is mediated by the expression of BZLF1 and BRLF1 viral transactivator proteins and triggers the expression of viral genes (Young and Murray, 2003) and certain cellular promoters. This leads to the activation of genes involved in DNA replication and metabolism, followed by genes coding for viral structural proteins. In this lytic cycle, the EBV genome is amplified more than hundredfold by the viral replication machinery (Tsurumi *et al*., 2005). Latent genes are also down-regulated and this results in apoptosis and aids the release of infectious EBV virions.

1.5.2 Primary Infection

In the peripheral blood, EBV is present in the memory B cells and these cells express the latent gene products LMP-2A and EBNA-1 (Babcock *et al.,* 1998). Studies done by Joseph and colleagues showed that a subset of healthy tonsils contains EBV positive naïve B cells which show an activated phenotype, thus suggesting that they have been directly infected (Joseph *et al*., 2000). These naïve B cells are either eliminated by CTLs or differentiate to memory B cells which leave the tonsil. Some of these memory B cells will terminally differentiate into plasma cells where they may initiate replication and replenish the memory B cell pool (Murray and Young, 2001).

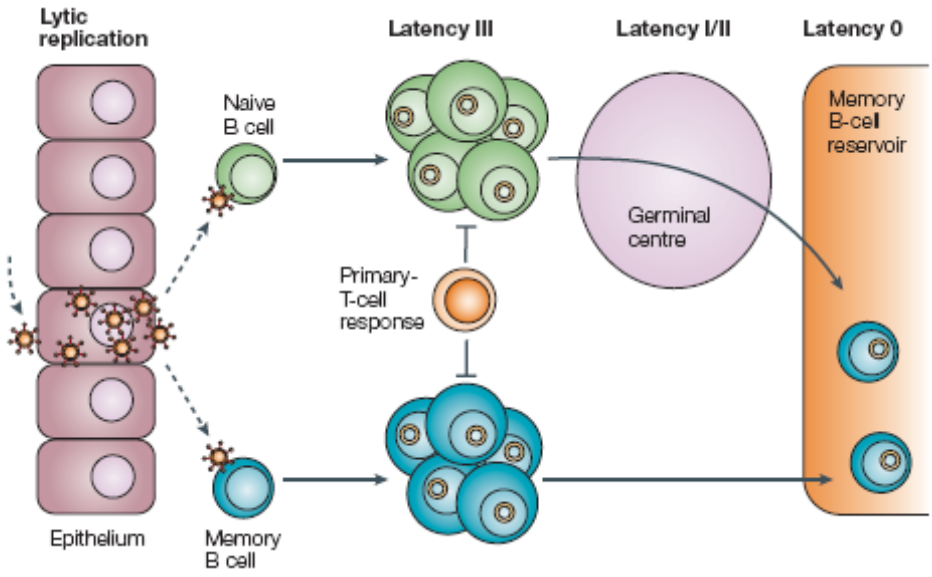


Figure 1.3 Primary Infection of EBV
Possible pathways that take place during primary infection.
(Young and Rickinson, 2004)

Figure 1.3 above is a summary of the possible pathways that can take place during primary infection of EBV. The exact entry site of EBV is still uncertain and several views prevail. The virus may infect the epithelium via oral transmission and establish lytic replication producing infectious EBV particles that infects either naïve or memory B cells. Many of the B cells are eliminated by the cytotoxic primary T cell response but some may escape by down-regulating antigen expression to establish a reservoir of positive memory B cells with suppressed antigen presentation (Latency 0). In an alternative view, naïve B cells are the main targets of EBV infection. After infection, they undergo germinal centre differentiation and become memory B cells (Liu and Arpin, 1997).

1.5.3 Persistent Infection

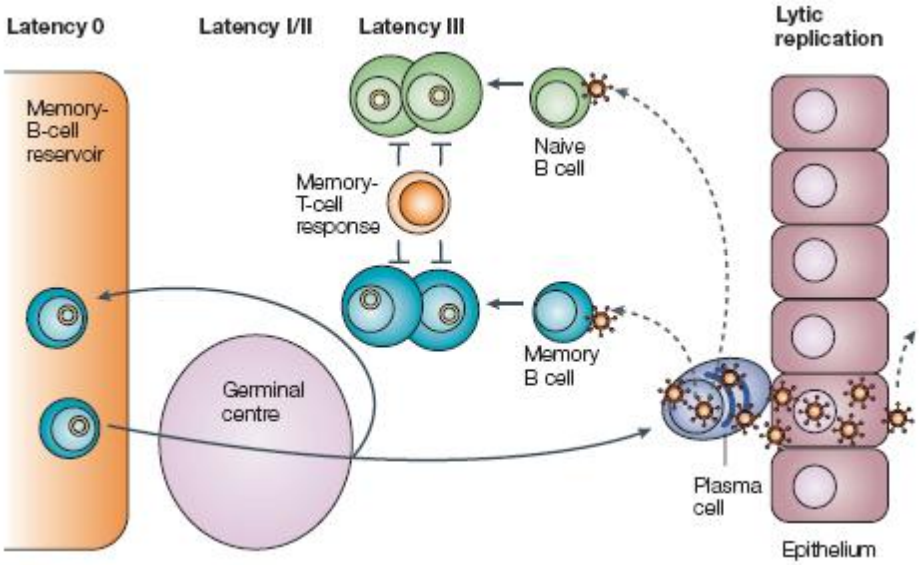


Figure 1.4 Persistent infection Of EBV
Possible pathways that take place during persistent infection.
(Young and Rickinson, 2004)

After primary infection, EBV persists in a person for life. The B cells harbour the virus in the latent form and there is a low level continuous or intermittent production of virus which is secreted in the saliva (Yao *et al*., 1985). To sustain this persistent infection, EBV has evolved a highly successful way to evade the immune system. Although the exact mechanism is still unclear, Thorley-Lawson and Babcock have done studies that suggest that EBV gains access into the memory B cells where it can reside undetected by immuno-surveillance mechanisms for a long time (Thorley-Lawson and Babcock, 1999). However, B cells require two survival signals to survive in the lymph node. Firstly, the B cell receptor (BCR) has to bind to an antigen on the surface of dendritic cells and secondly, the B cell surface co-receptor molecule such as CD40, has to bind to their ligands on CD4 T cells (MacLennan, 1994).

In EBV-infected B cells, the virus induces the expression of LMP-1 and LMP-2A, which have functions similar to CD40 and BCR, respectively. Thus, the infected cells survive and differentiate

into plasma cells or memory B cells (Anagnostopoulos *et al*., 1995; Babcock *et al.*, 1998). The EBV-infected plasma cells may then migrate to the epithelial cells and activate the lytic cycle, releasing low levels of virus particles in the saliva. In addition, the virus particles produced could infect new memory or naïve B cells which replenish the B cell reservoir or are eliminated by the established memory T cell response as seen in Figure 1.4 (Young and Rickinson, 2004).

1.6 Latent Proteins

1.6.1 Epstein-Barr Virus Nuclear Antigen 1 (EBNA-1)

In our study, we focused upon EBNA-1, thus we shall look at this latent protein in greater detail. EBNA-1 is a DNA-binding protein that is expressed in all EBV-infected cells. It is essential for replication and maintenance of the EBV genome and does this by a sequence-specific binding of EBNA-1 to the origin of replication, OriP. EBNA-1 also functions as a transcriptional activator and upregulates the expression of Cp and LMP-1 (Kieff and Rickinson, 2001). In addition, EBNA-1 can also negatively regulate its own expression by interacting with two *cis* regulatory sites downstream of the promoter Qp (Nonkwelo *et al*., 1996).

EBNA-1 contains a glycine-glycine-alanine (Gly-Gly-Ala) repeat sequence that separates the protein into amino- and carboxy-terminal domains. This repeat sequence varies in length in different EBV isolates. Previous studies have suggested that this acts as a *cis*-acting inhibitor and may inhibit antigen processing via the ubiquitin-proteosome MHC class I pathway (Levitskaya *et al*., 1995). Hence, EBNA-1 peptides are not presented on MHC class I molecules, protecting it from $CD8^+$ T cell responses.

Blake and colleagues isolated human CD8 T cell clones that recognised EBNA-1-specific peptides on HLA-B3501 and HLA-A203. The T cells only recognised and presented EBNA-1 without the Gly-Ala repeat domains and were unable to present full length EBNA-1. However, when full length EBNA-1 was introduced exogenously, the protein could be processed and presented on MHC class I via a (transporter associated with antigen processing) TAP-independent pathway (Blake *et al*., 1997). In a subsequent study by Blake *et al.*, an alternative pathway known as cross-presentation or cross-priming was proposed involving the antigen presenting cells (APCs) as intermediates. They demonstrated that specialised cells such as dendritic cells (DCs), could take up full length EBNA-1 and present them exogenously to $CD8^+$ T cells thus eliciting responses that were as strong as the immunodominant epitopes of EBNA-3 proteins (Blake *et al*., 2000).

This finding remains controversial as it remains difficult to translate cross-presentation on DCs to normal processing of full-length EBNA-1 in infected B cells. Gene knockout studies carried out by Humme and colleagues indicate that EBNA-1 does not have a crucial role in *in vitro* B cell transformation beyond the maintenance of the viral genome (Humme *et al*., 2003). On the other hand, EBNA-1 may have a direct role in oncogenesis as directing EBNA-1 expression to B cells results in B cell lymphomas in transgenic mice (Wilson *et al*., 1996). In addition, EBNA-1 may also have a role in the survival of Burkitt's lymphoma cells *in vitro* (Kennedy *et al*., 2003).

The proposed functions of the rest of the latent proteins are summarised in the table below.

Latent Proteins	Functions	References
EBV Nuclear Antigen 2 (EBNA-2)	• Transformation of B cells • Transcriptional activator of cellular and viral genes	• Hammerschmidt and Sugden, 1989 • Kieff and Rickinson, 2001 • Wang *et al*., 1987
EBV Nuclear Antigen 3 (EBNA-3) • **EBNA-3A** • **EBNA-3B** • **EBNA-3C**	• 3A and 3C: Transformation of B cells • 3B: Induces the expression of vementin and CD40 • Repress EBNA-2 mediated transactivation	• Robertson, 1997
Latent Membrane Protein 1 (LMP-1)	• Induces the expression of cell-surface adhesion molecules, activation antigens • Upregulates anti-apoptotic proteins (BCL2 and A20) and cytokine production (IL6, IL8) • Function similar to CD40 - provides growth and differentiation signals to B cells	• Wang *et al*., 1990 • Eliopoulos *et al*., 1997, 1999 • Uchida *et al*., 1999
Latent Membrane Protein 2 (LMP-2) • **LMP-2A** • **LMP-2B**	• 2A: Modifies normal B cell development and favours the maintenance of EBV latency • 2A: Prevents inappropriate activation of the EBV lytic cycle. • 2B: Regulates LMP-2A	• Longnecker, 2000 • Caldwell *et al*., 1998
Small Non-polyadenylated RNAs (EBERs)	• Disrupts the activity of interferon alpha and confers cellular resistance against viral infections	• Nanbo *et al*., 2002

Table 1.2 Summary Of The Functions Of Latent EBV Genes

1.7 Structure Of HLA Class I Complex And Peptide Binding

The human leucocyte antigen (HLA) is the human version of the major histocompatibility complex (MHC) and it consists of class I and class II. HLA class I is expressed on most somatic cells while class II is expressed on a subgroup of immune cells such as B cells, T cells, dendritic cells, macrophages and some endothelial/epithelial cells (Klein, 1986). As seen from Figure 1.5, HLA class I complex comprises of a heavy and light chain. The heavy chain consists of α1 and α2 which are peptide binding domains; α3, an immunoglobulin-like domain; the transmembrane region (TM); and the cytoplasmic tail while the light chain consists of β2-microglobulin (β_2m).

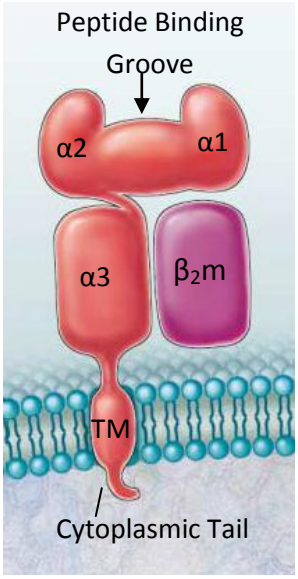


Figure 1.5 Structure of HLA Class I Molecule (Klein, 1986)

The peptide binding region consists of a floor formed by a β-pleated sheet and two walls formed by α helixes. The MHC class I peptide binding groove is closed as the ends converge (Bjorkman, 1987). As such it contains shorter peptide sequences (7 to 15 residues long) as compared to the open groove of the MHC class II molecule. In each peptide sequence, some amino acids in the middle of the peptide may protrude out of the groove while most of the other amino acids point into the groove and are housed in small pockets in the floor of the groove. Most MHC class I molecules have six pockets and these determine the specificity of peptides that can bind. Two of the pockets at positions two (P2) and nine (P9) are particularly important and they are known as anchor residues. In HLA-A2, there is a preference for isoleucine, leucine or methionine at P2 and leucine or valine at P9 (Batalia and Collins, 1997).

1.8 Immune Response

Antigens are processed through two different pathways – MHC classes I and II. Endogenous antigens are processed by the MHC class I pathway while exogenous antigens are processed by the MHC class II pathway. Our study focuses on tumour antigens which are endogenous and hence are processed through the MHC class I pathway.

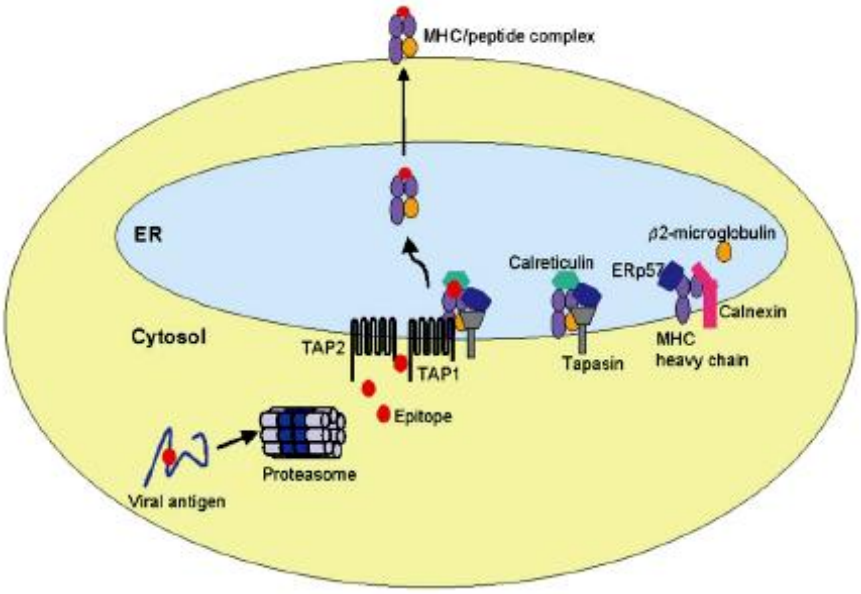


Figure 1.6 The MHC Class I Pathway
The molecules involved in the various steps of MHC class I presentation.
(Lautscham *et al*., 2003)

Endogenous antigens are degraded in the cytosol by the proteosome complex into peptide fragments. As seen in Figure 1.6, these are transported into the endoplasmic reticulum (ER) in an ATP-dependent manner by the transporter associated with antigen processing (TAP). TAP is a heterodimer that comprises of TAP1 and TAP2 subunits. It extends through the ER membrane and transports peptides that are 8-16 amino acids in length, a size suitable for the binding of peptides to the groove of the MHC class I molecule.

In the ER, the newly synthesized MHC class I heavy chains associate with calnexin and with the help of ERp57, the heavy chain refolds and binds to free β_2m. The MHC complex then associates with tapasin, and calnexin is exchanged for calreticulin. When this MHC complex associates with TAP, the peptide binds to the MHC complex and migrates to the surface of the cell where the peptide is presented to CD8 cytotoxic T lymphocytes (Lautscham *et al*., 2003).

1.8.1 Identified Cytotoxic T Lymphocyte Epitopes Expressed By EBNA-1

EBNA-1 was traditionally thought to be an immunologically invisible latent protein produced by EBV to evade the host immune system. However, many studies have since shown that EBNA-1 can be processed and presented to CD8 and CD4 T cells on MHC classes I and II. In addition, the C terminal tail is the most immunogenic portion of the protein. Currently, cytotoxic T lymphocytes (CTL) epitopes on EBNA-1 are still poorly characterised as it is not as immunodominant as other EBNAs such as EBNA-3. As mentioned before, Blake and colleagues isolated human CD8 CTL clones that recognised EBNA-1 peptides on HLA-B3501 and HLA-A203. HLA-B3501 recognised the 11-mer peptide sequence HPVGEADYFEY (EBNA-1 407-417) while HLA-A203 recognised the 9-mer sequence VLKDAAIKDAL (EBNA-1 574-582) (Blake *et al.,* 1997).

In subsequent studies, it was discovered that strong responses to exogenous EBNA-1 was not limited to these two HLA molecules. An additional HLA-B7 and HLA-B53 specific for EBNA-1 was identified after screening for interferon gamma (IFN-γ) responses with ELISPOT. These MHC class I molecules were specific for EBNA-1 peptides and elicited CTL responses that were as strong as immunodominant protein, EBNA-3. Both recognised a 9-mer sequence. HLA-B7 recognised IPQCRLTPL (EBNA-1 528-536) while HLA-B53 recognised HPVGEADYF which was interestingly, within the sequence of HLA-B3501 (Blake *et al*., 2000). Another study by Stuber *et*

al. using MHC stabilisation assay, also showed that this exact sequence bound well to HLA-B7. Besides this, synthetic 9-mer sequences were also shown to bind well to HLA-A201 (Stuber *et al*., 1995).

1.9 HLA Allele Frequencies

The allelic frequencies of the HLAs are also important to determine how widespread each allele is and the ethnic groups that represent them. For a TCR-like antibody to gain widespread use, the targeted HLA allele should be one that is common. HLA-A201 is second most common allele found in 11 regions in the world (Solberg *et al*., 2008). Figure 1.7 below shows the distribution of HLA-A201 around the world.

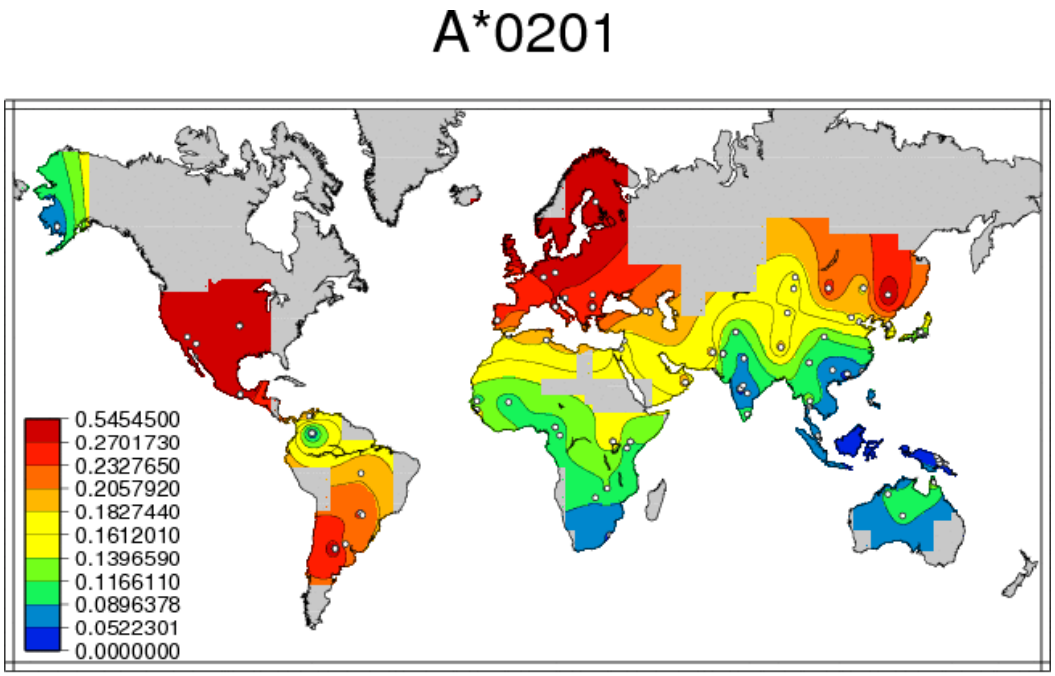


Figure 1.7 A Geographical Interpolation of HLA-A201 Allele Frequencies

In the Singaporean Chinese population, the HLA-A2 allele is one of the most common alleles with various subtypes such as HLA-A203, HLA-A206 and HLA-A207. The allelic frequencies of the various subtypes are shown in Table 1.3 below. Previously, our laboratory has also generated an antibody that is HLA-A201 restricted. Hence, we decided to focus our study on this particular HLA-A201 allele, specific for the EBNA-1 epitope (FMVFLQTHI) identified by Stuber and colleagues (Stuber *et al*., 1995).

HLA Allele	Frequency in Singapore (%)
HLA-A201	10.4
HLA-A203	6.7
HLA-A206	4
HLA-A207	13.1

Table 1.3 Allelic Frequencies Of HLA-A2 In The Singaporean Chinese Population
(Middleton et al., 2000)

1.10 EBV Malignancies

1.10.1 Burkitt's Lymphoma (BL)

Burkitt's lymphoma (BL) is a highly proliferative B cell tumour in which EBV was first identified (Epstein *et al*., 1965). There are three forms of BL known as the endemic, sporadic and immunodeficiency related form. The endemic variant is the high-incidence form of BL that occurs in malaria endemic areas of Africa and Papua New Guinea, with an annual incidence of about 5-10 cases per 100 000 children. It usually involves the facial bones such as the jaw, and other organs like the kidney, ovaries, or the breast. The sporadic variant is a low-incidence form of BL which takes place worldwide, mostly in developed countries but with a much lower incidence rate. Although EBV is present in all cases of endemic BL, it is found in only 15% of the

sporadic cases and these involve mainly the lymphoid tissue of the gut and the upper respiratory tract (Yustein and Dang, 2007). The immunodeficiency related variant is mainly observed in HIV patients, frequently occurring before the development of full-blown AIDS. About 30-40% of the cases are related to EBV infection (Kieff and Rickinson, 2001; Young and Rickinson, 2004).

A feature characteristic of BL tumours is the chromosomal translocation of chromosome 8 (8q24) in the region of c-myc proto-oncogene and either chromosome 14 of the immunoglobulin heavy chain or chromosome 2 or 22 of the immunoglobulin light chain (Murray and Young, 2001). This results in the constitutive expression of c-myc oncogene which causes continuous proliferation and inhibits differentiation (Zech *et al*., 1976). In addition, many tumours also have mutations in the tumour suppressor gene p53 which is the most common gene mutation in human cancers (Farrell *et al*., 1991).

The examination of BL tumour tissues shows that they are monoclonal; suggesting that EBV infection is an early event preceding the proliferation of B cells (Neri *et al*., 1991; Raab-Traub and Flynn, 1986). The origin of BL may be the germinal centroblasts as studies have shown that BL cells resemble centroblast cells. Proliferating centroblasts are subject to somatic hypermutation and c-myc translocation may have occurred as an error during this process (Chapman *et al*., 1995; Klein *et al*., 1995; Harris *et al*., 2001). An analysis of viral gene expression of BL cells revealed EBNA-1 (Kelly *et al*., 2002) as well as EBERs and *Bam*HI transcripts (Crawford, 2001). Although the exact role of EBV in the pathogenesis of BL is still unclear, EBNA-1 may play a role in maintenance of the viral genome (Humme *et al*., 2003) while EBERs induce the

expression of IL10 to increase cell survival and reduce immune reactivity (Takada and Nanbo, 2001).

Malaria and HIV can act as chronic stimuli of B cell production and in HIV; exaggerated germinal centre activity may occur (Boshoff and Weiss, 2002). This would in turn increase the chances of c-myc translocation (Young and Rickinson, 2004). Both these diseases possess immunosuppressive effects that may decrease the levels of EBV-specific cytotoxic T cell response (Whittle *et al*., 1984).

1.10.2 Hodgkin's Lymphoma (HL)

Whilst Hodgkin's lymphoma (HL) is not geographically restricted, its association with EBV is geographically restricted. In a study done by Glaser and colleagues, it was shown that EBV associated HL was twice as prevalent in less developed countries than the developed countries (Glaser *et al.*, 1997). A review by Hsu and Glaser also showed that 90-100% of HL cases in developing countries are EBV associated as compared to a minority of cases in developed countries such as the USA and Europe (Hsu and Glaser, 2000). Besides geographical location, EBV association with HL also varies with sex, age, ethnicity and histological subtype (Flavell and Murray, 2000). Hodgkin's lymphoma is characterised by the presence of large multinucleate Reed-Sternberg cells (RSC) (Figure 1.8) and Hodgkin's cells which takes up only 1-2% of the tumour tissue but together result in malignancy.

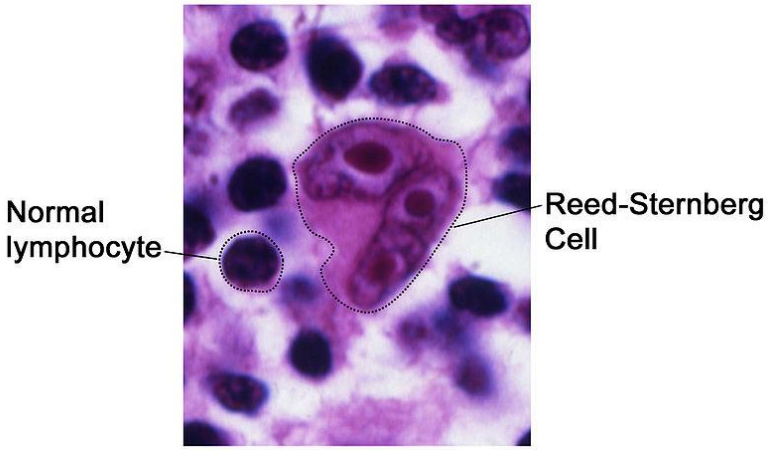


Figure 1.8 Morphology Of Cells Found In Hodgkin's Lymphoma.
Normal lymphocytes compared with a Reed-Sternberg cell, which is large and multinucleate.
(National Cancer Institute)

By studying their immunoglobulin (Ig) genes, the origins of these cells were found to derive from B cells and are clonal. This indicates that EBV infection occurred before clonal expansion (Anagnostopoulos *et al*., 1989). The association of EBV with HL was indicated by high level of antibody titres to EBV antigens in HL patients as compared to other lymphoma patients. It was also shown that these high levels preceded the development of HL by several years. In addition, there were findings of a threefold increase in risk of HL with patients who had a history of IM (Gutensohn and Cole, 1980).

In EBV associated HL, the viral genome is present in every cell but its expression of EBV latent genes is restricted to EBNA-1, LMP-1, LMP-2A, EBERs and *Bam*HI transcripts (Deacon *et al*., 1993). LMP-1 may provide growth and survival signals while LMP-2A may provide the BCR signals required for survival of the malignant cells (Thorley-Lawson and Babcock, 1999). To obtain an accurate diagnosis of HL, multiple biopsies may have to be taken to identify the characteristic RSCs since they are present in a very small percentage of cells and tissues (Ansell *et al*., 2006).

1.10.3 Nasopharyngeal Carcinoma (NPC)

Nasopharyngeal carcinoma (NPC) is a tumour of the squamous epithelium in the post nasal space (Crawford, 2001). The undifferentiated form of NPC – World Health Organisation (WHO) type III – shows the most consistent association with EBV. This tumour is most common in areas of China and South-east Asia, having an incidence of around 20-30 cases per 100 000 (Yu and Yuan, 2002). Individuals of Chinese descent, especially Cantonese males, have high incidence rates, regardless of where they live. Besides this genetic predisposition, environmental and dietary factors such as ingestion of salted fish and traditional herbal remedies may contribute to NPC (Yu *et al*., 1986; Armstrong *et al*., 1998).

The undifferentiated form of NPC is characterised by a prominent lymphocytic infiltrate and this interaction between the tumour cells and the lymphocytes may be important for the continued growth of the malignant cells. The association of EBV and NPC was first established through serological studies (Old *et al*., 1966). EBV DNA is consistently detected in all NPC regardless of the geographical location and incidence rate. The viral DNA from NPC tissues have been found to be monoclonal which suggests that EBV infection of a single cell occurred before the clonal expansion of malignant cells (Raab-Traub and Flynn, 1986). Some EBV latent genes such as EBNA-1, LMP-1, LMP-2A, LMP-2B, EBERs and *Bam*HI transcripts, are detected in NPC tumour cells (Brooks *et al*., 1992). Out of these, LMP-1 and LMP-2 which are involved in cellular gene expression and growth may contribute to the highly invasive and malignant growth of the tumour (Raab-Traub, 2002). NPC produces few symptoms in the early stages with innocuous lesions in the post nasal cavity. As such most cases are already advanced when detected. Early detection is extremely difficult, even by an experienced ortholaryngologist (Leong *et al.,* 1999).

1.10.4 Current Therapies For EBV Malignancies

The current therapies of Burkitt's lymphoma and Hodgkin's lymphoma involve radiotherapy and short courses of high intensity chemotherapy together with cyclophosphamide, vincristine, doxorubicin and methotrexate (Magrath *et al*., 1996). Other alternatives include biological agents such as rituximab, an anti-CD20 monoclonal antibody that activates antibody-dependent cell cytotoxicity and cell-dependent cytotoxicity. The addition of rituximab to chemotherapy was shown to improve outcome of BL especially in the elderly (Thomas *et al*., 2006). However, the drawback of chemotherapy is its side effects such as haematological toxicity and neurotoxocities. The most effective therapy for NPC is radiation therapy as it is usually advanced at presentation and the lesions may be poorly accessible to surgeons. Combination therapies that involve chemotherapy and surgery are alternatives dependent on the stage of the disease (Psyrri *et al*., 2006). A major disadvantage in the current therapies is the lack of specificity in the targeting of tumour cells which may destroy normal bone marrow and result in severe side effects. In addition, the use of toxins in chemotherapy may inhibit the growth of immune cells and leave patients immunocompromised. Hence, a therapeutic agent that targets tumour cells specifically without adverse side effects would be ideal.

1.11 Aims Of Our Project

In our project, we aimed to produce a monoclonal antibody that recognises latent EBV protein, EBNA-1, in the context of HLA-A201. EBNA-1 is present in 100% of EBV associated tumours and thus has the potential to be a useful tool in a diagnostic and therapeutic setting for EBV associated malignancies. To achieve this, HLA-A201/EBNA-1 monomers were produced and used as reagents to produce the monoclonal specific antibodies in mice. Fusion was carried out with the spleenocytes taken from the mice and myeloma cells to produce hybridoma cell lines. This

was followed by screening of the cells via flow cytometry to establish its specific binding. The monoclonal was then characterised and tested against EBV transformed B-lymphoblastoid cell lines (BLCLs), tumour cell lines and NPC biopsies.

MATERIALS AND METHODS

Chapter 2

Media, Buffers, Solutions And Chemicals

All media, buffers, solutions and chemicals used in this study are listed in the Appendix.

2.1 Tissue Culture

2.1.1 HLA-A2 Cell Lines

The cell lines used were T2, THP-1, U937 (A2), RPMI-6666 and CCRF-SB (all purchased from ATCC, USA). All were cultured in RPMI-1640 medium (Hyclone, Thermo Fisher Scientific, Roskilde, Denmark) with 10% foetal bovine serum (FBS) (Gibco, Invitrogen, Carlsbad, CA USA) and 1% penicillin/streptomycin (pen/strep) (Gibco). T2 is a human lymphoblast suspension cell line; THP-1 is a human monocytic leukaemic suspension cell line while U937 is a human monocytic suspension cell line. U937 cells were stably transfected with HLA-A201. RPMI-6666 and CCRF-SB are tumour cell lines. RPMI-6666 is a human lymphoblast suspension cell line derived from Hodgkin's lymphoma while CCRF-SB is a human lymphoblast suspension cell line derived from acute lymphoblastic leukaemia. These two cell lines expressed human leucocyte antigen A2 (HLA-A2) and are EBNA-1 positive.

2.1.2 Culture Of HLA-A2 Cell Lines

Cells were grown in 25 cm^2 (T25) or 75 cm^2 (T75) sterile plastic tissue culture flasks (Nunc Thermo Fisher Scientific, Roskilde, Denmark) and cell densities were maintained between 3 x 10^5 and 1 x 10^6 cells per ml according to the protocol on the ATCC website. Once the cell density exceeded 10^6, half the cell suspension was discarded. This was then replaced with the same volume of RPMI-1640 media (Hyclone) with 10% FBS and 1% penicillin/streptomycin (R10). Cells were pipetted gently to minimise cell clumping and sub-cultured every 2 to 3 days and grown at 37^oC in a 5% CO_2 incubator (Sanyo, Wood Dale, USA).

2.1.3 Culture Of B-Lymphoblastoid Cell Lines (BLCLs)

The EBV transformed B-lymphoblastoid cell lines were obtained from various patients and volunteers and stored frozen in liquid nitrogen at WHO Immunology Centre, Singapore.

2.1.3.1 Growth Of BLCLs

BLCLs were thawed at 37^{o}C in a water bath and resuspended in 10 ml of R10 under sterile conditions. The cells were centrifuged at 350 g for 5 mins and the supernatant discarded. The pellet is resuspended in 5 ml of R10 and transferred to a T25 culture flask. The cell culture was then incubated in a CO_2 incubator at 37^{o}C. The density of the cells was monitored and culture medium added when necessary. When the volume exceeded 15 ml, 80% of the cell culture was transferred to a T75 culture flask and 20 ml of fresh R10 medium was added. To the remaining cell suspension, 5 ml of fresh R10 medium was added in the T25 flask. The cell cultures were incubated at 37^{o}C in a CO_2 incubator until ready for freezing or harvesting.

2.1.3.2 Freezing Of BLCLs

Once the cells achieved 80% confluent growth, the cell suspension was centrifuged at 350 g for 5 mins and the supernatant was discarded. The pellet was resuspended in 1 ml cold freezing mixture and transferred to a labelled cryo-vial (Thermo Fisher Scientific, Nunc) and kept at -80^{o}C for a day before being transferred to liquid nitrogen.

2.2 Antibody Production

2.2.1 Peptides

EBNA-1 (FMVFLQTHI) (Stuber *et al*., 1995) and M1 peptides (GILGFVFTL) were synthesised by Mimotopes (Mimotopes, Victoria, Australia) and its purity was analysed by electrospray mass spectrometry.

2.2.2 Preparation Of Competent Cells

E. coli strain BL21 (DE3) (Novagen, Madison, WI, USA), used for plasmid amplification and recombinant protein expression, was prepared for chemical transformation.

Stock cells were streaked onto Luria-Bertani agar (LA) plates and incubated at 37^{o}C overnight. A single colony was inoculated into 10 ml Luria-Bertani broth (LB) and incubated at 37^{o}C overnight with vigorous shaking at 220 rpm. The next day, the 10 ml culture was inoculated into 500 ml LB medium and incubated at 37^{o}C for 3-4 hrs with vigorous shaking until the optical density (OD_{600}) reached 0.6. The cell culture was centrifuged at 3000 rpm for 15 mins. The supernatant was discarded and the pellet was resuspended in 10 ml transformation buffer I (TfBI) and centrifuged at 3000 rpm for 8 mins. The supernatant was discarded and the pellet was resuspended gently in 2 ml cold transformation buffer II (TfBII). The competent cells were aliquoted into 1.5 ml tubes, frozen in liquid nitrogen and stored at -80^{o}C.

2.2.3 Transformation

E. coli strain BL21 competent cells were thawed on ice and 100 µl of cells were aliquoted into a 2 ml tube. 100 ng pET30-HLA construct was added to the cells and incubated on ice for 30 mins. After incubation, the cells were heat-shocked for 30 s at 42^{o}C and put on ice for 1 min. Next, 250

µl of SOC media (Invitrogen) was added and the suspension was incubated at 37°C for 1 hr with shaking. The suspension was put on ice for 1 min and 50 µl and 200 µl of the suspension was spread on each LK plate (LB with 30 µg/ml kanamycin). The plates were then left to incubate overnight at 37°C.

2.2.4 Inoculation

The next morning, the plates were taken out from the incubator and stored at 4°C. In the evening, a single colony was inoculated into 15 ml of LB media with 30 µg/ml kanamycin (LK) and incubated at 37°C overnight with shaking.

2.2.5 Protein Expression

5 ml of culture was inoculated into 500 ml of LK media and the flask was incubated at 37°C for 1½ hr with shaking. Mini-prep (Qiagen, GmbH, Germany) was carried out for the remainder of the culture according to the manufacturer's instructions. The OD_{600} was checked every half an hour. Once the OD_{600} reached between 0.6 and 0.8, 1 mM isopropyl β-D-I-thiogalactopyranoside (IPTG) (Sigma-Aldrich Inc, St. Louis, MO, USA) was added into each flask to induce protein expression at mid-log phase. The culture was left to incubate in the dark at 37°C for 4 hrs with shaking. Thereafter, the cells were centrifuged at 4°C, 5000 rpm for 10 mins to harvest the cells. Supernatant was discarded and the pellet was resuspended in 5 ml of resuspension buffer with 10 mM dithiothreitol (DTT) (Sigma-Aldrich), 0.2 mM phenylmethylsulphonylfluoride (PMSF) (Sigma-Aldrich), and 5 µl 1 mg/ml pepstatin A (Sigma-Aldrich). The resuspended pellet was stored at -80°C overnight.

2.2.6 Inclusion Bodies Preparation

The inclusion bodies were thawed at room temperature. To every 5 ml of resuspended culture, 12.5 ml of lysis buffer was added together with 0.5 ml 1 mg/ml DNase I (Sigma Aldrich), 10 mM DTT and 5 mM $MgCl_2$. The mixture was transferred into 30 ml centrifuge bottles and rocked vigorously on ice for 20 mins. 10 mM sodium ethylenediaminetetraacetate (NaEDTA; pH 8.0) was added to each bottle. The cells were further lysed by sonication which was carried out for 5 cycles, each consisting of 30 s pulsing followed by 30 s break. After that, the mixture was centrifuged at 10 000 rpm, 4^{o}C for 15 mins. The supernatant was discarded and the pellet was resupended in 15 ml of wash buffer I with 1 mM DTT, 0.2 mM PMSF and 15 µl 1 mg/ml pepstatin A. After resuspension, the mixture was homogenised for 30 s and centrifuged at 10 000 rpm, 4^{o}C for 15 mins. This wash step was repeated thrice. Next, the pellet was resuspended in 10 ml of wash buffer II together with 1 mM DTT, 0.2 mM PMSF and 10 µl 1 mg/ml pepstatin A. The mixture was homogenised for 30 s and centrifuged at 10 000 rpm, 4^{o}C for 15 mins. The supernatant was discarded and 200 µl of water was added to the pellet to form a white paste. 10 ml of 8 M urea buffer was then added to the paste with 0.1 mM DTT, 0.2 mM PMSF and 10 µl 1 mg/ml pepstatin A. The mixture was left to shake for 1 hr at room temperature and centrifuged at 10 000 rpm, 4^{o}C for 1 hr. The supernatant was collected into a 50 ml falcon tube and 0.2 mM PMSF and 10 µl 1 mg/ml pepstatin A was added. This was then aliquoted and into 1.5 ml centrifuge tubes and stored at -80^{o}C.

2.2.7 SDS-PAGE Gel

2.2.7.1 Preparation of Polyacrylamide Gel

Gel electrophoresis was carried out to quantitate the concentration of inclusion bodies. 10% separating gels were used for the heavy chain samples while 15% separating gels were used for the β_2m samples. Both used 4% stacking gels. Gels were cast using the Bio-Rad Mini-PROTEAN

Electrophoresis System (Bio-Rad, Hercules, CA, USA). The following reagents were added to form two 1.5 mm thick gels.

	10% Separating Gel	15% Separating Gel	4% Stacking Gel
RO Water	8 ml	5 ml	6 ml
30% Acrylamide (Bio-Rad)	7 ml	10 ml	1.5 ml
Resolving Buffer (pH 8.8)	5 ml	5 ml	-
Stacking Gel Buffer (pH 6.8)	-	-	2.5 ml
10% APS	100 µl	100 µl	100 µl
TEMED (Bio-Rad)	15 µl	15 µl	10 µl

Table 2.1 Polyacrylamide Gel Recipes

2.2.7.2 Sample Preparation And Electrophoresis

Samples were prepared in 0.5 ml tubes and 10 µl 5x Lammeli sample buffer (Bio-Rad) was added into each tube. Samples and standards were mixed with sample buffer. Samples of heavy chain and β_2m were added in increasing volumes of 0.5, 1, 2, 4 µl while the standards were added in increasing concentrations of 1, 2, 4, 8 µg. Samples with buffer were boiled at 98^{o}C for 5 mins. Before loading into the wells, the samples were pulsed down. Samples and standards were loaded together with a protein marker (Bio-Rad Precision Plus Protein Standards). Electrophoresis was carried out in the presence of 1x denaturing running buffer under a constant voltage of 200 V for about 45 mins until the dye front reached the bottom of the gel. Proteins separated on a SDS-PAGE gel were simultaneously fixed and stained with Coomassie blue staining solution. The gel was immersed in the staining solution for 20 mins with gentle rocking. It was then destained with destaining buffer overnight with gentle rocking. Subsequently, the gel was soaked in water for 2 days and dried in a gel dryer (GelAir Drying

System, Bio-Rad). The concentrations of the samples were determined by a comparison against the known concentrations of the standards.

2.2.8 Refolding Of HLA- β_2m-Peptide Monomers

Once the concentrations of the samples were determined, refolding of HLA monomers was carried out. Refolding buffer was prepared in a 500 ml bottle according to the volumes listed.

Compounds	Final	Amount (400 ml)
Tris	100 mM	40 ml of 1 M Tris (pH 8.0)
NaEDTA	2 mM	1.6 ml of 0.5 M EDTA (pH 8.0)
L-Arginine Monohydrochloride	400 mM	33.6g
L-Gluthathione Oxidised	0.5 mM	0.122g
L-Gluthathione Reduced	5 mM	0.614g
RO H_2O	-	Top up to 400 ml

Table 2.2 Refolding Buffer

To the refolding buffer, 0.2 mM PMSF and 400 µl 1 mg/ml pepstatin A was added and the solution was cooled at 4^{o}C. 4 mg of peptide was dissolved in 25 µl DMSO and added to the refolding buffer. In a 15 ml tube, 12 mg heavy chain and 8 mg β_2m was mixed. An additional 3 mg of refolded β_2m was added to increase yield. Using a peristaltic pump (Peristar, WPI, Stevenage, UK), the mixture of heavy chain and β_2m was pumped into the refolding buffer at a constant flow rate. The bottle was placed on a magnetic stirrer and left in the cold room. Further injections of heavy chains were added directly into the refolding buffer at 8 mg every 24 hrs over a span of 72 hrs.

2.2.9 Dialysis

After refolding, the samples were dialysed before further purification. A large 5 L beaker was filled with 20 mM Tris-HCl (pH 8.0). The Spectra/Por Dialysis membranes (Spectrum Laboratories Inc, Rancho Dominguez, CA, USA) were soaked in MilliQ water for 5 mins and rinsed with Tris. One end of the tubing was sealed with a plastic clip and using a funnel, the tube was filled with the refolding mixture from the other end and sealed. The tubing was placed into the beaker and left to stir at 4^{o}C for 12-16 hrs. Subsequently, the dialysed solution was filtered through a 0.22 µm filter unit and the solution was stored at 4^{o}C.

2.2.10 Anion Exchange Chromatography

Pumps A and B were washed with MilliQ water. With the flow rate set at 1 ml/min, the HiPrep 16/10 DEAE FF column (AKTA, GE Healthcare, Uppsala, Sweden) was attached to the AKTA FPLC System (GE Healthcare). Column equilibration was carried out with 5 column volumes (CV) MilliQ water followed by 5 CV buffer A, 5 CV buffer B and 5 CV buffer A. Buffer A is 20 mM Tris (pH 8.0) while buffer B is 20 mM Tris/1 M NaCl (pH 8.0). The dialysed solution was loaded into the column after equilibration at a flow rate of 5 ml/min and run according to a stepwise gradient programme. Proteins were eluted in increasing salt concentrations (buffer B) at a flow rate of 5 ml/min and 1 ml fractions were collected. The stepwise gradient programme used is shown below (Figure 2.1).

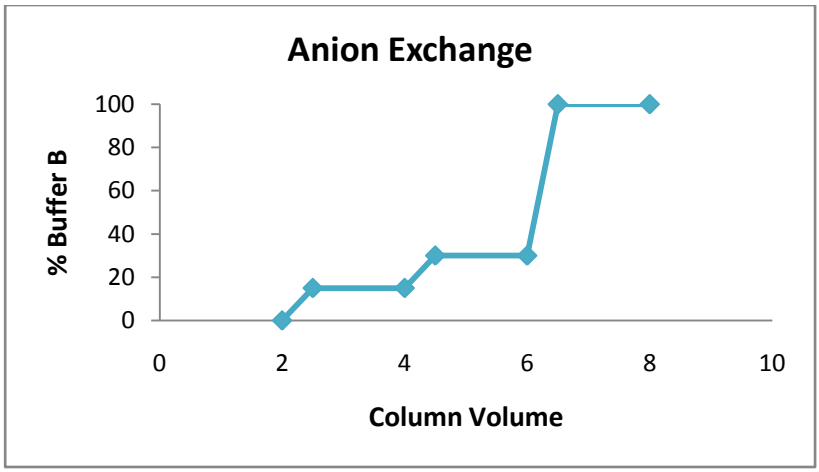


Figure 2.1 Profile Of Gradient Programme Used For Anion Exchange Chromatography

HLA monomers were eluted in approximately 15% buffer B while the other proteins were eluted at higher concentrations of buffer B. After anion exchange, selected samples were analysed on a SDS-PAGE gel. 10 µl of each sample was run on a 15% SDS-PAGE gel and analysed. Fractions which had both the heavy chain (35 kDa) and β_2m (12 kDa) were pooled together and concentrated to 200-500 µl using the Centricon Centrifugal Filter Unit with Ultracel YM-30 membrane (Millipore Corporation, Billerica, MA, USA). The purified fraction was stored at 4^{o}C before further purification.

2.2.11 Superdex (Gel Filtration)

The Superdex HR 10/30 75 column (GE Healthcare) was attached to the AKTA FPLC System (GE Healthcare) and column equilibration was carried out with 5 CV of MilliQ water and 5 CV buffer A. 200 µl of the above purified HLA monomers was injected into the sample loop using a syringe and subsequently loaded into the column and proteins were eluted using an isocratic gradient with a flow rate of 0.8 ml/min in 20 mM Tris (pH 8.0). Fractions of 0.5 ml were collected and analysed by 15% SDS-PAGE. Those which had both the heavy chain (35 kDa) and β_2m (12 kDa)

were pooled together and concentrated to 500 µl using the Centricon YM-10 (Millipore). The purified monomers were then stored at -80^{o}C.

2.2.12 Biotinylation Of HLA Monomers

70 µl of purified monomer was biotinylated by incubating with 10 µl of biomix A, 10 µl of biomix B, 10 µl of supplemental biotin, 0.2 µl 3 mg/ml BirA enzyme (Avidity LLC, CO, USA), 0.2 µl 0.1 M PMSF and 0.1 µl 1 mg/ml pepstatin A at 28^{o}C for 12-18 hrs at a final volume of 100 µl. 1 ml 10 mM Tris was added to the biotinylated complex, mixed and transferred to a Centricon YM-30 tube. This was centrifuged at 3500 rpm for 10 mins. The washing step was repeated twice and the retentate collected.

The amount of biotinylated product was quantitated by immunoprecipitation using streptavidin beads (Sigma-Aldrich). 10 µl of the beads were first washed with 1 ml of 10 mM Tris, 150 mM NaCl, 0.1% Triton-X100 and pulsed at 8000 rpm. This step was repeated twice. The supernatant was discarded and 20 µg of cleaned-up biotinylated HLA complexes was added into a 1.5 ml centrifuge tube with 500 µl 10 mM Tris, 2 µl 0.1 M PMSF and 1 µl 1 mg/ml pepstatin A. The mixture was left to shake at 4^{o}C for 16 hrs. After incubation, the beads were washed with 1 ml of 10 mM Tris, 150 mM NaCl, 0.1% Triton-X100 and pulsed at 8000 rpm. This was repeated twice. The supernatant was discarded and the sample was subjected to 15% SDS-PAGE and visualised with Coomassie blue staining. The quantity of biotinylated HLA complexes was compared against bovine serum albumin (BSA) (Sigma-Aldrich) standards of known concentrations. The percentage of biotinylated HLA complexes was determined by comparing it against biotinylated HLA complexes that were non-immunoprecipitated. A negative control of streptavidin beads was used as well. The cleaned-up biotinylated HLA-complexes was aliquoted and stored at -80^{o}C.

2.3 Immunisation Of Mice

Immunisation was carried out over a span of 45 days on 4 Balb/C female mice that were 6-8 weeks old. On day 0, 100 µg monomer in an emulsion of 500 µl 1x phosphate buffered saline (PBS) and 500 µl complete Freund's adjuvant (Sigma-Aldrich) was prepared and equal volumes were injected intra-peritoneally into each mouse. This was repeated on days 21 and 35 with 100 µg monomer in an emulsion of 500 µl 1x PBS and 500 µl incomplete Freund's adjuvant each. On day 42, 100 µg monomer was dissolved in 500 µl 1x PBS and equal volumes were injected into each mouse intra-veinously via the tail vein. The mice were sacrificed on day 45 and their spleens harvested.

2.3.1 Preparation Of Feeder Layer

Balb/C female mice were sacrificed and their fur around the peritoneal cavity removed to reveal the peritoneal membrane. A small incision was made and 5 ml cold RPMI medium was injected into each mouse. The mouse was massaged to dislodge the macrophages in the intra-peritoneal cavity. RPMI medium was then pipetted out and collected in a 50 ml Falcon tube. This process was repeated 4 times to ensure that most of the macrophages were collected. Next, a cell count was done to ensure that there were sufficient cells for use. The cell suspension was centrifuged at 1600 rpm for 5 mins. The supernatant was discarded and the pellet was resuspended in an appropriate amount of hypoxanthine-aminopterin-thymidine (HAT) media (Sigma). 100 µl of cell suspension was pipetted into each well of a 96-well plate. The plates were incubated overnight at 37^{o}C in a CO_2 incubator.

2.3.2 Harvesting Of NS-1 Cells

NS-1 myeloma cells were transferred into round-bottomed tubes and centrifuged at 1200 rpm for 5 mins. The supernatant was discarded and the pellet resuspended in 10 ml warm RPMI medium. A cell count was done and the tubes were incubated at 37^{o}C in a CO_2 incubator.

2.3.3 Harvesting Splenocytes From Mice

The mice were sacrificed and their spleens removed under sterile conditions. The spleens were washed in 1x PBS and homogenised through a sterile cell strainer (BD Biosciences, San Jose, CA USA). RPMI medium was added to obtain a single cell suspension. This was centrifuged at 1100 rpm for 5 mins. The supernatant was discarded and the pellet was resuspended in 10 ml RPMI and split into 2 tubes for fusion.

2.3.4 Hybridoma Fusion Without MACS Beads Selection

The splenocytes were added to the NS1 myeloma cells and centrifuged at 1100 rpm for 3 mins. The supernatant was discarded and the cells were resuspended. 1 ml of warm polyethylene glycol (PEG) (Sigma-Aldrich) was added slowly over 1 min. The tube was then incubated for 1 min at 37^{o}C. Following that, 3 ml of RPMI medium was added at a rate of 1 ml/min. It was topped up with an additional 5 ml of RPMI medium and the tube was centrifuged at 1100 rpm for 3 mins. The supernatant was discarded and the pellet was resuspended. 20 ml of warm HAT media was added and the tube was incubated at 37^{o}C in a CO_2 incubator for 2 hrs. After incubation, the cell suspension was topped up with an appropriate volume of HAT media and 100 µl of cell suspension was pipetted into each well of a 96-well plate that had previously been incubated with the feeder layer. The plates were incubated at 37^{o}C in a CO_2 incubator and each

well was topped up with an additional 100 µl of HAT on day 7. The plates were observed 8-10 days later when colonies were visible. On day 14, cells were fed with HT media instead.

2.3.5 Hybridoma Fusion With MACS Beads Selection

30 µg of biotinylated monomer was added to the splenocytes and incubated at 4^{o}C for 20 mins. Thereafter, 5 ml of cold PBS was added and centrifuged at 1100 rpm for 5 mins to wash. Next, 100 µl anti-biotin microbeads (Miltenyi Biotec GmbH, Germany) was added to the cells and incubated at 4^{o}C for 20 mins. The mixture was washed with 5 ml of cold PBS and centrifuged at 1100 rpm for 5 mins. In the meantime, the LS columns (Miltenyi Biotec) were pre-wet with 1 ml of cold PBS and 1 ml of RPMI medium. After centrifuge, the supernatant was discarded and the cells were resuspended in 1 ml of RPMI medium. The cells were then added to the column. After all the cells have been adsorbed on the column, 1 ml of RPMI medium was added to each column to wash out the unbound cells. This process was repeated twice. Following that, the LS column was taken out of the magnetic board and put into a 15 ml tube where the cells were eluted with 3 ml of RPMI medium. The elution process was repeated with another 3 ml of RPMI medium.

The eluted cells were added to NS1 cells and centrifuged at 1100 rpm for 3 mins. The supernatant was discarded and the cells were resuspended. 1 ml of warm PEG was added slowly over 1 min. The tube was then incubated for 1 min at 37^{o}C. Following that, 3 ml of RPMI was added at a rate of 1 ml/min. 5 ml of RPMI was added and the tube was centrifuged at 1100 rpm for 3 mins. The supernatant was discarded and the pellet was resuspended. 20 ml of warm HAT media was added and the tube was incubated at 37^{o}C in a CO_2 incubator for 2 hrs. After incubation, the cell suspension was topped up with an appropriate volume of HAT media and

100 µl of cell suspension was pipetted into each well of a 96-well plate that had previously been incubated with the feeder layer. The plates were incubated at 37^{o}C in a CO_2 incubator and the wells were topped up with an additional 100 µl of HAT on day 7. The plates were observed 8-10 days later when colonies were visible. On day 14, the cells were fed with HT media instead. The clones were scored and screened by flow cytometry for specific binding.

2.4 Screening Of The Clones By Flow Cytometry

Screening of the clones was done by flow cytometry using T2 cells. Once the clones were visible, 100 µl of supernatant was pipetted out and transferred into individually labelled flow cytometry tubes (BD). An appropriate volume of T2 cells were harvested and one set was incubated with M1 peptide (Mimotopes) while the other set was incubated with EBNA-1 peptide (Mimotopes) for 45 mins at 37^{o}C in the CO_2 incubator. After incubation, an equal amount of cells was pipetted into each tube. Following that, the tubes were incubated at 4^{o}C for 35 mins. To wash away the excess supernatant, 4 ml PBS was added to each tube and centrifuged at 350 g for 5 mins. The supernatant was discarded and 10 µl diluted goat anti-mouse AlexaFluor 488 (FITC) (Invitrogen) was added to each tube. The tubes were incubated in the dark at room temperature for 25 mins. After incubation, 4 ml PBS was added to each tube and centrifuged at 350 g for 5 mins. The supernatant was discarded and the cells were resuspended. To fix the cells, 500 µl 4% paraformaldehyde (PFA) (Thermo Fisher Scientific) was added to each tube and the tubes were now ready to be analysed with CellQuest Pro Software on a BD Calibur Machine (BD).

2.4.1 Sub-cloning Of Hybridoma Cells

After analysis, clones that showed a positive shift as compared to the controls were marked. The cells were transferred into a 24-well plate and incubated at 37^{o}C in the CO_2 incubator. Once the cells had achieved confluency, the supernatant was harvested and another round of flow cytometry was carried out to confirm that the antibody still had the ability to bind to the peptide. Clones which showed a good specificity to the peptide was cultured and frozen in liquid nitrogen.

2.4.2 Limiting Dilution Of Hybridoma Cells

Limiting dilution was performed to ensure that single cell per well was attained so as to increase the chances of getting a monoclonal antibody. The cells were transferred from the 24-well plate into a 15 ml tube and centrifuged at 350 g for 5 mins. The supernatant was discarded and the cells were resuspended in 10 ml R10 medium. A cell count was done and the cell suspension was diluted to 10^4 per ml. Tenfold dilutions were done until the required cell dilutions were obtained (0.5 and 1 cell per well). 100 µl of cells were then pipetted into each well on a 96-well plate and incubated at 37^{o}C in a CO_2 incubator. The clones were scored after 8-10 days and screened by flow cytometry for specific binding.

2.5 Clonality Check

To determine if the antibody obtained was indeed monoclonal, a clonality check was done using the Novagen IgG primer kit. RNA extraction was carried out according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). To obtain the cDNA, RT-PCR was carried out with the appropriate primers from the kit. Following that, the samples were analysed by gel

electrophoresis on a 1% agarose gel at 120 V for 45 mins. The bands were visualized with the Chemidoc machine (Vilber Lourmat, France).

2.6 *In Vivo* Ascites

Once a monoclonal clone with a high specificity was obtained, it was expanded in T75 flasks. Every 3-4 days, half the volume was pipetted out and centrifuged at 350 g for 5 mins. The supernatant was collected and stored at 4^{o}C. The same volume of R10 medium was then added back into the T75 flask.

2.7 Purifying Monoclonals By FPLC

The supernatant collected was filtered with a 0.22 µm filter and purified by fast protein liquid chromatography (FPLC) using Sepharose G beads. The concentration of purified antibody was measured by the Bradford Protein Assay.

2.8 Bradford Protein Assay

In a 96-well plate, 10 µl of the standards were added in increasing concentrations. Next, samples were added in duplicates for each volume. Following that, 300 µl of Bradford solution (Pierce, Thermo Fisher Scientific, Roskilde, Denmark) was added into each well. Once a colour change from brown to blue was observed, the plate was analysed with a microplate reader (Bio-Rad).

2.9 Storage Of Purified Antibody

After quantification, the antibody was diluted with glycerol to a final concentration of 1 mg/ml. The antibody was aliquoted into 1.5 ml tubes and stored at -80^{o}C.

2.10 Antibody Isotyping

Hybridoma supernatant was collected and 150 µl of the sample was placed into each development tube. The tubes were incubated at room temperature for 30 s and vortexed briefly to completely resuspend the coloured latex. Next, one isotyping strip was placed into each tube. The strips were incubated for 5-10 mins until the positive bands appeared. A blue band indicates the class or subclass and light-chain composition of the monoclonal antibody (Isostrip Mouse Monoclonal Isotyping Kit, Roche).

2.11 Enzyme-Linked ImmunoSorbent Assay (ELISA)

To estimate the affinity of the monoclonal antibody, a sandwich ELISA was used. A 96-well Maxisorp plate (NUNC) was coated with 100 µl of polyclonal rabbit anti-β_2m antibody (Dako, Denmark) diluted 2000x in 0.05 M sodium carbonate buffer (pH 9.6) and incubated overnight at room temperature. The next day, the plate was washed 3 times with PBS/0.05% Tween. To block non-specific binding, 300 µl 1% bovine serum albumin (BSA) was added to each well and incubated at room temperature for 1 hr. The plate was washed 3 times with PBS/0.05% Tween and incubated with 100 µl EBNA-1/HLA-A201 monomer at room temperature for 2 hrs. The monomers were diluted in 1% BSA. After incubation, the plate was washed 3 times with PBS/0.05% Tween and incubated with 100 µl monoclonal antibody at room temperature for 4 hrs. After washing 3 times with PBS/0.05% Tween, 100 µl of goat anti-mouse horseradish peroxidise (HRP) (Abcam, Cambridge, USA) diluted 10 000x was added to each well and incubated for 1 hr at room temperature. The plate was then washed 3 times with PBS/0.05% Tween. Following the wash, 100 µl pre-warmed 3,3´,5,5´-tetramethylbenzidine (TMB) substrate (BD) was added to each well and incubated until a colour change was observed. Thereafter, 50 µl of 2M sulphuric acid was added to stop the reaction. A colour change of blue to yellow was

observed. The intensity of the colour was measured with an ELISA reader (Bio-Rad) at a wavelength of 450 nm.

2.12 Native Gel Electrophoresis

2.12.1 Preparation Of Native Polyacrylamide Gel

Separating gels with 10% acrylamide concentrations were used together with stacking gels of 2.5%. Gels were cast using the Bio-Rad Mini-PROTEAN Electrophoresis System (Bio-Rad) according to the manufacturer's instructions. The following reagents were added to form two 1.5 mm thick gels.

Reagents	10% Separating Gel	2.5% Stacking Gel
Water	7.5 ml	5 ml
30% Acrylamide/Bis	6 ml	-
4x Separating Gel Buffer	4.5 ml	-
10% Acrylamide/Bis	-	2 ml
8x Stacking Gel Buffer	-	1 ml
10% APS	120 µl	50 µl

Table 2.3 Native Gel Recipes

The separating gel was prepared first and left to polymerise for 1 hr at room temperature. Thereafter, the stacking gel was added above it and left to polymerise for 2 hr at room temperature before use.

2.12.2 Sample Preparation And Electrophoresis

Each sample was mixed with 15 µl of native sample buffer (Bio-Rad) and loaded into the wells. A pre-stained protein marker was added as well (Bio-Rad Precision Plus Protein Standards). Electrophoresis was carried out in the presence of 1x non-denaturing running buffer under a constant current of 25 mA for 5-6 hrs at 4^{o}C.

2.12.3 Western Blotting

After gel electrophoresis, the gel was transferred to a Polyvinylidene Difluoride (PVDF) membrane (Bio-Rad) using a Mini Trans-Blot Cell (Bio-Rad). The PVDF membrane was first pre-wet with 100% ethanol and rinsed with MilliQ water. Following this, the gel, PVDF membrane, filter paper and fibre pads were equilibrated in 1x transfer buffer for 10 mins. A gel sandwich was assembled by putting together a fibre pad onto the cathode side of the cassette followed by a filter paper, the gel, PVDF membrane, another filter paper and fibre pad. Precautions were taken to ensure that no air bubbles were trapped in between the layers. The membrane was also cut to distinguish between different sample lanes. After the sandwich was assembled, gel electrophoresis was carried out in the presence of 1x transfer buffer at a constant voltage of 350 mA for 2 hrs at 4^{o}C.

2.12.4 Staining Of PVDF Membrane

The PVDF membrane was removed from the sandwich, wet with methanol and dried in a 56^{o}C oven for 10 mins for blocking. The membrane was then washed with TTBS for 10 mins thrice with vigorous shaking. Following that, the membrane was incubated with W6/32, a conformational specific monoclonal antibody, at a 1:500 dilution in TTBS for 1 hr with shaking. After incubation, it was washed with TTBS for 10 mins thrice with vigorous shaking. A goat anti-

mouse HRP (Pierce) was incubated with the membrane at a dilution of 1:5000 in TTBS for 1 hr. After washing with TTBS for 10 mins thrice with vigorous shaking, a developing solution (Western Lightning Chemiluminescence, Perkin Elmer, MA, USA) was added to the membrane for 2 mins. The signals were then developed and visualised on a film (Amersham Biosciences, GE Healthcare).

2.13 Immunohistochemistry

2.13.1 Embedding And Cutting

A drop of embedding liquid was placed on the metal bowl that was in a styrofoam box of dry ice. A tissue was then placed on top of it and covered immediately with another drop of embedding media. After it had solidified, the tissue was stored at -80^{o}C. The tissue samples were cut using the cryostat machine, placed on glass slides and stored in a slide box at -80^{o}C.

2.13.2 Staining

After the slides had been left to dry at room temperature for 1 hr, the slides were fixed in ice-cold methanol for 10 mins and washed in PBS thrice. To prevent unspecific binding, 1% BSA was used as a blocking agent for 10 mins. After washing with PBS thrice, the slides were stained with primary antibody and incubated overnight at room temperature. The next day, the slides were washed and one drop of peroxidise block (Dako) was added to the slides for 5 mins at room temperature (RT). The slides were washed and another drop of Dako labelled polymer was added and incubated for 30 mins at RT. The slides were washed and 100 µl diluted 3,3-diaminobenzidine (DAB) was added to each sample. The slides were washed and dipped in Mayer's haematoxylin. After washing in water, 2% ammonium hydroxide was added. Following this, the slides were washed in water and dipped into 70% ethanol for 5 mins, 95% ethanol for 5

mins thrice, absolute ethanol for 5 mins thrice and xylene for 5 mins four times. The slides were then mounted with Clarion mounting media and were ready for viewing.

RESULTS

Chapter 3

3.1 Antibody Generation And Characterisation

3.1.1 HLA-A2 Expression And Purification

The heavy chain HLA-A201 and the light chain β_2m were separately transformed in *E. coli* strain BL21 competent cells and expressed as inclusion bodies. The *E. coli* cells were then lysed by sonication and the inclusion bodies were isolated and purified by repeated washings. To determine the concentrations of heavy or light chains obtained, the samples were analysed on SDS-PAGE and a comparison was done with standards of known concentrations (stock concentration of 1 mg/ml). As seen in Figure 3.1 below, a range of concentrations was analysed on the SDS-PAGE, the density and thickness of the bands were compared to the standards and the final concentration obtained was deduced to be 32 mg/ml.

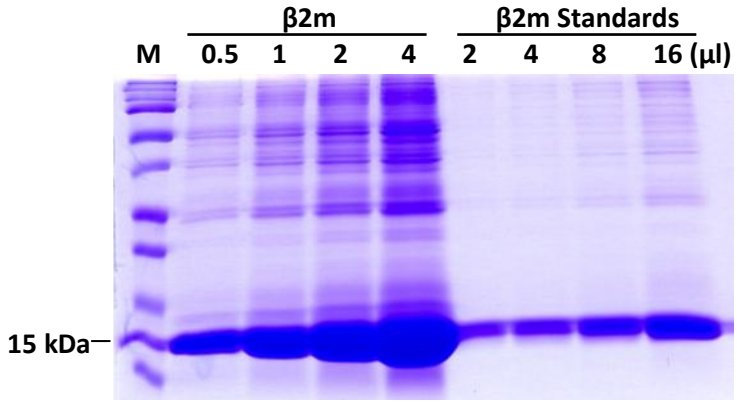


Figure 3.1 SDS-PAGE Of Light Chain β_2m.
To determine the concentration of light chain obtained, a comparison was done with standards of known concentrations (stock concentration of 1 mg/ml). Increasing volumes of both were added and the density and thickness of the bands between the test sample and standard was compared. The concentration of β_2m obtained is 32 mg/ml.

To obtain the monomer, the heavy and light chains were refolded in the presence of the peptide over 72 hours with the addition of heavy chain every 24 hours.

In our study, a peptide sequence was chosen from Epstein-Barr virus nuclear antigen 1 (EBNA-1) protein. EBNA-1 is essential for replication and maintenance of the EBV genome and is expressed in all EBV-infected cells. A peptide prediction software, ProPred1, was used to predict the binding of HLA-A201 to the whole EBNA-1 sequence (Singh and Raghava, 2003). The peptide sequence FMVFLQTHI (position 562-570) was chosen as it showed potentially good binding and it is a well-defined CTL epitope (Bihl *et al*., 2005).

3.1.2 Purification Of Monomer And Mouse Immunisation

After refolding, the monomer was dialysed overnight and purified through anion exchange chromatography followed by size exclusion. Anion exchange chromatography separates molecules according to their charge. The monomer which is negatively charged will adsorp to the matrix that contains positively charged molecules – diethylaminoethyl (DEAE). The monomer will then be eluted by an increasing salt concentration in a step-wise gradient (green line in Figure 3.2 below).

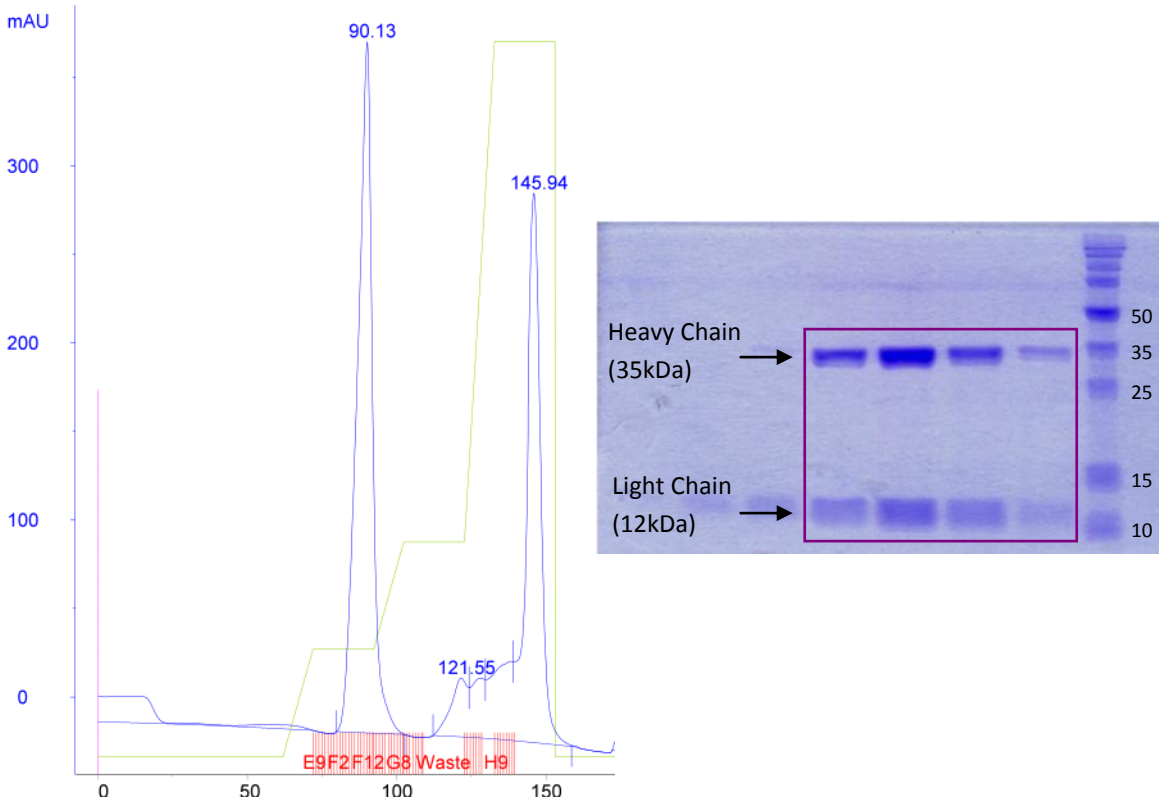


Figure 3.2 DEAE Anion Exchange Profile And SDS-PAGE.
Fractions from the two peaks were collected and analysed with SDS-PAGE. Only fractions that contain both the heavy and light chains were collected for further purification. The boxed section shows lanes that contain both the heavy (35 kDa) and light chains (12 kDa).

Figure 3.2 above shows a typical DEAE anion exchange profile. Selected fractions were taken from each peak and analysed on SDS-PAGE. As seen in Figure 3.2, two bands were obtained for each fraction, one for the heavy chain and the other for the light chain. Only fractions that had both the heavy and light chains were collected and concentrated to a volume of 200-500 µl for further purification by size exclusion (Superdex). Size exclusion involves separation of molecules based on their size and the time it takes to travel through the pores in the column. Hence, larger molecules take less time to travel though the column and are eluted first. A typical size exclusion profile generated from our experiments is shown in Figure 3.3.

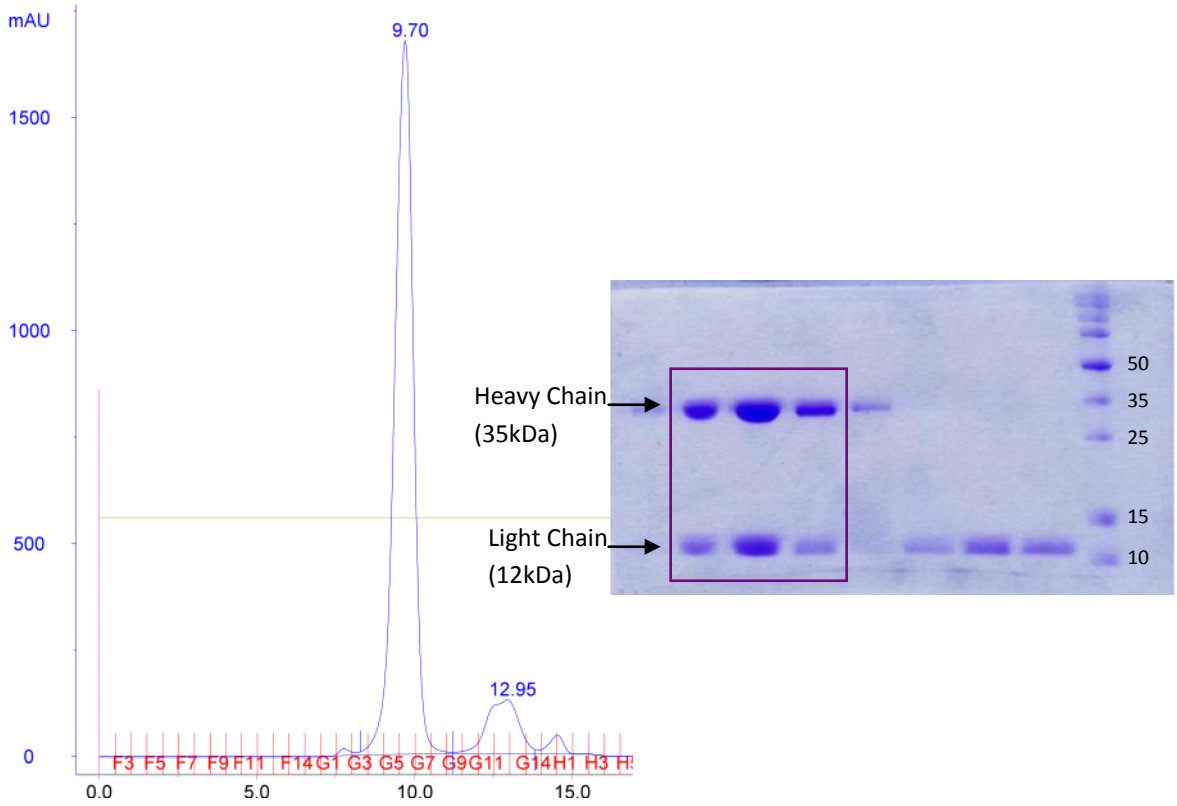


Figure 3.3 Superdex Profile And SDS-PAGE.
Fractions from the two peaks were collected and analysed with SDS-PAGE. Only fractions that contain both the heavy and light chains were collected and concentrated. The boxed section shows lanes that contain both the heavy (35 kDa) and light chains (12 kDa).

All fractions collected were analysed on an SDS-PAGE. Similar to the anion exchange profile, only those fractions that have both the heavy and light chains were collected and concentrated. Thereafter, a Bradford protein assay was carried out to determine the concentration of the monomer obtained. The HLA-A201/EBNA-1 monomer had a concentration of 5 mg/ml. These were aliquoted and stored at -80^{o}C.

3.1.3 Biotinylation Of HLA Monomers

The purified monomer was biotinylated with the incubation of BirA enzyme mix at 28^{o}C for 12-18 hours. The mixture was washed and concentrated in a Centricon YM-30 tube. The amount of biotinylated product was quantitated by immunoprecipitation using streptavidin beads followed

by analysis on a 15% SDS-PAGE (Figure 3.4). The concentration of biotinylated monomers was estimated by a comparison with the BSA standards of known concentrations (1 mg/ml) while the percentage of biotinylation of HLA monomers was determined by comparing it against biotinylated HLA monomers that were non-immunoprecipitated. From the results obtained, approximately 60% of HLA monomer was biotinylated.

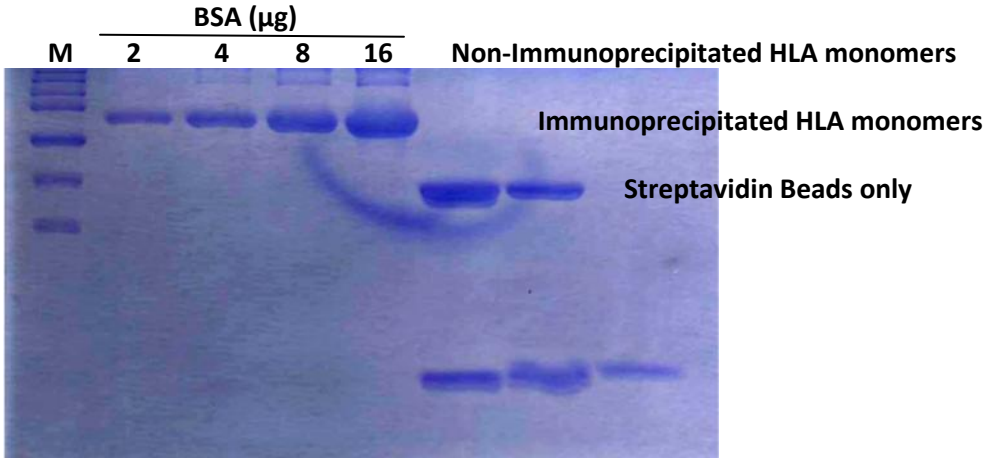


Figure 3.4 SDS-PAGE Of Biotinylated HLA Monomer.
The concentration of biotinylated monomers was estimated by a comparison with the BSA standards of known concentrations (1 mg/ml) while the percentage of biotinylation of HLA monomers was determined by comparing it against biotinylated HLA monomers that were non-immunoprecipitated. Streptavidin beads act as a negative control.

3.1.4 Analysis Of Monomer On Native Gels

To confirm that the monomer had folded properly, purified monomer was analysed on a non-denaturing native gel and immunoblotted with the conformational specific monoclonal antibody (W6/32) (Parham *et al*., 1979). This antibody binds HLA complexes when they are folded into a tertiary complex with the peptide. In Figure 3.5, the band observed shows that the monomer is properly folded and can be used as an immunogen for making HLA-A201/EBNA-1 antibodies in mice.

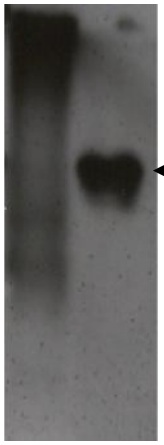


Figure 3.5 Analysis Of Purified Monomer Using Non-Denaturing Native Gel.
The gel was immunoblotted with anti-HLA conformational specific monoclonal W6/32 and the single band obtained confirmed that the purified monomer was folded correctly.

Balb/C female mice were immunised with the HLA-A201/EBNA-1 monomer in complete Freund's adjuvant. Over a period of 45 days, two more boosts were given on days 21 and 35 with incomplete Freund's adjuvant and the final boost was given intra-veinously on day 42 via the tail vein with HLA-A201/EBNA-1 monomer in PBS only. The mice were sacrificed on day 45 and their spleens harvested. A portion of the splenocytes were fused with NS-1 myeloma cells to form hybridomas while the rest underwent an additional selection process. This involved pulsing the splenocytes with biotinylated monomers followed by immunomagnetic selection using anti-biotin microbeads. The selected cells were then fused to NS-1 myeloma cells. The hybridomas were plated onto 96-well plates and observed 8-10 days later when colonies were visible. The clones were then scored and screened by flow cytometry for specific binding.

3.1.5 Antibody Screening

After 8-10 days, once the growing colonies were visible by eye, 100 µl supernatant was harvested and screened with T2 cells. T2 cells which are EBV/EBNA-1 negative and TAP deficient are not able to process and cleave endogenous peptides. Without these short peptide fragments binding to the MHC class I molecule, they are unstable and do not present the MHC complex on the surface of cells. However, when an exogenous peptide is added, it binds to the

MHC class I molecule and stabilises it, thus allowing the MHC/peptide complex to be presented on the surface of cells to cytotoxic T cells. Our antibody will then recognise this MHC/peptide complex and bind.

Firstly, T2 cells were incubated with the EBNA-1 peptide before the supernatant, which contained the antibodies, was added. The cells were stained with FITC and analysed by flow cytometry. An irrelevant M1 influenza peptide (GILGFVFTL) was used as a negative control. This peptide was chosen as it is a well characterized, HLA-A201 restricted peptide. This control helps us to differentiate those antibodies that bind to HLA-A201 only versus those that bind to both HLA-A201 and the EBNA-1 peptide. Below is a representative flow cytometry histogram (Figure 3.6) for antibodies that are only specific for HLA-A201. It is observed that the antibody binds equally well to the relevant EBNA-1 (Blue) and irrelevant M1 peptide (Red) and show similar shifts for both peptides. This shows that the antibody binds to HLA-A201 and does not specifically recognises the HLA-A201/EBNA-1 peptide complex. Since we are only interested in antibodies with T-cell receptor-like specificity that bind to both HLA-A201 and the specific peptide EBNA-1, we assume that antibodies with the profile described above are negative.

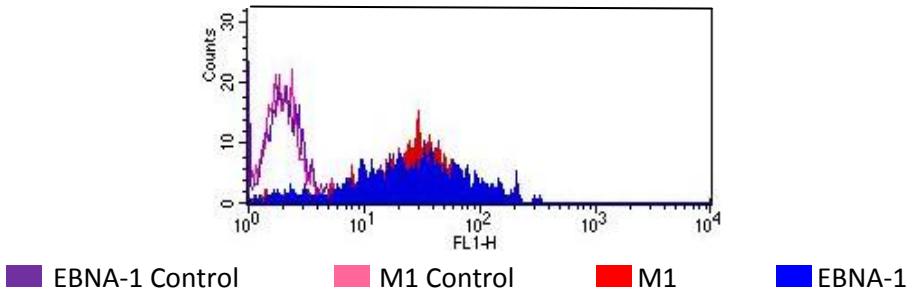


Figure 3.6 Profile Of Hybridoma Screening For Clones That Are HLA-A201 Specific Only.
T2 cells were used for screening. The control irrelevant M1 peptide (Red) shows a similar shift as the EBNA-1 peptide (Blue), thus showing that the antibody is specific for HLA-A201 but not the EBNA-1 peptide. Secondary controls (FITC only without primary antibody) pulsed with M1 peptide (Pink) and EBNA-1 peptide (Purple) are shown.

Figure 3.7 shows the flow cytometry profile of a positive result obtained from an antibody with specific binding. As seen in the histogram, a one log shift to the right (Blue) was observed as compared to the irrelevant peptide (Red). This shows that the antibody is able to bind specifically to the HLA-A201/EBNA-1 epitopes only and do not bind to either HLA-A201 or the irrelevant M1 peptide.

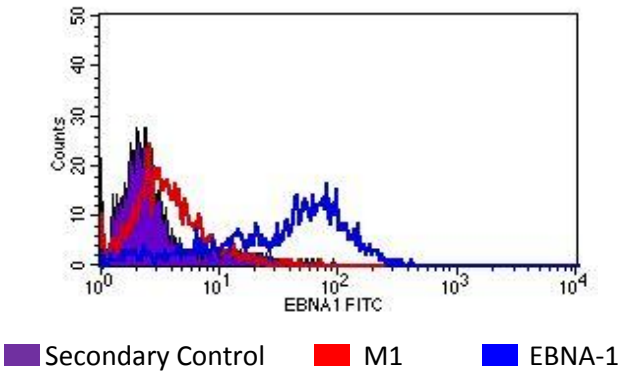


Figure 3.7 Profile Of A Positive Result That Shows Specific Binding.
Results obtained from a hybridoma clone that binds specifically to both the HLA-A201 and EBNA-1 peptide as seen by a one log shift of the EBNA-1 (Blue) curve as compared to the M1 (Red) curve. The secondary control (Purple) is shown.

Figure 3.8 shows a summary of the hybridomas that were screened after undergoing an additional biotinylated monomer based selection step. 6% of the clones obtained were specific for both the HLA-A201 and the EBNA-1 peptide while 13% were positive for just HLA-A201 alone. This is in contrast to the hybridomas that did not undergo the biotin selection. None of the clones screened were positive for HLA-A201 and EBNA-1 while only 1% were HLA-A201 positive. This shows that this method is crucial for the production of antibodies that bind specifically to both the HLA and the peptide.

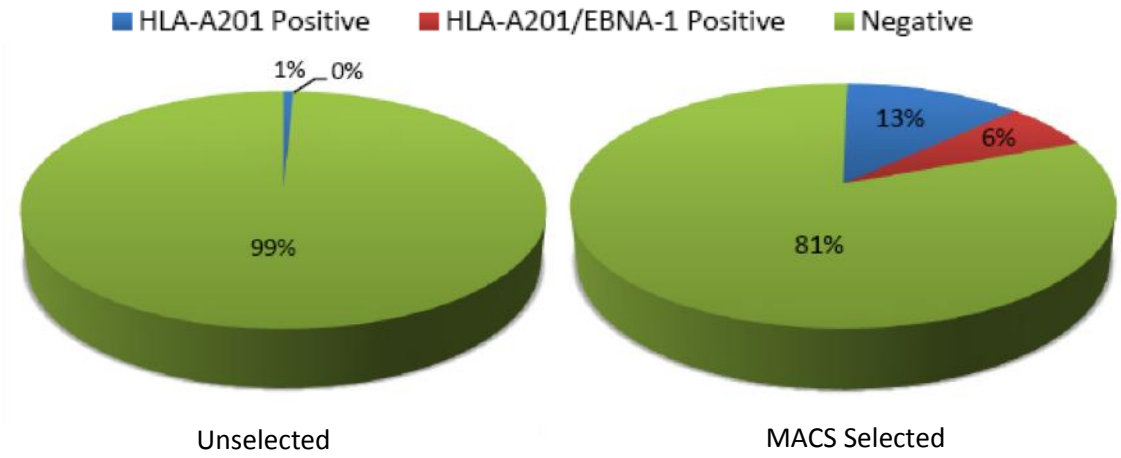


Figure 3.8 Pre-Selection Of B Cells For The Required Specificity Significantly Enhances The Percentage Of HLA-A201/EBNA-1 Specific Hybridomas Versus Unselected Splenocytes. Unselected splenocytes were compared with splenocytes selected on the basis of their binding to HLA-A201/EBNA-1 biotinylated monomers prior to fusion. Unselected splenocytes did not generate any HLA-A201/EBNA-1 specific hybridomas compared to splenocytes selected for their binding capacity to the monomer. Moreover, the selected cells were found to have a stable phenotype with better overall specificities compared to positive clones from the unselected hybridoma pool.

3.1.6 Limiting Dilution

To obtain a monoclonal antibody, the hybridoma cells above were cultured and subcloned twice. Subcloning involves limiting dilution at 0.5 or 1 cell per well. This was done to ensure that single cell per well was attained so as to increase the chances of getting the correct monoclonal antibody. At each round, the clones were screened by flow cytometry to confirm their binding specificities. Figure 3.9 shows the flow cytometry profile of a clone obtained after two rounds of limiting dilution.

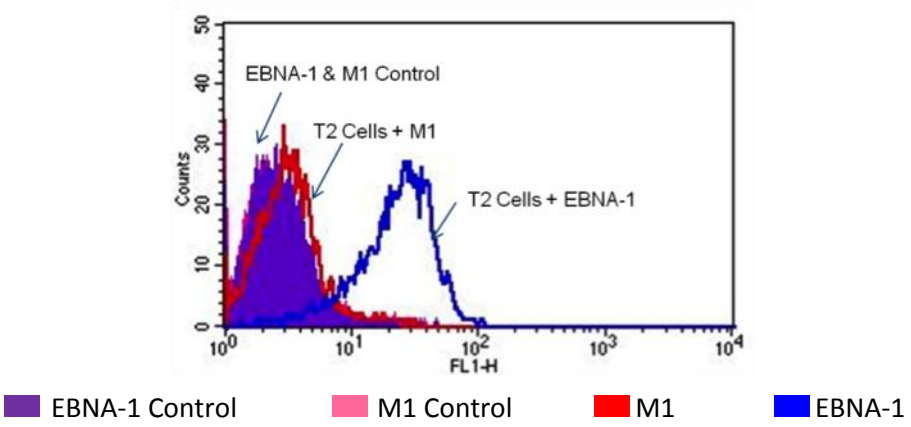


Figure 3.9 Screening B Cell Hybridomas For HLA-A201/EBNA-1 Specificity.
T2 cells were used to test the specificity of the hybridoma clones generated from mice immunised with HLA-A201/EBNA-1 peptide complexes. T2 cells were pulsed with EBNA-1 peptide (FMVFLQTHI) (5 µM) (Blue) and compared by flow cytometry with T2 cells pulsed with the HLA-A201 restricted influenza A peptide M1 (GILGFVFTL) (5 µM) (Red). Secondary controls (FITC only without primary antibody) pulsed with M1 (Pink) and EBNA-1 (Purple) peptides are shown.

3.1.7 Isotype Test And Clonality Check

To test the isotype of the antibody, an isotyping strip from Roche was used. In each strip, two bands were observed after incubation. One is for the positive control and the other is for the antibody. As seen in Figure 3.10 below, our antibody is specific for IgG1 and κ light chain.

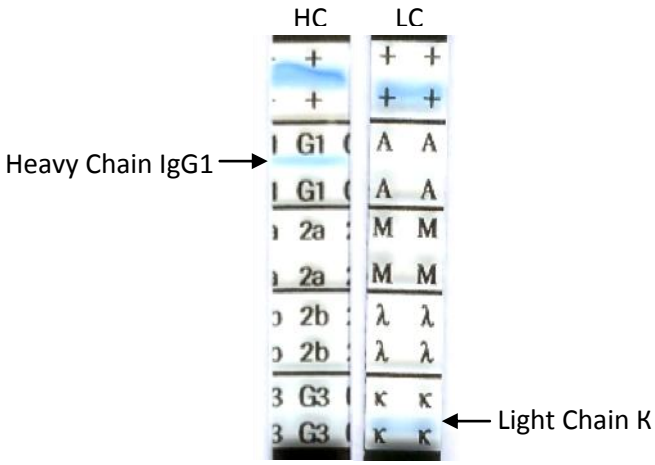


Figure 3.10 Isotype Test For Anti-HLA-A201/EBNA-1.
The isostrip was incubated in 150 µl supernatant for 10 mins at room temperature. The isotype profile for anti-HLA-A201/EBNA-1 is IgG1 and κ light chain. HC=Heavy Chain, LC=Light Chain

An additional test was carried out to confirm its monoclonality. RNA was extracted from the hybridoma cells and a reverse-transcription PCR (RT-PCR) was carried out with primers specific for the variable regions of the heavy chain IgG1 and κ light chain (Novagen IgG Primer kit). The 3' Ig primers provided were complementary to the conserved region adjacent to the variable regions and thus were specific to the various heavy chain IgG (A-F) and κ light chain subtypes (A-G). Hence, these primer sets enabled these regions to be amplified by PCR.

The results were analysed by agarose gel electrophoresis. From the gel photo (Figure 3.11A), it is seen that our antibody is specific for subtype C of the heavy chain and A, B, C, D and F of the light chain. One reason for the multiple bands for the light chain is that the fusion myeloma NS-1

cells also variably express aberrant κ transcripts. Only clones that showed a single band in the heavy chain were chosen. A positive control PCR template provided in the kit was used to ensure the suitability of PCR conditions used (Figure 3.11B).

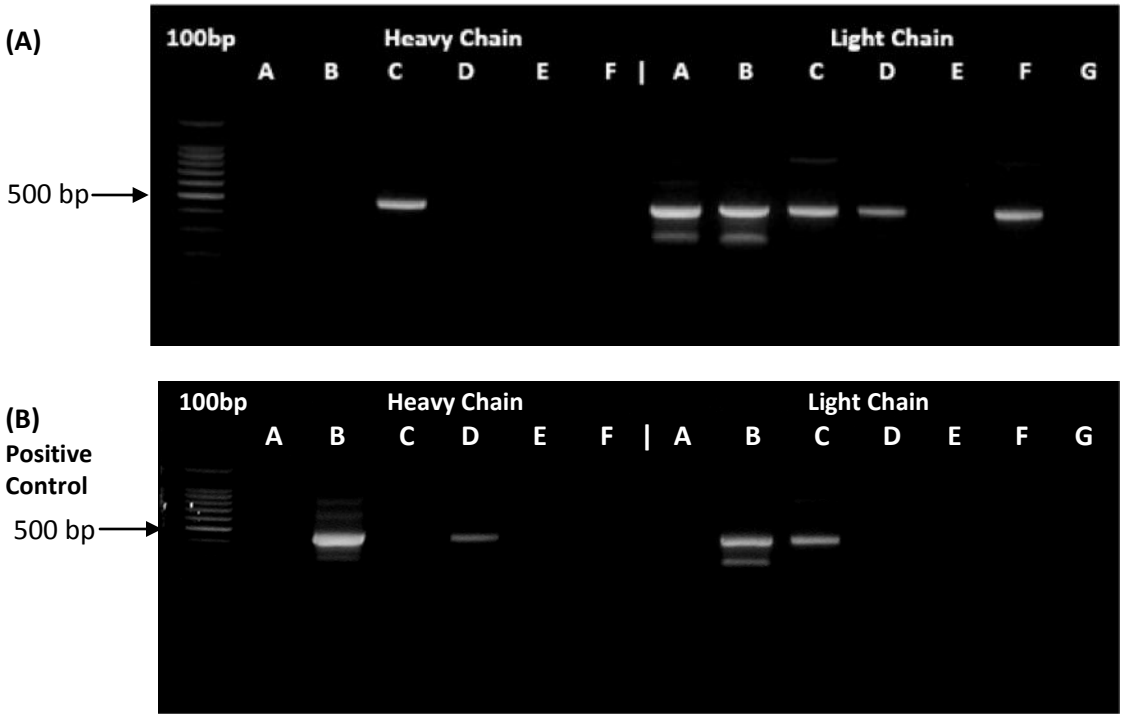


Figure 3.11 Immunoglobulin Clonality For Anti-HLA-A201/EBNA-1.
RNA was extracted from the hybridoma cells and RT-PCR was carried out using primers specific for the subtypes of heavy and light chains. The results were analysed by agarose gel electrophoresis. The subtypes for heavy chain and light chain are (A-F) and (A-G) respectively. Only clones that showed a single band in the heavy chain were chosen. (A) is an example of a positive clone while (B) is the positive control provided by the kit. For the positive control, there should be no bands for heavy chain A,C, E and F and light chain A, D, E and F.

3.1.8 *In Vitro* Ascites And Purification of Antibody

This clone was cultured and produced on a larger scale using an *in vitro* ascites method. Supernatant containing the antibodies was collected over a period of time and purified through FPLC using Separose G beads. The FPLC trace shows a distinct peak at 100 ml of buffer B with a value of about 2400 mAU. The fractions collected were analysed by SDS-PAGE. Two bands were observed, one represents the heavy chain (about 50 kDa) while the other represents the light chain (about 20 kDa) (Figure 3.12).

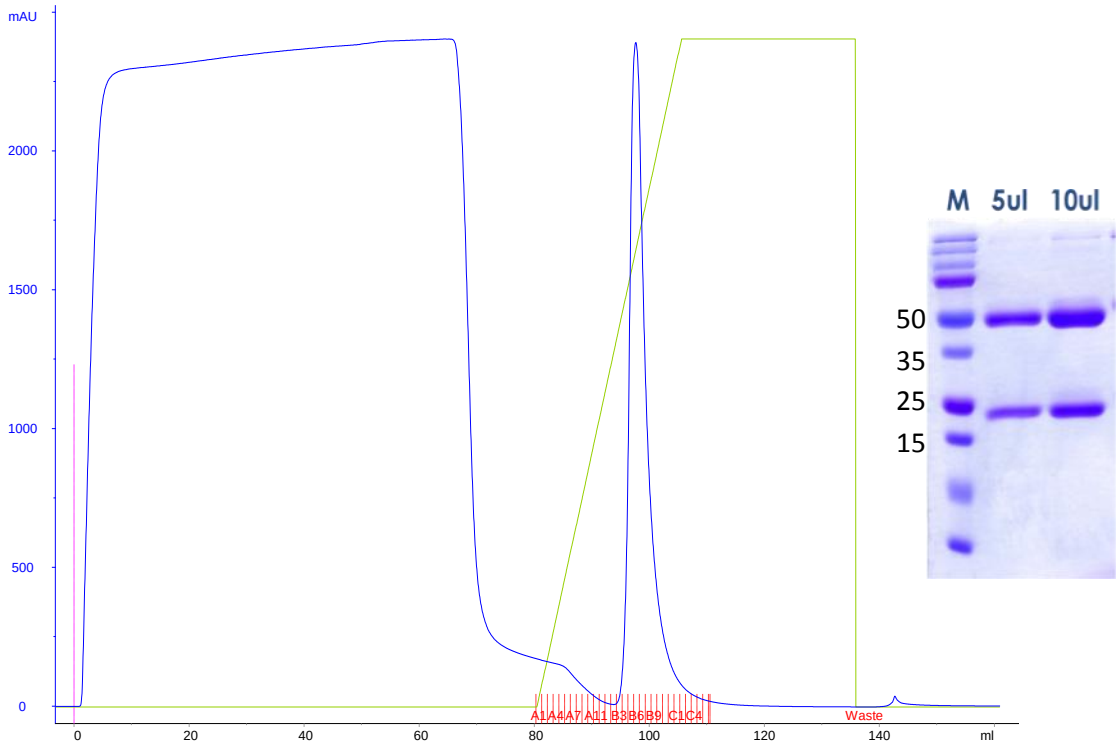


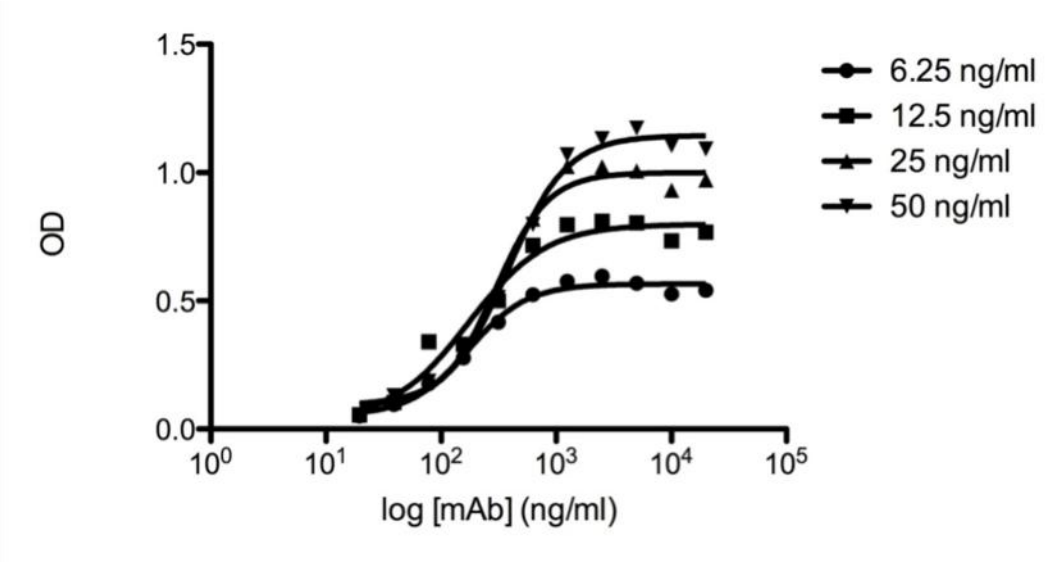
Figure 3.12 FPLC Trace And SDS-PAGE Analysis Of Purified Antibody.
The collected supernatant was purified by FPLC. The fractions corresponding to the peak was collected and analysed by SDS-PAGE. A heavy chain (50kDa) and light chain (20 kDa) was observed.

3.1.9 Antibody Affinity

To test the affinity of the monoclonal to the monomer, an established ELISA method was carried out (Beatty *et al*., 1987). A titration of the monomer (antigen) was done to obtain a standard curve in the presence of excess antibody. Thereafter, the range of monomer concentrations on the linear portion of the standard curve was used for the actual affinity ELISA test. The range of monomer used was between 6.25 ng/ml and 50 ng/ml. The OD was measured and a graph was plotted of OD (y-axis) verses log (antibody concentration) (x-axis) (Figure 3.13).

The range of monoclonal used was between 0 ng/ml and 20 000 ng/ml. A sigmoid curve was obtained with the best fit curve. As the concentration of monomer increases, the curve is more pronounced. The formula listed was used to calculate the approximate affinity (K_{aff}) of the antibody. The affinity obtained for our antibody (1.544×10^{-9} M) is comparable to an established antibody BB7.2, which is an antibody specific for HLA-A2 (Parham and Brodsky, 1981).

Affinity Curves of HLA-A201/EBNA-1 Monoclonal Antibody



Formula used to calculate K_{aff}:

$$K_{aff} = \frac{n - 1}{2[Ab'] - [Ab]}$$

n = Dilution factor of antigen
Ab' = OD_{50} of antibody at diluted concentration
Ab = OD_{50} of antibody at less diluted concentration

$K_{aff} = 1.544 \times 10^{-9}$ M

Figure 3.13 Experimental Dose-Response Curves For Anti-HLA-A201/EBNA-1 At Increasing Concentrations Of Antigen.
The affinity of the antibody for the antigen was determined with ELISA and calculated based on the above formula.

3.1.10 Peptide Titration And Specificity Of Anti-HLA-A201/EBNA-1

To test the sensitivity of the monoclonal to EBNA-1 peptide, a titration of the peptide was tested with T2 cells. T2 cells were pulsed with the various concentrations of EBNA-1 peptide, incubated with the monoclonal and analysed by flow cytometry. As seen from the plot (Figure 3.14), there is an increase in binding as the concentration of peptide increases and the monoclonal can be

used to detect HLA-A201/EBNA-1 peptide complexes on the cell surface down to a concentration of 0.01 µM.

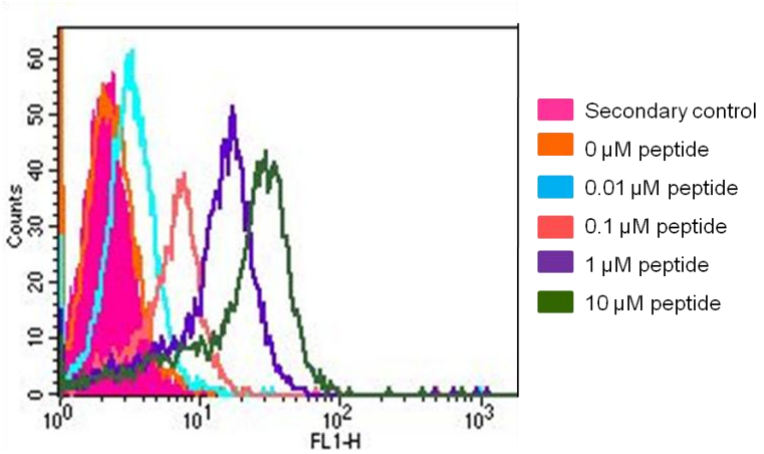


Figure 3.14 Binding Of Anti-HLA-A201/EBNA-1 Over A Range Of Peptide Concentrations.
T2 cells were pulsed with increasing concentrations of EBNA-1 peptide. Peptide loaded cells were incubated with anti-HLA-A201/EBNA-1 and detection of binding was with goat anti-mouse FITC. The results were analysed using flow cytometry. Binding of anti-HLA-A201/EBNA-1 to peptide complexes was observed with a concentration as low as 0.01 µM peptide.

To test the specificity of the monoclonal to the HLA-A201/EBNA-1 complex, T2 cells were pulsed with various HLA-A201 restricted peptides and the binding was analysed by flow cytometry. These include EBV peptides such as latent membrane protein 1 (LMP-1) and Cytomegalovirus (CMV) peptides such as internal matrix protein (pp65) and immediate-early protein 1 (IE1). M1, an influenza peptide was used as a negative control. From Figure 3.15, it is observed that specific binding was seen for HLA-A201/EBNA-1 complexes only (Blue) and not for the rest of the HLA-A201 peptide complexes.

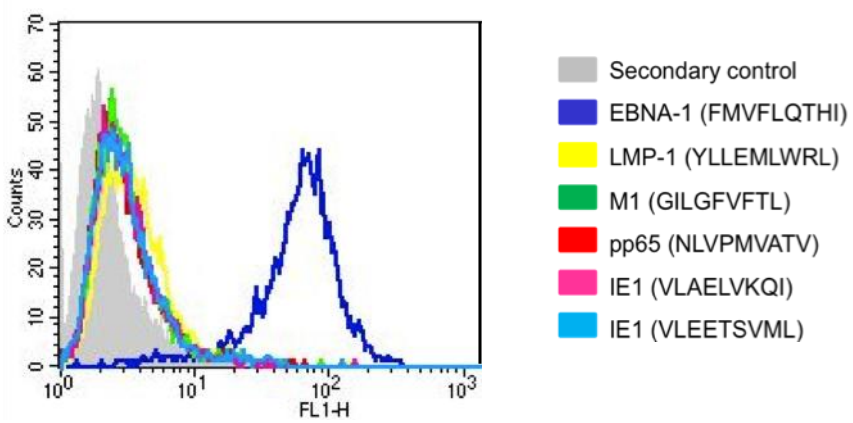


Figure 3.15 Anti-HLA-A201/EBNA-1 Binds Exclusively To HLA-A201/EBNA-1 Complexes And Not To Other HLA-A201 Peptide Complexes.
T2 cells were pulsed with 5 µM of the various HLA-A201 restricted peptides. Peptide loaded cells were incubated with anti-HLA-A201/EBNA-1 and detection of binding was with goat anti-mouse FITC. The results were analysed using flow cytometry. Specific binding was observed for HLA-A201/EBNA-1 complexes only (Blue) and not the other HLA-A201 peptide complexes.

3.1.11 Testing Monoclonal Against HLA-A201 Cell Lines

Next, we tested the monoclonal against the HLA-A201 positive B-lymphoblastoid cell lines (BLCLs) (CF 986, CM 936 and CM 800). These BLCLs were obtained from various patients and volunteers and stored frozen in liquid nitrogen in the WHO Immunology Centre, NUS. The cells were pulsed with EBNA-1 peptide before the addition of the monoclonal, and analysed with flow cytometry. In BLCLs, peptide loading is facilitated by the exchange of endogenously derived peptides with EBNA-1 peptides that were introduced externally. Our results show that the monoclonal was able to detect HLA-A201/EBNA-1 complexes on the surface of the BLCLs (Figure 3.16A). Interestingly, all three BLCLs also showed a degree of binding when pulsed with the irrelevant M1 peptide (Red). An explanation could be that since BLCLs are EBV transformed, they would express EBV latent proteins. Hence it can be deduced that our monoclonal is able to detect constitutive levels of HLA-A201/EBNA-1 complexes.

To test this further, the monoclonal was tested on cell lines that are HLA-A201 expressing but not EBV transformed. Hence there should not be any EBV proteins present constitutively. The

three cell lines used were T2, THP-1 and U937-A2. T2 cells are human lymphoblast suspension cells, THP-1 are human monocytic leukemic suspension cells and U937-A2 are human monocytic suspension cells that are stably transfected with HLA-A201. The results show that when the cells were pulsed with M1 influenza peptide, incubated with the monoclonal and analysed by flow cytometry, no shift was observed (Figure 3.16B). However, when the cells were pulsed with EBNA-1 peptide, a shift was observed and this shows that the monoclonal is able to bind specifically to HLA-A201/EBNA-1 peptide complexes on the cell surface and not to HLA-A201 or the irrelevant peptide. It also confirms the previous observation that the monoclonal is able to detect constitutive levels of HLA-A201/EBNA-1 complexes.

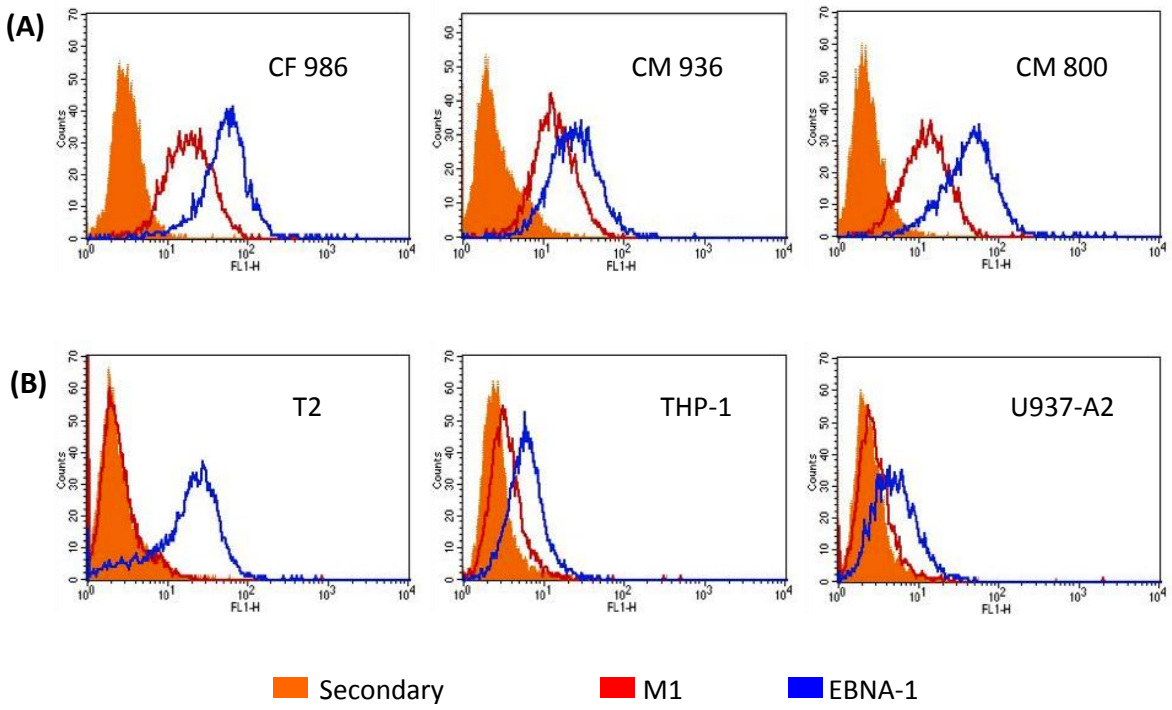


Figure 3.16 Anti-HLA-A201/EBNA-1 Recognises Constitutively Expressed EBNA-1 In EBV Transformed HLA-A201 BLCLs.

(A) EBV transformed BLCLs that are HLA-A201 restricted (CF986, CM936, CM800). Cells were pulsed with peptides and analysed by flow cytometry. Anti-HLA-A201/EBNA-1 was able to recognise constitutively expressed EBNA-1 in EBV immortalised BLCLs as shown by the shifts observed when these BLCLs were pulsed with a control influenza peptide M1 (Red).

(B) Non-EBV transformed cell lines which are HLA-A201 restricted (T2, THP-1, U937-A2). Cells were pulsed with peptides and analysed by flow cytometry. No shift was observed in the control M1 peptide pulsed cells (Red) while a shift was observed in EBNA-1 pulsed cells (Blue). Controls are shown in orange.

3.2 Monoclonal Specificity For EBV Tumours

3.2.1 Testing Of Monoclonal Against HLA-A2 Subtypes

The monoclonal was tested against other subtypes of HLA-A2 and its ability to bind was determined by flow cytometry. The other subtypes tested were from BLCLs that expressed either HLA-A203, HLA-A206 or HLA-A207. The peptide epitopes presented by HLA-A201 may be similar to that presented by HLA-A203, HLA-A206 and HLA-A207. When these BLCLs were pulsed with EBNA-1 peptide, there was some binding observed as shown by the shifts in the blue histograms (Figure 3.17).

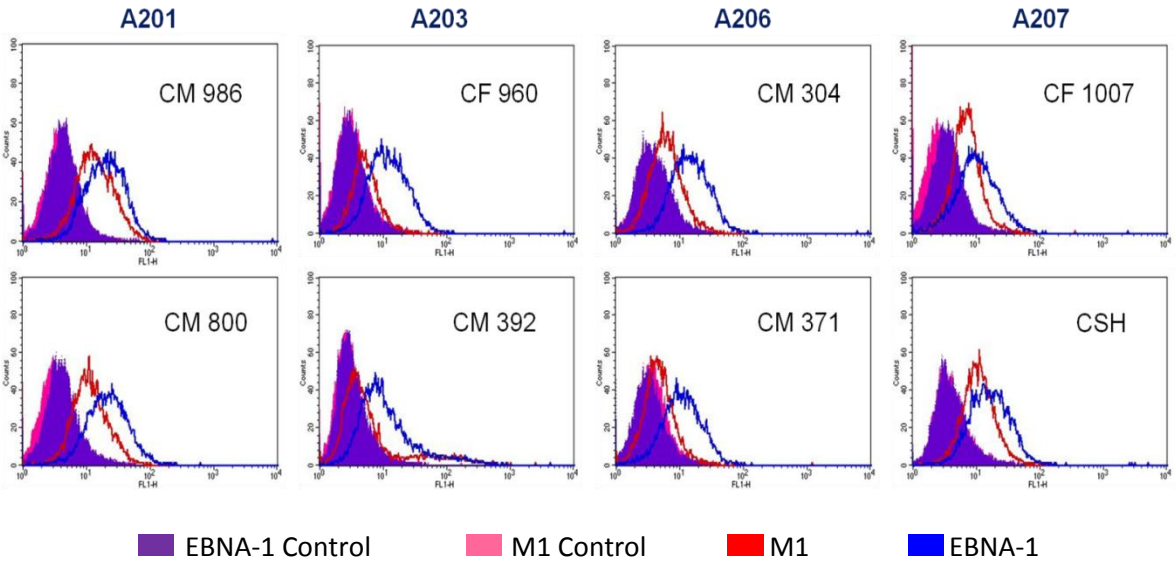


Figure 3.17 BLCLs Taken From Human Donors And Pulsed With Peptides. Flow cytometry was used to analyse binding of anti-HLA-A201/EBNA-1 to peptide complexes on BLCLs of various subtypes. A degree of binding was seen in both the EBNA-1 peptide pulsed cells (Blue) and the influenza peptide M1 pulsed cells (Red), suggesting that anti-HLA-A201/EBNA-1 can bind to constitutively expressed levels of EBNA-1. Secondary controls pulsed with EBNA-1 (Purple) and M1 (Pink) are shown.

However, the degree of binding for HLA-A201 and HLA-A207 was more similar as compared to HLA-A203 and HLA-A206. This could be due to the fact that there is only one base difference between HLA-A201 and HLA-A207 with an amino acid change from tyrosine to cysteine. On the other hand, there are two and three base changes for HLA-A206 and HLA-A203 respectively.

Table 3.1 below gives a summary of the amino acid changes. Certain base changes may affect the binding of the peptide to the groove on the HLA molecule thus affecting the binding efficiency of the monoclonal to it.

HLA	Nucleotide	Amino Acid	HLA	Nucleotide	Amino Acid
A201	**G**CG	Alanine	**A203**	**A**CG	Threonine
	G**T**G	Valine		G**A**G	Glutamic Acid
	T**T**G	Leucine		T**G**G	Tryptophan
	T**T**C	Phenylalanine	**A206**	T**A**C	Tyrosine
	AC**A**	Threonine		AC**C**	Threonine
	T**A**T	Tyrosine	**A207**	T**G**T	Cysteine

Table 3.1 Base Changes Between HLA-A201 And HLA-A203, HLA-A206 And HLA-A207

These BLCLs are all EBV transformed cells thus they have constitutive levels of EBNA-1 as seen in the previous experiment. Our monoclonal is thus able to detect these constitutive levels of EBNA-1, resulting in a shift even when the cells are pulsed with an irrelevant peptide such as M1. Similarly, the degree of shift observed in M1 may vary depending on the HLA-A2 subtype of the cells.

This has potentially important implications for the use of these antibodies. According to a study by Solberg who conducted a meta-analytic review on 497 population studies, HLA-A201 is the second most common allele and is found in 11 regions in the world (Solberg *et al.*, 2008). In the Singaporean Chinese population, HLA-A201 is also one of the more common alleles with a frequency of 10.4%. The frequencies of the other HLA-A2 subtypes in the Singaporean Chinese population are as follows, 6.7% for HLA-A203, 4.0% for HLA-A206 and 13.1% for HLA-A207 (Middleton *et al.*, 2000) (Figure 3.18). Hence, this monoclonal is applicable for a larger

population as it can detect HLA-A2/EBNA-1 complexes on the surface of the other HLA-A2 subtypes as well.

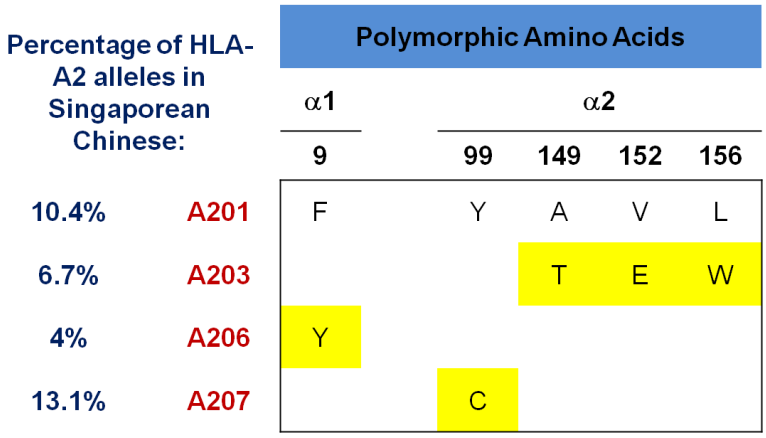


Figure 3.18 Percentages Of HLA-A2 Alleles In Singaporean Chinese Population And The Amino Acid Changes Between The Different HLA-A2 Subtypes.

3.2.2 Binding Of Monoclonal To Tumour Cells

To test the binding of the monoclonal to naturally processed EBNA-1 epitopes in tumour cells, two tumour cell lines were chosen. CCRF-SB is a human lymphoblast suspension cell line derived from acute lymphoblastic leukaemia while RPMI-6666 is a human lymphoblast suspension cell line derived from Hodgkin's lymphoma. Both cell lines are EBNA-1 positive and express HLA-A201 as seen by their staining with BB7.2 which is a HLA-A2 monoclonal antibody (Figure 3.19). These cells give a more accurate representation of the normal levels of HLA-A201/EBNA-1 complexes that are expected to be present on the cell surface as compared to the peptide loaded samples. The cells were incubated with the monoclonal, stained with goat anti-mouse FITC and analysed by flow cytometry. The results showed a clear binding of the monoclonal to EBNA-1 epitopes in a natural state without the addition of exogenous peptide.

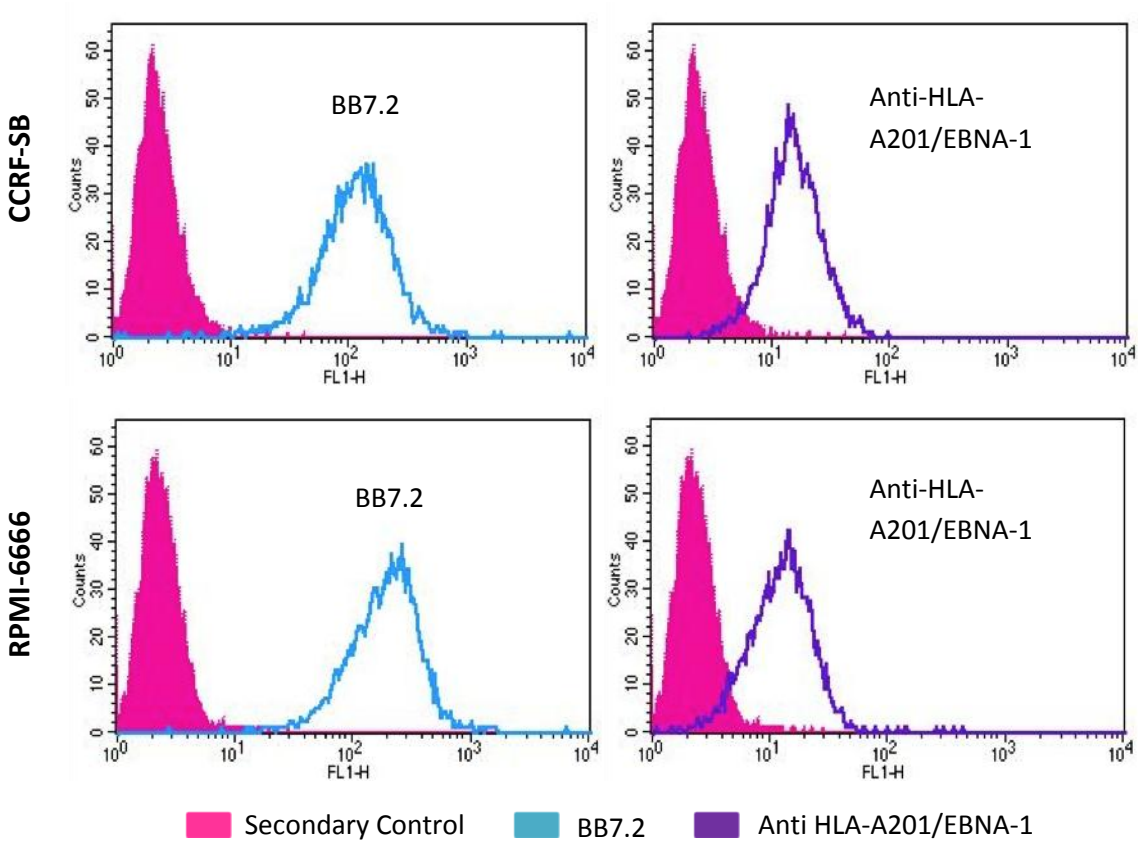


Figure 3.19 Anti-HLA-A201/EBNA-1 Detects Constitutive Levels Of EBNA-1 In HLA-A2 Restricted Tumour Cell Lines.

Cells were incubated with BB7.2 and anti-HLA-A201/EBNA-1 and detection of binding was shown by flow cytometry. CCRF-SB is a human lymphoblast derived from acute lymphoblastic leukaemia while RPMI-6666 is a human lymphoblast derived from Hodgkin's lymphoma. Both cell lines express HLA-A201 as seen by the positive staining with a HLA-A2 specific antibody, BB7.2. Anti-HLA-A201/EBNA-1 was able to recognise constitutively expressed EBNA-1 in both cell lines (Purple). Controls are shown in pink.

3.2.3 Staining Of NPC Biopsies With The Monoclonal

Frozen cryosectioned NPC biopsies were obtained from patients and stained with our monoclonal. Figures 3.20A and 3.20D show the negative controls (secondary antibodies only) at 10x and 100x magnification respectively. Figures 3.20B and 3.20E are the positive controls that were stained for HLA-A2. As compared to the negative control, Figures 3.20C and 3.20F show positive staining. This data shows the ability of the monoclonal to detect HLA-A201/EBNA-1 complexes on the surface of these biopsies.

There is the potential for this monoclonal to be used to detect EBV infected cells *in situ* without the need for biopsies to be carried out in the future. This could involve tagging the antibody with a fluorescent label and viewing infected cells via *in vivo* whole animal imaging technology such as the xenogen system. This would be useful for diseases such as NPC where the lesions may be situated in spots that are hard to reach and biopsy.

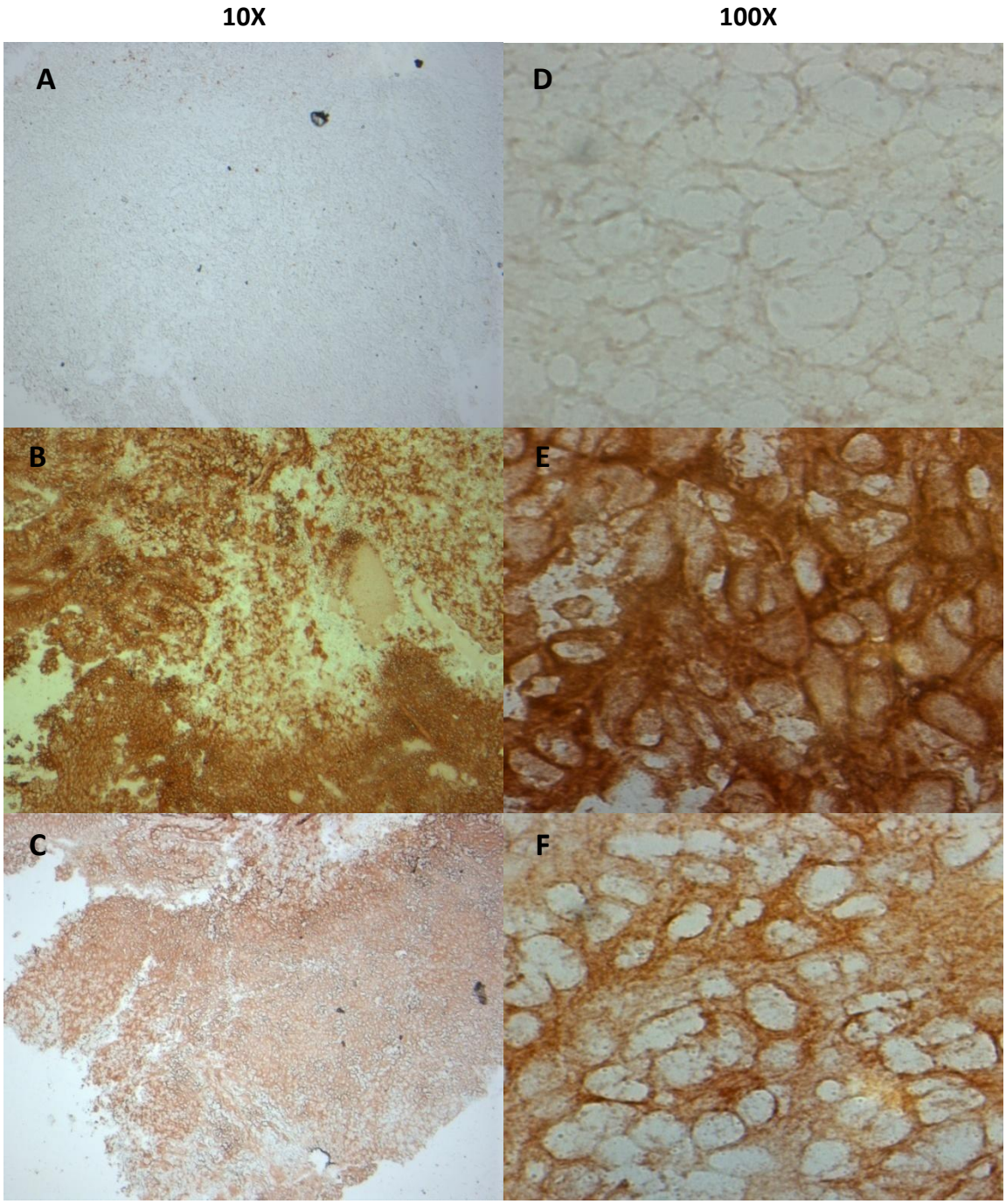


Figure 3.20 Immunohistological Staining Of Nasopharyngeal Carcinoma (NPC) Biopsy With The Monoclonal.

Sections B and E were stained with BB7.2, an antibody specific for HLA-A2 while sections C and F were stained with anti-HLA-A201/EBNA-1. Positive staining was observed for both as seen by the reddish brown colour. Anti-HLA-A201/EBNA-1 can bind to HLA-A201/EBNA-1 complexes on infected tissue samples. Sections A and D were control slides stained with secondary antibodies only. (Done by Song Zhenying)

Discussion

Chapter 4

It is now well established that tumour cells express antigens that can be recognised by cytotoxic T lymphocytes (CTLs) derived from cancer patients (Renkvist *et al*., 2001; Rosenberg 2001). These CTLs recognise antigens that are in complex with MHC class I molecules. However, several studies have shown that using CTL for immunotherapy may not work in a clinical setting (Perez-Diez and Marincola, 2002; Huang *et al*., 2007). To enhance the immune response, a high affinity antibody that can mimic the specificity of the T cell and specifically recognise MHC/peptide complexes represents an attractive alternative strategy (Figure 4.1).

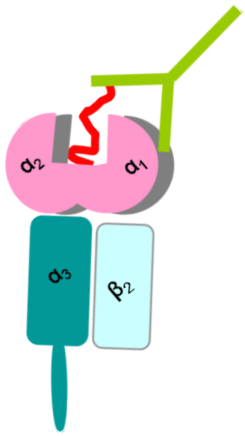


Figure 4.1 TCR-like Monoclonal. The monoclonal (green) recognises the MHC (pink) and peptide (red) complexes.

In our study, we have generated a TCR-like monoclonal antibody which targets EBNA-1 in the context of HLA-A201. EBNA-1 is a latent protein of EBV and it is a DNA binding protein that is expressed in all EBV-infected cells. Using a novel technique of pre-selecting the hybridoma cells using biotinylated monomers, we have demonstrated that this step is crucial to increase the probability of obtaining a MHC/peptide specific antibody. This antibody was also shown to be monoclonal with a single IgG1 heavy chain and K light chain.

In contrast to low affinity T cell receptors (Davis *et al*., 1998), our monoclonal has a higher affinity and yet is able to maintain T cell like specificity. The affinity of our monoclonal is comparable to that produced in other labs. A HLA-A2 monoclonal specific for Mage3, produced by Bernardeau *et al.* has an affinity of 2.37×10^{-9} (Bernardeau et al., 2005) while another HLA-A201 monoclonal specific for eIF4G has an affinity of 6×10^{-9} (Weidanz et al., 2007).

The monoclonal exhibits a specific binding pattern and binds only to HLA-A2/EBNA-1 complexes and not to other HLA-A201 restricted peptide complexes. Our monoclonal is also sensitive enough to detect peptide complexes on the cell surface at a concentration of 0.01 µM by flow cytometry.

In addition, our monoclonal is able to detect constitutive levels of HLA-A201/EBNA-1 complexes on the surface of the BLCLs, including tumour cells from Hodgkin's lymphoma and acute lymphoblastic leukaemia. Using immunohistochemical methods, we have also shown the ability to detect HLA-A201/EBNA-1 complexes in NPC biopsy samples. Our monoclonal is thus able to detect MHC/peptide complexes after processing of antigens. Hence, it may be useful as a tool to detect and analyse tumour-specific antigens and study antigen presentation in EBV related diseases.

Currently, some diseases associated with EBV include Hodgkin's lymphoma (HL), Burkitt's lymphoma (BL) and nasopharyngeal carcinoma (NPC). Although other groups have developed similar MHC/peptide specific monoclonal antibodies, none have been specific for EBV peptides and they have not been used for diagnostic or therapeutic purposes.

Hence, our monoclonal could potentially be applied in a clinical setting both for diagnostic and therapeutic purposes. In Singapore, the incidence of NPC among Chinese ranks among the highest in Asia. It is also among the top ten cancers in men in Singapore (Trends in Cancer Incidence in Singapore 2001-2005, Singapore Cancer Registry). This monoclonal could be used to detect EBV infected cells *in situ* without the need for biopsies to be carried out. This could involve tagging the monoclonal with a fluorescent label and viewing infected cells via *in vivo*

whole animal imaging technology such as the xenogen system. This would be useful for diseases such as NPC where the lesions may be situated in spots that are hard to reach and biopsy. This would also aid in the early diagnosis of diseases and lead to better prognosis and treatment.

In a therapeutic setting, humanised versions of these monoclonals can be applied to all cells that have the EBV target protein and the right HLA type. Cytotoxic drugs and radionuclides could be tagged to the monoclonals and used to target specific tumour cells. The monoclonals could also be used to identify patients with a specific tumour peptide complex before attempting therapeutic vaccinations and following that, to track the survival of cells lacking the target antigen (Porgador *et al*., 1997).

An important consideration for the effectiveness of the use of the monoclonal in a therapeutic setting is the concentration and density of the target peptide complex in the tumour cells. To overcome this problem, we can use a combination of a panel of antibodies targeted at different EBV proteins. Previously, our laboratory has generated monoclonals targeted at other EBV proteins such as latent membrane protein 1 (LMP-1) and latent membrane protein 2 (LMP-2). Two other approaches to increase the sensitivity of the antibody involve antibody engineering strategies. Firstly, the avidity of the antibody can be enhanced by making it multivalent and tagging it with a fluorescent probe. This has been shown by Cohen *et al*. to dramatically increase the binding by two logs as compared to the monovalent molecule measured by flow cytometry. In addition, the use of such fluorescent multivalent antibodies requires only a single staining step (Cohen *et al*., 2003).

Secondly, affinity maturation strategies can increase the affinity of the antibody without altering the TCR-like specificity. This involves a combination of L chain shuffling, H chain-targeted mutagenesis and *in vitro* selection of phage display libraries (Chames *et al*., 2002; Chowdhury *et al*., 1999). A combination of these strategies will thus result in antibodies that are more sensitive and specific for therapeutic uses.

Our monoclonal can also be used to quantify specific HLA/peptide complexes on the surface of cells produced by endogenous processing pathways. This can be done by flow cytometry staining and comparing the fluorescent intensity with that of calibration beads with known numbers of fluorescent molecules per bead. This is a simple and straight-forward method to obtain quantitative data on specific HLA/peptide complex expression and may aid in our understanding of certain disease phenotypes. Previously, to quantify HLA/peptide complexes, biochemical isolation techniques were used. This was a laborious and expensive method that produced multiple experimental artefacts. In addition, it could not distinguish between intracellular pools of peptide complexes and those on the cell surface (Cohen *et al*., 2003).

Another use of our monoclonal could be in the area of localising the sites of peptide interaction with the MHC class I molecules and to trace the trafficking of such MHC/peptide complexes in cells by methods such as confocal microscopy. Through this, we would be able to determine the organelles in which the peptides are being loaded onto MHC class I molecules and study these pathways in greater detail.

The use of this monoclonal to study antigen presentation in cells has interesting implications for EBNA-1 as the exact mechanism for EBNA-1 presentation has not been well characterised. As

mentioned earlier, EBNA-1 contains a glycine-glycine-alanine repeat sequence and studies have suggested that this acts as a *cis*-inhibitor to inhibit antigen processing via the ubiquitin-proteosome MHC class I pathway (Levitskaya *et al*., 1995). Hence EBNA-1 peptides are not presented on MHC class I molecules, protecting it from $CD8^+$ responses. However, Blake and colleagues proved otherwise when they isolated some human CD8 T cell clones that recognised EBNA-1 specific peptides (Blake *et al*., 1997).

A study by Lee and colleagues showed that EBV infected cells could directly present EBNA-1 epitopes to T cells via a proteosome/TAP-dependent pathway (Lee *et al.,* 2004). Another study also confirmed the finding that defective ribosomal products (DRiPs) and not full length EBNA-1, were the major source of antigens for endogenously processed EBNA-1 $CD8^+$ T cell epitopes. Treatment of cells with citrate buffer blocked the endogenous presentation of epitopes from newly synthesised EBNA-1 and significantly decreased EBNA-1-specific T cells (Tellam *et al*., 2004).

Using this monoclonal, we may be able to answer questions at a molecular detail not possible previously. The ease with which quantification can be carried out with this monoclonal would also make it an important tool in the study of antigen presentation and processing. This will shed some light on the pathway of EBNA-1 processing in the cell, its location at different time points, its density and pattern of distribution.

The usefulness and widespread application of this monoclonal lies in its target protein EBNA-1 which is a latent protein that is present in all EBV-infected cells. Moreover, HLA-A201 is not only common in Singaporean Chinese but is the most common allele in Caucasians as well. In

Singaporean Chinese, there are many HLA-A2 subtypes and as seen from our data, the monoclonal may be able to detect them in complex with the EBNA-1 peptide as well. To gain a better understanding of the exact amino acids that bind to the monoclonal, an alanine scan could be carried out. This may provide some insight as to the differential binding abilities of the monoclonal to the various HLA-A2 subtypes.

From our study, we have developed a TCR-like monoclonal antibody that is specific for HLA-A2/EBNA-1 using conventional hybridoma techniques with a high degree of specificity. This monoclonal has been shown to bind specifically with high affinity to HLA-A2/EBNA-1 complexes on the surface of EBV transformed BLCLs, tumour cell lines and in NPC biopsies. The monoclonal is also able to detect constitutive levels of HLA-A2/EBNA-1 complexes as seen in EBV transformed BLCLs. Currently, we are in the midst of humanising this monoclonal in collaboration with Prof. David Lane, University of Dundee. Given the well established association between EBV and various human malignancies, the development of this monoclonal will lead to exciting findings as a new EBV laboratory reagent and as a diagnostic and therapeutic tool.

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APPENDIX

Chapter 6

6.1 Reagents for Tissue Culture

6.1.1 R10 Medium

Reagents	Amount (500 ml)
RPMI-1640	500 ml
FBS	50 ml
Pen/strep	5 ml

FBS and pen/strep was added to the RPMI-1640 medium and stored at 4^{o}C.

6.1.2 Freeze Mix

Reagents	Amount
FBS	9 ml
DMSO	1 ml

FBS and DMSO was mixed and stored at -20^{o}C.

6.2 Reagents For Antibody Production

6.2.1 Luria-Bertani Agar (LA)

Reagents	Amount (1L)
Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
Agarose	15 g
RO water	Top up to 1 L

The reagents were dissolved in 1 L of RO water, autoclaved and stored at room temperature.

6.2.2 Luria-Bertani Broth (LB)

Reagents	Amount (1L)
Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
RO water	Top up to 1 L

The reagents were dissolved in 1 L of RO water, autoclaved and stored at room temperature.

6.2.2 Transformation Buffer I (TfBI)

Reagents	Final	Amount (200 ml)
KAc	30 mM	0.589 g
KCl	100 mM	1.49 g
$CaCl_2.H_2O$	10 mM	0.294 g
Glycerol	15% (v/v)	30 ml
$MnCl_2$.2H2O	50 mM	1.62 g
RO water	-	Top up to 200 ml

The reagents were dissolved in 200 ml of RO water, filtered through a 0.22 µm filter and stored at 4^{o}C.

6.2.3 Transformation Buffer II (TfBII)

Reagents	Final	Amount (100 ml)
NaMOPS	10 mM	50 ml
$CaCl_2.H_2O$	75 mM	1.103 g
KCl	10 mM	0.075 g
Glycerol	15% (v/v)	15 ml
RO water	-	Top up to 100 ml

50 ml of MOPS was titrated with 1 drop of 10 M NaOH followed by 1 M NaOH until pH 7.0 was reached. This together with the rest of the compounds were added to 100 ml of RO water, filtered through a 0.22 µm filter and stored at 4^{o}C.

6.2.4 Kanamycin

Reagents	Amount (60 ml)
Kanamycin	60 mg
RO water	Top up to 60 ml

Kanamycin was dissolved in RO water and filtered through a 0.22 µm filter. It was then aliquoted and stored at -20^{o}C.

6.2.5 Isopropyl β-D-I-thiogalactopyranoside (IPTG)

Reagents	Amount (50 ml)
IPTG	11.915 g
RO water	50 ml

IPTG was dissolved in RO water and filtered through a 0.22 µm filter. It was then aliquoted and stored at -20^{o}C.

6.2.6 Dithiothreitol (DTT)

Reagents	Amount (50 ml)
DTT	3.858 g
RO water	50 ml

DTT was dissolved in RO water and filtered through a 0.22 µm filter. It was then aliquoted and stored at -20^{o}C.

6.2.7 Phenylmethylsulphonylfluoride (PMSF)

Reagents	Amount (50 ml)
PMSF	0.871 g
Isopropanol	50 ml

PMSF was dissolved in isopropanol, aliquoted and stored at -20^{o}C.

6.2.8 Pepstatin A

Reagents	Amount (50 ml)
Pepstatin A	50 mg
Ethanol	50 ml

Pepstatin A was dissolved in ethanol, aliquoted and stored at -20^{o}C.

6.2.9 Resuspension Buffer (pH 8.0)

Reagents	Final	Amount (1 L)
Tris-HCl	50 mM	50 ml
Sucrose	25% (w/v)	250 g
NaEDTA	1 mM	2 ml
RO water	-	Top up to 1 L

The reagents were added and pH was adjusted to 8.0. RO water was added to obtain a final volume of 1L. It was stored at 4^{o}C. DTT (10 mM) was added prior to use.

6.2.10 Lysis Buffer

Reagents	Final	Amount (1 L)
Tris-HCl	50 mM	50 ml
Triton X-100	1% (v/v)	10 ml
Sodium deoxycholate	1% (v/v)	10 g
NaCl	100 mM	5.84 g
Sodium azide	0.1% (w/v)	1 g
RO water	-	Top up to 1 L

All reagents were added except Triton X-100 to 900 ml of RO water. Following that, Triton X-100 was added and the buffer was topped up to 1 L. The lysis buffer was stored at 4^{o}C. DTT (10mM) was added prior to use.

6.2.11 DNase I Stock

Reagents	Final	Amount (10 ml)
DNase	1 mg/ml	10 mg
Tris-HCl	20 mM	200 µl
$MgCl_2$	10 mM	100 µl
Glycerol	50% (v/v)	5 ml
RO water	-	Top up to 10 ml

The above reagents were dissolved in 10 ml RO water, aliquoted and stored at -20^{o}C.

6.2.12 Wash Buffer I (pH 8.0)

Reagents	Final	Amount (2 L)
Tris-HCl	50 mM	100 ml
Triton X-100	1% (v/v)	10 ml
Sodium deoxycholate	1% (v/v)	10 g
NaCl	100 mM	5.84 g
Sodium azide	0.1% (w/v)	1 g
RO water	-	Top up to 2 L

All reagents were added except Triton X-100 to 1900 ml of RO water. Following that, Triton X-100 was added and the pH was adjusted to 8.0. RO water was added to obtain a final volume of 2 L. The wash buffer was stored at 4^{o}C. DTT (1mM) was added prior to use.

6.2.13 Wash Buffer II (pH 8.0)

Reagents	Final	Amount (1 L)
Tris-HCl	50 mM	50 ml
NaEDTA	1 mM	2 ml
Sodium azide	0.1% (w/v)	1 g
RO water	-	Top up to 1 L

The above reagents were dissolved in 900 ml of RO water and the pH was adjusted to 8.0. RO water was added to obtain a final volume of 1 L. DTT (1 mM) was added prior to use.

6.2.14 Urea Buffer (pH 6.0)

Reagents	Final	Amount (500 ml)
4-Morpholineethanesulfonic acid (MES)	25 mM	2.44 g
Urea	8 M	240.24 g
NaEDTA	10 mM	10 ml
RO water	-	Top up to 500 ml

The above reagents were dissolved in 480 ml of RO water and the pH was adjusted to 6.0. RO water was added to obtain a final volume of 500 ml. DTT (0.1 mM) was added prior to use.

6.3 Reagents For SDS-PAGE Gel

6.3.1 Resolving Buffer (pH 8.8)

Reagents	Final	Amount (1 L)
Tris base	1.5 M	181.7 g
RO water	-	Top up to 1 L

Tris base was dissolved in 950 ml of RO water and pH was adjusted to 8.8. RO water was added to obtain a final volume of 1 L.

6.3.2 Stacking Gel Buffer (pH 6.8)

Reagents	Final	Amount (500 ml)
Tris base	500 mM	30.3 g
RO water	-	Top up to 500 ml

Tris base was dissolved in 450 ml of RO water and pH was adjusted to 6.8. RO water was added to obtain a final volume of 500 ml.

6.3.3 Ammonium Persulphate (APS)

Reagents	Amount (1 ml)
APS	0.1 g
RO water	1 ml

APS was dissolved in 1 ml of RO water, filtered through a 0.22 µm filter and stored at $4^{o}C$.

6.3.4 Coomassie Blue Staining Solution

Reagents	Amount (500 ml)
Coomassie Blue R250	0.6 g
Methanol	250 ml
Glacial acetic acid	50 ml
RO water	200 ml

Coomassie Blue was dissolved in the above reagents and mixed well.

6.3.5 Destaining Solution

Reagents	Amount (880 ml)
Methanol	400 ml
Glacial acetic acid	40 ml
RO water	400 ml

6.4 Reagents For Immunisation Of Mice

6.4.1 Polyethylene Glycol (PEG)

Reagents	Amount
PEG-4000	4 g
RPMI-1640	6 ml

PEG was dissolved in RPMI and the pH was adjusted with sterile 2 M NaOH til a pink colour was obtained. The solution was then filtered through a 0.22 µm filter.

6.4.2 100x HAT

HAT was purchased as a 50x powder from Sigma-Aldrich, reconstituted with 10 ml of media to make 50x HAT solution and stored at -20^{o}C.

6.4.3 100x HT

Reagents	Amount
Hypoxanthine	136.1 mg
Thymidine	38.7 mg
RO water	100 ml

The above reagents were dissolved in RO water and stored as 5 ml aliquots in -20^{o}C. One aliquot was used for every 500 ml of RPMI-1640 medium.

6.4.4 HAT Medium

Reagents	Amount
RPMI-1640	500 ml
FBS	50 ml
Pen/strep	5 ml
100x HAT	10 ml

6.4.5 HT Medium

Reagents	Amount
RPMI-1640	500 ml
FBS	50 ml
Pen/strep	5 ml
100x HT	5 ml

6.5 Reagents For Flow Cytometry

6.5.1 4% Paraformaldehyde (PFA)

Reagents	Amount
PFA	20 g
1x PBS	500 ml

PFA was dissolved in 1x PBS in a 56^{o}C water bath and stored at 4^{o}C.

6.6 Reagents For ELISA

6.6.1 Sodium Carbonate Buffer (pH 9.6)

Reagents	Amount
Sodium carbonate	0.53 g
RO water	100 ml

Sodium carbonate was dissolved in RO water and the pH was adjusted to 9.6.

6.6.2 PBS/0.05% Tween

Reagents	Amount (1 L)
Tween-20	500 µl
1x PBS	1 L

Tween-20 was added to 1 L of 1x PBS and mixed well.

6.6.3 1% BSA

Reagents	Amount (200 ml)
BSA	2 g
1x PBS	200 ml

6.7 Reagents For Native Gel Electrophoresis

6.7.1 10% Acrylamide Solution

Reagents	Amount
Acrylamide	10 g
N,N-methylenebisacrylamide	2.5 g
RO water	Top up to 100 ml

The reagents were dissolved in 100 ml of RO water, filtered and stored at 4^{o}C.

6.7.2 4x Non-Denaturing Separating Gel Buffer (pH 8.9)

Reagents	Final	Amount
Tris base	1.5 M	18.2 g
TEMED	-	0.23 ml
RO water	-	Top up to 100 ml

Tris base and TEMED was dissolved in 90 ml of RO water and the pH was adjusted to 8.9. RO water was added to obtain a final volume of 100 ml. The buffer was wrapped in aluminium foil to protect it from light and stored at room temperature.

6.7.3 8x Non-Denaturing Stacking Gel Buffer (pH 6.8)

Reagents	Final	Amount
Tris base	0.47 M	5.7 g
TEMED	-	0.46 ml
RO water	-	Top up to 100 ml

Tris base and TEMED was dissolved in 90 ml of RO water and the pH was adjusted to 6.8. RO water was added to obtain a final volume of 100 ml. The buffer was wrapped in aluminium foil to protect it from light and stored at room temperature.

6.7.4 10x Non-Denaturing Gel Running Buffer (pH 8.3)

Reagents	Final	Amount
Tris base	50 mM	6.06 g
Glycine	384 mM	28.8 g
RO water	-	Top up to 1 L

Tris base and glycine was dissolved in 950 ml of RO water and the pH was adjusted to 8.3. RO water was added to obtain a final volume of 1 L.

6.8 Reagents for Western Blot

6.8.1 10x Transfer Buffer (pH 8.3)

Reagents	Final	Amount
Tris base	250 mM	30.3 g
Glycine	1.92 M	144 g
RO water	-	Top up to 1 L

Tris base and glycine was dissolved in 950 ml of RO water and the pH was adjusted to 8.3. RO water was added to obtain a final volume of 1 L. The buffer was stored at 4^{o}C.

6.8.2 1x Transfer Buffer

Reagents	Amount
10x Transfer Buffer	100 ml
Methanol	100 ml
RO water	800 ml

6.8.3 TTBS

Reagents	Final	Amount
Tris	20 mM	20 ml
NaCl	150 mM	30 ml
Tween-20	-	1 ml
RO water	-	Top up to 1 L