The role of idiotype-specific immunity in antigen receptor diversity

by

David George Millar

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Abstract

Lymphocytes express antigen receptors which are formed by re-arrangement of gene segments. Mutations acquired during this process, predominantly in the complementarity determining regions (CDRs), result in generation of non-germline sequences. Through analysing the CDR3 sequence, this study attempts to determine whether editing of the lymphocyte repertoire is present in an HLA-dependant manner.

Data presented demonstrates a decrease frequency of CDR3-derived HLA-A2 binding peptides in HLA-$A2^+$ donors (0.03% (SYFPEITHI) and 0.35% (BIMAS)) compared with HLA-$A2^-$ donors (0.24% (SYFPEITHI, $p=0.01$) and 0.54% (BIMAS, $p=0.19$)). Trends similar to those seen in HLA-A2 were observed in other HLA alleles as well suggesting that there may be a process by which potentially dangerous B cell populations are edited from the B cell repertoire. Similar analysis of the TCR CDR3 did not reveal any such process in all of the HLA alleles tested suggesting that there is no immunoediting of the T cell repertoire.

Simultaneously, this study attempts to determine the processing and presentation of CDR3-derived peptides at the cell surface using lymphocyte antigen receptor models containing CDR3-encoded viral epitopes. The apparent presence of these peptides on the cell surface leads to the hypothesis that antibodies enter the antigen processing pathway and potentially deliver an immunogenic peptide to a target cell. Using antibodies specific for B cells, this study has shown that cells labelled with an antibody-peptide complex are targeted and lysed by cytotoxic $CD4^+$ T cells in a peptide-specific manner. The use of such technology in antibody immunotherapy may be of considerable therapeutic benefit.

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List of Antibodies Used

Target	Company	Clone
CD1a	BD Pharmingen	HI149
CD3	Mabtec	ZL7-4
CD4	BD Pharmingen	RPA-T4
CD8 (FITC)	BD Pharmingen	G42-8
CD8 (PE)	BD Pharmingen	HIT8a
CD19	BD Bioscience	4G7
CD19	Birmingham University	BU12
CD20	BD Bioscience	L27
CD21	Birmingham University	BU32
CD21	Birmingham University	BU33
CD21	Dako	1F8
CD45 (FITC)	BD Pharmingen	HI30
CD45 (APC)	BD Bioscience	2D1
CD70	Birmingham University	BU69
CD74	eBioscience	LN2
CD79a	Serotec	ZL7-4
CD79b	Serotec	ZL9-2
CD80	BD Pharmingen	BB1
CD83	BD Pharmingen	HB15e
CD86	BD Pharmingen	IT2.2
Herceptin	Genentech	N/A
IgG	Birmingham University	A57H
IgG	Birmingham University	R8Z1E9
TCRVβ7	Abcam	TR310
TCRVβ14	Beckman Coulter	CAS1.1.3
TCRVβ17	Beckman Coulter	E17.5F3.15.13

Antibodies produced in University of Birmingham kindly donated by Dr Margaret Goodall.

List of Reagents Prepared In-House

Reagent	Company	Preparation
PBS	Oxoid	One tablet in 100ml deionised H_2O and filter sterilised. NaCl - 8.0g/L, KCl - 0.2g/l, Na_2HPO_4 - 1.15g/L & KH_2PO_4 - 0.2g/L; pH 7.4.
RPMI	Sigma	103.9g in 10 litres deionised H_2O and filter sterilised. Addition of 100 units/ml penicillin, 100µg/ml streptomycin and 2mM L-Glutamine (all Sigma, UK).
TBE	–	108g Tris base, 55g Boric acid, 9.3g EDTA in 1 litre deionised H_2O (all Melford Labs, UK). Dilute 1:10 in deionised H_2O when preparing DNA agarose gel.
PBS Tween (0.05%)	-	250µl Tween 20 (Melford Labs, UK) in 1000ml PBS.
MACS Buffer	-	1ml FCS + 1mM EDTA in 500ml PBS, filter sterilised.

List of Abbreviations

aa	Amino acid
Ab	Antibody
APC	Antigen presenting cell
APC	Allophycocyanin
BCR	B cell receptor
bp	Base pair
BSA	Bovine serum albumin
CD	Cluster of differentiation
cDNA	Complementary DNA
CMV	Cytomegalovirus
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DMSO	Dimethyl sulphoxide
DNA	Deoxyrionucleic acid
dNTP	Deoxyribonucleotide triphosphate
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
HLA	Human leukocyte antigen
HuS	Human serum
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
Kb	Kilobase pairs
LCL	Lymphoblastoid cell line
M	Molar
mAb	Monoclonal antibody
mg	milligram

MHC	Major histocompatibility complex
ml	Millilitre
mM	Millimolar
mRNA	Messenger RNA
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
RNA	Ribonucleic acid
ROC	Receiver operator curve analysis
SMCC	Sulfosuccinimidyl 4-[*N*-maleimidomethyl] cyclohexane-1-carboxylate
TAP	Transporter associated with antigen processing
TCR	T cell receptor
T_H1/T_H2	T helper 1 or 2
U	Units
µg	Microgram
µl	Microlitre
µM	Micromolar

Chapter 1 - Introduction

1.1 The Immune System

The immune system is a complex network consisting of many different cell types and soluble factors. Conventionally, it has been divided into two distinct sections, the innate and the adaptive immune systems, however, there is considerable overlap between these two systems and they function seamlessly in concert with each other.

The innate immune system is often seen as the first line of defence against invading pathogens and consists of cells which recognise proteins and molecules only present on pathogens, termed pathogen associated molecular patterns. It also consists of many soluble factors such as lipid mediators of inflammation (prostaglandins and leukotrienes) and the kinin system (bradykinins), which help to prevent the spread of invading pathogens. Although the innate immune system is very quick to react to the presence of pathogens within the body, sometimes it is not enough to prevent the pathogen spreading throughout the body. Within 48-96 hours after infection, the adaptive immune system begins to play an active role in controlling the invading pathogen. The adaptive immune system consists of soluble factors as well as two distinct cell types, B cells and T cells. The soluble factors mainly comprise of cytokines, small proteins produced by cells involved in signalling and cell maturation, and antibodies - secreted by stimulated B cells. The adaptive immune system is extremely sensitive and specific for invading pathogens, and can recognise either short, processed peptide fragments or whole, unprocessed proteins. As well as differences in the kinetics of the different innate and adaptive immune response, they differ in terms of magnitude, with the adaptive arm able to generate stronger and

faster secondary immune responses, a hallmark of immunological memory. The innate immune system is set before birth and cannot be altered and therefore, every challenge with the same antigen will mount a similar immune response. However, the adaptive immune system has the ability to retain memory B and T cells, generated after the first immune response against the pathogen, which can be called upon the next time the host is infected with the same pathogen. This results in a faster, stronger, and therefore more effective, immune response on subsequent re-exposure to a given pathogen, typically aborting infection.

1.2 Adaptive Immune Responses

1.2.1 B Cells

B cells are generated and mature in the bone marrow and those which develop earliest, typically expressing CD5 and produce IgM natural antibodies, are termed B1 cells. The surface immunoglobulin expressed by these B1 cells is thought to recognise receptors on many common pathogens, but with a lower affinity for their antigen (Hayakawa et al., 1999 and Youinou et al, 1999) and are thought to be less diverse than the other B cells.

B2 cells (termed B cells from here on), which lack CD5 expression, develop slightly later and are the B cells that produce the majority of the IgG, IgA and IgE antibodies found in the body. B cells recognise antigen, either conformational or linear epitopes, through their surface immunoglobulin receptor. Mature, naïve B cells co-express IgM and IgD on the cell surface but can switch their immunoglobulin heavy chain to IgA, IgE or IgG after stimulation with their cognate antigen and before they become memory B cells. Upon activation and maturation, B cells become antibody producing

cells termed plasma cells. The antibody produced by the B cell contains exactly the same specificity as the surface immunoglobulin receptor. There are a few differences between the two including the absence of a transmembrane region and short cytoplasmic tail in the secreted antibody and the presence of a joining (J) chain which links multiple IgM and IgA antibodies into multimeric structures. Antibodies function in many different ways, dependent upon the heavy chain used, to prevent the spread and to aid in the killing of the pathogen.

Antibodies may also limit pathogen infection by binding to pathogen receptors and neutralising secreted toxins. In addition, antibodies potently opsonise invading pathogens leading to killing via phagocytosis or through complement dependent cytotoxicity (CDC). Furthermore, another mechanism to control viral infections is termed antibody dependant cellular cytotoxicity (ADCC) whereby infected host cells are coated by viral-specific antibodies and lead to engagement with cytotoxic lymphocytes such as natural killer (NK) cells.

1.2.2 T Cells

T cells are derived from haematopoietic stem cells generated in the bone marrow which then migrate to the thymus as common lymphoid progenitors. Unlike B cells, the T cell receptor is only expressed in a surface bound form. T cells can be classified according to the TCR receptor family used, either αβ or γδ. αβ T cells recognise their cognate antigen, peptide bound to either MHC class-I or II, through their T cell receptor (TCR) whereas γδ T cells are thought to recognise non-peptidic ligands such as bacterial lipids (Pfeffer et al, 1990) which could be presented by CD1c (Leslie et al, 2002). MHC class-I (Spits et al, 1990), MHC class-II (Bosnes et al, 1990), the

MHC class-I like molecule CD1c (Faure et al, 1990) and the non-classical MHC-I molecules MICA and MICB (Groh et al, 1998) are thought to present non-peptide ligands to γδ T cells. αβ T cells are further classified into groups determined by which MHC class molecule they recognise and which surface co-receptor they express. T cells which recognise peptide-loaded MHC class-I molecules are known as "cytotoxic" T lymphocytes (CTLs) and express the CD8 co-receptor. Those T cells which recognise peptide-loaded MHC class-II molecules are referred to as "helper" T cells (T_H) and these express the CD4 co-receptor. For CTLs, lysis of the target cell can be achieved by degranulation of cytotoxic granules containing perforin and granzymes, or by mobilisation of CD178 (Fas ligand (FasL)) to the surface of the CTL. FasL binds to CD95 (Fas), expressed on the target cell, and causes activation of the caspase pathway leading to apoptosis of target cell (Podack, 1995). $CD8^+$ T cells also secrete a variety of cytokines, such as interferon γ (IFN-γ), which promotes antigen processing in all cells thus preventing the spread of infection (Mosmann et al, 1997).

Helper T cells secrete cytokines and can either help to amplify different components of or down-regulate the immune response when they recognise their peptide bound to MHC class-II. Helper T cells can be further divided into different subsets depending on which cytokines are secreted. T helper 1 cells (T_H1) are generally thought to be pro-inflammatory cells helping to amplify the T cell response and secreting interleukin 2 (IL-2) and IFN-γ. T helper 2 cells (T_H2) secrete IL-4, IL-5 and IL-6 and are thought to be anti-inflammatory cells which help to amplify the humoral B cell response. Regulatory T helper cells (T_{reg}) are thought to suppress activation of other T cells by production of IL-10 and transforming growth factor-β (TGF-β) (Zheng et

al, 2004) as well as by cell-to-cell contact (Takahashi et al, 1998). More recently identified are the T_H17 subset of helper T cells - defined by their production of IL-17, are thought to play a major role in autoimmunity and inflammation (Rouvier et al, 1993; Betteli et al, 2007). The most recent subset of $CD4^+$ T cells, follicular T helper cells (T_{FH}), would appear to play a dominant role in controlling T-dependant B cell immune responses (Velardi et al, 1986; Vinuesa et al, 2005). Although T_H cells are primarily thought to produce cytokines and aid in the generation of the immune response, there are reports that have demonstrated cytotoxic activity from $CD4^+$ T cells (Wagner et al, 1977, Swain et al, 1981, Tite & Janeway Jr, 1984, Nakamura et al, 1986, Williams & Engelhard, 1997 and Appay et al, 2002).

1.2.3 Co-Stimulation of Lymphocytes

Naïve T cells require at least 2 signals to become activated, 'Signal 1' through the T cell receptor and 'Signal 2' via co-stimulatory molecules (Figure 1.1). These signals, which come from professional antigen presenting cells, prevent T cells from becoming non-functional. The best characterised co-stimulation signal is the interaction of CD28 with CD80 (B7.1) and CD86 (B7.2). These have been shown to stimulate the clonal expansion of T cells and blocking CD80 and CD86 using antibodies leads to inhibition of T cell expansion (Gonzalo et al., 2001). After activation, T cells begin to express further co-stimulatory markers including CD154 (CD40 ligand) which helps to drive the expansion of T cells. Interaction of CD154 with its ligand CD40 stimulates the T cell and also stimulates the antigen presenting cell to upregulate co-stimulatory molecules (Grewal et al, 1995). Another set of co-stimulatory molecules are CD137 (4-1BB) on T cells and CD137 ligand (4-1BBL) on the APC. Similar to CD40, interaction of 4-1BB with its ligand produces stimulatory

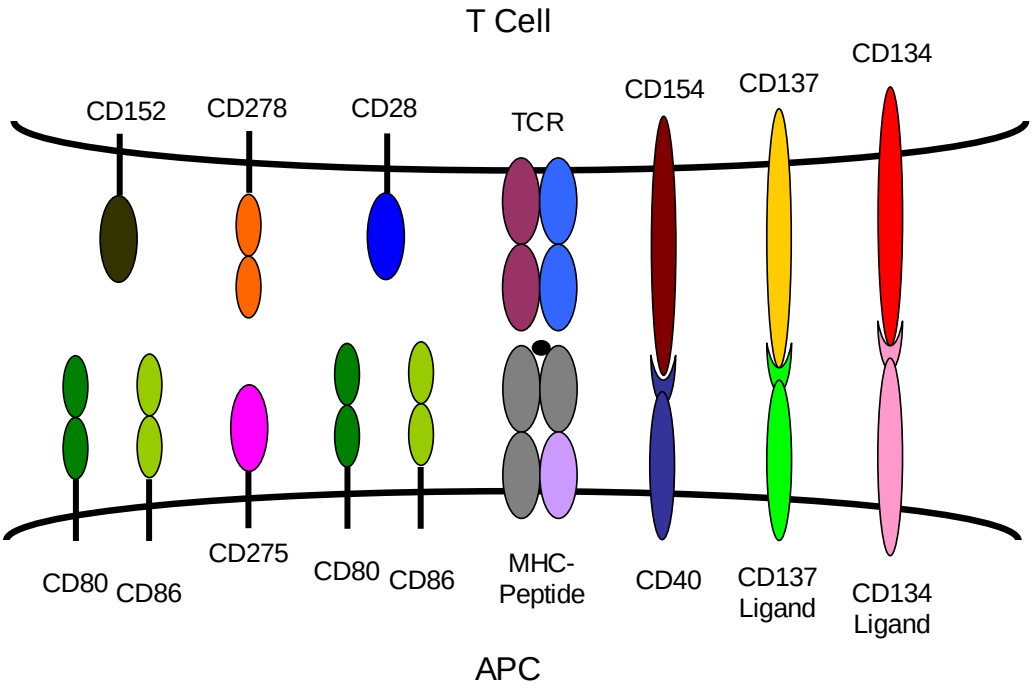


Figure 1.1 Co-stimulation of T cells

Naïve T cells require co-stimulation from professional antigen presenting cells for activation. Engagement of TCR and peptide-MHC is known as signal 1 and the co-stimulatory signals are known as signal 2. Interaction of ligands activates T cells and stimulates the T cell immune response. CD80/CD86 can interact with two different ligands, CD28, an immuno-stimulatory signal, and CD152, an immuno-inhibitory signal. The interaction with CD152 takes place with a much higher affinity and is a mechanism in place to dampen the T cell immune response.

signals for both the T cell and the APC (DeBenedette et al., 1995). A third related co-stimulation protein expressed on T cells is CD278 (inducible co-stimulator (ICOS)) which binds to CD275 (ICOS ligand) on APCs. Interaction of ICOS with is ligand is thought to stimulate T cell growth (Gonzalo et al., 2001). CD134 (OX40), and its ligand CD134 ligand (4-1BB ligand), also participate in promoting T cell activation and survival (Gramaglia et al, 1998).

CD80 and CD86, as well as binding CD28 also bind to CD152 (cytotoxic T lymphocyte antigen 4 (CTLA-4)). CTLA-4 has a similar structure to CD28 but binds to CD80/CD86 with a greater affinity (Green, 2000) and therefore sequesters CD80/CD86 molecules during a T cell immune response. CTLA-4 is known to limit the proliferation of T cells and prevents lymphoproliferative disorders, as seen in mice lacking functional CTLA-4 (Khattri et al, 1999). Absence of co-stimulatory signals can lead to peripheral tolerance through induction of an anergic state.

1.3 Lymphocyte Development

1.3.1 T Cell Development and Selection

T cells are derived from the common lymphoid progenitor which is initially produced in the bone marrow before migrating to the thymus to differentiate further (Figure 1.2 A). Lymphoid progenitor cells enter the thymic cortex as $CD4^-/CD8^-$, double negative (DN) cells. After entry into the thymus, T cell receptor (TCR) β-chain gene re-arrangement occurs along with the pre-TCRα chain. At this stage, only those T cells with in-frame gene re-arrangement will be permitted to continue to develop in the cortex (Raulet et al, 1985). Cells that successfully re-arrange the TCR move deeper into the cortex and begin to express CD4 and CD8 termed double positive

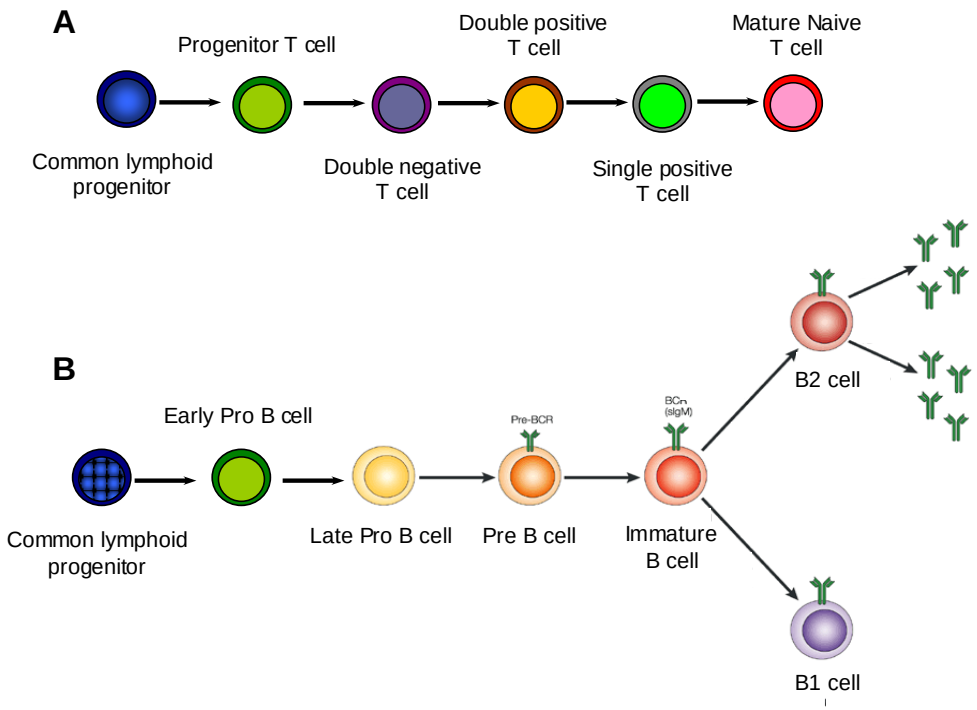


Figure 1.2 B and T Lymphocyte development

B and T lymphocytes develop in either the thymus (T cells) (A) or bone marrow (B cells) (B) (Okkenhaug and Vanhaesebroeck, 2003) from the common lymphoid progenitor. Antigen receptors are produced in many stages which allows continuous testing of the receptor to ensure that only lymphocytes with functional receptors continue to develop. All lymphocytes undergo stringent selection processes to prevent self recognition before they are fully developed. Mature naïve T cells exit the thymus and immature B cells exit the bone marrow to populate the periphery.

(DP) T cells. The newly produced DP T cells now undergo the processes of positive and negative selection, termed thymic education, before they will be permitted to leave the thymus and populate the periphery. There is a remarkable amount of cellular waste produced during the development of lymphocytes and it has been suggested that 97-99% of all T cells generated fail to make it past the selection stage within the thymus (Egerton et al, 1990 and Goldrath & Bevan, 1999). Within the thymus, T cells are highly motile and interact with peptide-MHC complexes on thymic stromal cells, still within the cortex, through their TCR (Bousso et al, 2002). During this stage, known as positive selection, T cells are programmed to die unless they are given survival signals by the stromal cells. T cells which interact with a high affinity for self-peptide-MHC complexes are given signals to die by apoptosis. T cells which do not recognise peptide-MHC complexes do not receive any survival signals and are left to "die by neglect". However, T cells which interact weakly with the peptide-MHC complexes are given survival signals by the stromal cells. These T cells further differentiate to become single positive T cells (SP) depending on which MHC class they recognise, T cells recognising MHC class-I lose CD4 expression and retain CD8 expression whereas T cells recognising MHC class-II molecules lose CD8 expression and retain CD4 expression. After differentiation into SP T cells, they begin to migrate from the thymic cortex towards the medulla where they will undergo the second round of selection, termed negative selection.

The primary function of negative selection is to remove from the repertoire T cells which contain a TCR reactive against a self protein. Allowing self-reactive T cells to escape the thymus could result in the induction of autoimmunity and negative selection mostly prevents this from happening. It has previously been shown that SP

T cells spend ~12 days within the thymic medulla before they are permitted to exit the thymus (Egerton et al, 1990). During this time, the thymic epithelial cells present self peptides in complex with MHC class-I and class-II molecules (Volkmann et al, 1997). T cells which recognise self-peptides in complex with MHC molecules are given signals to die by apoptosis and are therefore deleted from the T cell repertoire. However, T cells which do not recognise any self peptides within the thymus mature in the thymic medulla and can then populate the periphery. Although positive and negative selection is not 100% efficient, very few self-reactive T cells survive and reach the periphery, which helps to prevent the induction of auto-immune reactions.

1.3.2 B Cell Development and Selection

B cells are also derived from the common lymphoid progenitor (Figure 1.2 B) and mature within the bone marrow before migrating to the periphery. B cell precursors were discovered in 1991 (Hardy et al, 1991 and Li et al, 1996) and the earliest cells in the lineage were termed pre-pro B cells. At the stage of pre-pro-B cells, there is no recombination of immunoglobulin genes (Allman et al, 1999) and therefore, early development of B cells would appear to happen independently of the immunoglobulin. During the early stages of development, B cells begin rearranging their B cell receptor beginning with the recombination between heavy chain diversity (D) and junction (J) regions to form a D-J region. Recombination of the variable (V) region to the DJ rearrangement follows to form the V-DJ gene with the B cells now termed pro-B cells (Hardy et al, 1991 and Li et al, 1996). Late pro-B cells are prevented from further development unless they can express a functional heavy chain which can associate with the surrogate light chain to form the pre-B cell receptor (pre-BCR). B cells expressing a functional heavy chain are selected for further growth and

development whereas those cells expressing a non-functional heavy chain or a heavy chain that cannot associate with the surrogate light chain do not receive any further signals and will "die by neglect". It has been suggested that nearly half of all heavy chains produced cannot associate with the surrogate light chain (ten Boekel et al, 1997 & Kenya et al, 1995). The B cells move into the pre-B cell stage after the successful production of the pre-BCR and the production of surrogate light chain ceases (Grawunder et al., 1995). However, it is not known how they enter mitosis and progress down the pathway of their development. During mitosis, the expression of gene rearrangement machinery is downregulated until the cells stop proliferating when it is upregulated again. The light chain loci become accessible and this allows the cells to begin producing light chains that can attempt to associate with the functional heavy chain. During light chain rearrangement, allelic exclusion occurs allowing the expression of only one light chain therefore preserving the single specificity of the B cell. Pairing of the heavy chain with the newly synthesised light chain results in the generation of surface-bound IgM and this is a marker of an immature B cell. Importantly for this study, there is no published evidence of MHC class I protein expression in early B cell development.

It has been suggested that 50-75% of B cells contain a surface IgM that can cross-react with self proteins (Rousseau et al, 1989, Souroujon et al, 1988, Striebich et al, 1990 & Wardemann et al, 2003). Therefore, similar to T cells, B cells must undergo a process of selection to remove the auto-reactive B cells before they populate the periphery. B cells which survive the selection process in the bone marrow become mature naïve B cells and migrate to the peripheral lymphoid system. Previous studies have shown that B cells reactive against self proteins including double-stranded DNA

(Gay et al, 1993 and Chen et al., 1995) and membrane proteins from red blood cells (Okamoto et al., 1992) are deleted whilst still in the bone marrow. From these studies, it has been suggested that for B cells to be deleted in this selection process within the bone marrow, there must be cross-linking of the surface immunoglobulin followed by a strong signal from the immunoglobulin complex. Furthermore, signalling via a single immunoglobulin molecule is not thought to lead to deletion of the B cell (Nemazee & Burki, 1989). It is thought that although the B cell has recognised self protein, there is an intermediate stage in the development where the B cell can be rescued from death, possibly by T_H cells (Hartley et al., 1993). Signalling in the B cell is modulated by CD45, a membrane bound phosphatase, partly by de-phosphorylating the negative regulatory tyrosine kinase (Chan et al, 1994 and Trowbridge & Thomas, 1994). It has been shown that CD45-deficient B cells do not make the transition from immature to mature B cells after the selection process (Cyster et al., 1996).

1.4 Tolerance

Immunological tolerance prevents the recognition of self and therefore prevents autoimmune reactions. Tolerance is achieved through two distinct processes termed central and peripheral tolerance. Central tolerance is achieved during the selection of T cells within the thymus and for B cells by a poorly understood process within the bone marrow. The autoimmune regulator gene (AIRE) is present in the thymus and this regulates the expression of many self proteins in the thymus for expression on MHC molecules. Mutations in AIRE have been shown to lead to defects in the thymic selection process resulting in the peripheral migration of self-reactive T cells (Anderson et al., 2002 & Liston et al, 2003). However, it is unlikely that AIRE can

induce presentation of every possible self-antigen to T cells and therefore, there is the potential for some auto-reactive lymphocytes to escape from the bone marrow or thymus and populate the periphery. This requires further mechanisms to prevent auto-immune reactions and is collectively termed peripheral tolerance.

1.4.1 Peripheral T cell tolerance

In the thymus, T cells undergo positive selection where TCRs which cannot recognise MHC molecules are deleted whereas those which can recognise MHC molecules develop further and begin the process of negative selection. One of the problems with negative selection is the requirement for thymic epithelial cells to express every possible self antigen in complex with MHC molecules so that every possible self-reactive T cell can be deleted

Naïve T cells which recognise their cognate antigen on the surface of a cell, other than a professional antigen presenting cell, which does not express the correct co-stimulatory molecules are not activated but they are found in a state of unresponsiveness, or anergy. The lack of co-stimulation, usually provided by professional antigen presenting cells, means that the peptide recognised by the T cell may be a self-peptide expressed by a healthy cell and therefore the healthy cell is saved due to the T cell becoming anergic. This will then prevent the self-protein specific T cell from circulating and recognising the same peptide presented by another healthy cell, thus, preventing the induction of an auto immune reaction.

Weakly auto-reactive B cells that survived the selection pressure within the bone marrow are excluded from the lymphoid follicles by other, non-auto-reactive B cells

and are rapidly eliminated (Cyster et al, 1994). Further mechanisms of peripheral tolerance include generating a state of immunological ignorance and deletion of circulating T cells. Immunological ignorance occurs when self-protein specific T cells co-exist with their cognate antigen without becoming activated by it and can be a major barrier in immunotherapy. Deletion of self-reactive T cells in the periphery occurs after peptide-specific encounter (Carlow et al, 1992) but a recent study suggests that the induction of tolerance is not dependent on deletion of circulating T cells (Lees et al, 2006).

Peripheral tolerance also mediated by specialised tolerogenic $CD4^+$ T cells known as regulatory T cells (T_{reg}). T_{reg} actively suppress the immune response and help to prevent the induction of autoimmunity although the mechanism by which this is achieved is as yet unknown (Walsh et al, 2004). It may be due to cell-to-cell contact between the T_{reg} and the activated T cell or it may be due to the expression of suppressive cytokines such as TGF-β and IL-10 by the T_{reg} (Zheng et al, 2004) therefore generating a localised suppressive environment which prevents the activation of the T cell. Whatever the mechanism of action, it has been shown previously that a lack of T_{reg} cells results in an increased incidence of autoimmune reactions (Sakaguchi et al, 1995).

One other method of peripheral tolerance is barrier tolerance, whereby T cells are not permitted to enter immunologically privileged sites within the body such as the testis, brain and developing foetus (Janeway et al, 2005). These areas can be protected by several mechanisms including the induction of apoptosis by Fas-FasL interaction (Griffith et al, 1995) and blood-tissue-barrier which has tight junctions between

endothelial cells providing a physical barrier which prevents the T cells from crossing (Cines et al, 1998). All of these methods of peripheral tolerance are present because although the selection of T cells in the thymus is very good, it is not perfect and self-reactive T cells can mature and populate the periphery.

1.5 The T Cell Receptor and Structure

T cell receptors (TCRs) are heterodimeric, membrane bound proteins composed of the α (or γ) chain and the β (or δ) chain with 95% of peripheral blood T cells expressing an alpha-beta (αβ) TCR and the other 5% expressing the gamma-delta γδ TCR (Figure 1.3 A). The αβ T cell subset can be further separated into $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells. The protein chains are divided into the extracellular, transmembrane and (a short) intracellular cytoplasmic domains and demonstrate sequence variation at the amino terminus compared with a highly conserved sequence elsewhere. This difference in sequence variation led to the classification of these regions as variable (V) and constant (C) regions. These regions are similar in structure to the V and C regions which are found within immunoglobulin molecules. The V region of the TCR also contains three protein loops of hypervariable sequence, known as complementarity determining regions (CDR) 1-3.

T cells recognise short peptide sequences in complex with a membrane-bound protein known as major histocompatibility complex (MHC) molecules (Davis et al., 1998). The TCR protein forms loops at the end of which are the three CDR regions which form the antigen binding component of the TCR. The hypervariability of the protein loops, which interact with the peptide-MHC complex, provides the T cell repertoire with its extensive diversity. The CDR1 & 2 regions provide less variability than the

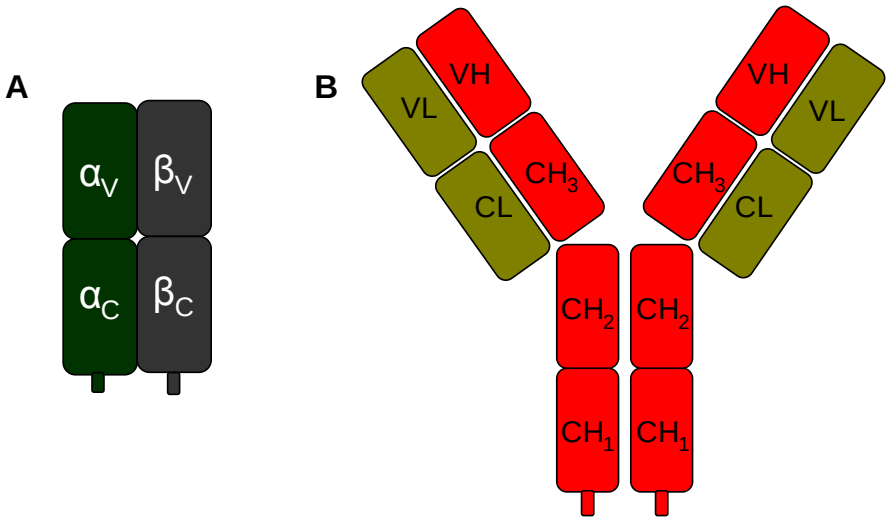


Figure 1.3 Structure of T cell receptor and B cell receptor

The T cell receptor (A) consists of 2 protein chains, the α and β chains. Both proteins are divided into two distinct domains depending on their sequence, the variable and constant regions. The variable region from the α and β chains forms the peptide-MHC binding and idiotype regions. The B cell receptor (B) consists of 4 protein chains, 2 identical heavy chains and 2 identical light chains. The heavy chains are formed of 4 domains, 3 constant domains and 1 variable domain, whereas the light chain consists of only 2 domains, 1 constant domain and 1 variable domain. The combination of the variable domains from both heavy and light chain form the antigen binding domain and idiotype region. Both receptors have a very short cytoplasmic and intracellular region as well, which is absent on the secreted form of B cell receptor (immunoglobulin).

CDR3 region and it is known that normally, the protein loops encoding the CDR1 & 2 regions largely bind to the MHC molecule (Wu et al, 2002). Accordingly, the CDR3 protein loop interacts and mediates recognition of the peptide whislt the CDR1 and 2 protein loops mediate binding to the polymorphic HLA alleles. $CD8^+$ T cells recognise short peptides presented by MHC class-I molecules, which are expressed on the surface of all nucleated cells. $CD4^+$ T cells recognise peptides which are slightly longer and are complexed with MHC class-II molecules. MHC class-II expression is restricted to professional antigen presenting cells such as dendritic cells, macrophages and B cells, although many cell types will also express class-II if exposed to IFNγ (Loughlin et al, 1993; Mach et al, 1996). TCRs interact with the protruding loops of the MHC molecule as well as the peptide presented and therefore are MHC restricted as well as peptide specific.

The TCR bears a very short cytoplasmic domain, insufficient to mediate signal transduction. Therefore, the TCR associates with the CD3 complex of proteins (Figure 1.4 A) that allow signal transduction. The complex consists of CD3γ, CD3ε, two molecules of CD3δ and a homodimer of a disulphide linked CD3ζ chain (Tunnacliffe, 1998). The CD3γ chain associates with one of the CD3δ chains and the CD3ε chain associates with the other CD3δ chain. Upon TCR ligation and either CD4 or CD8 signalling, an immune synapse forms between the T cell and the target cell. Co-stimulatory molecules (Figure 1.1) are recruited to the synapse and CD45 (tyrosine phosphatase) is excluded from the synapse due to its large size (Graf et al, 2007). Aggregation of the receptor complex within the immune synapse after TCR engagement leads to downstream signalling. Lck kinase is recruited to the complex (Iwashima et al, 1994; van Oers et al, 1996) and phosphorylation of the immuno-

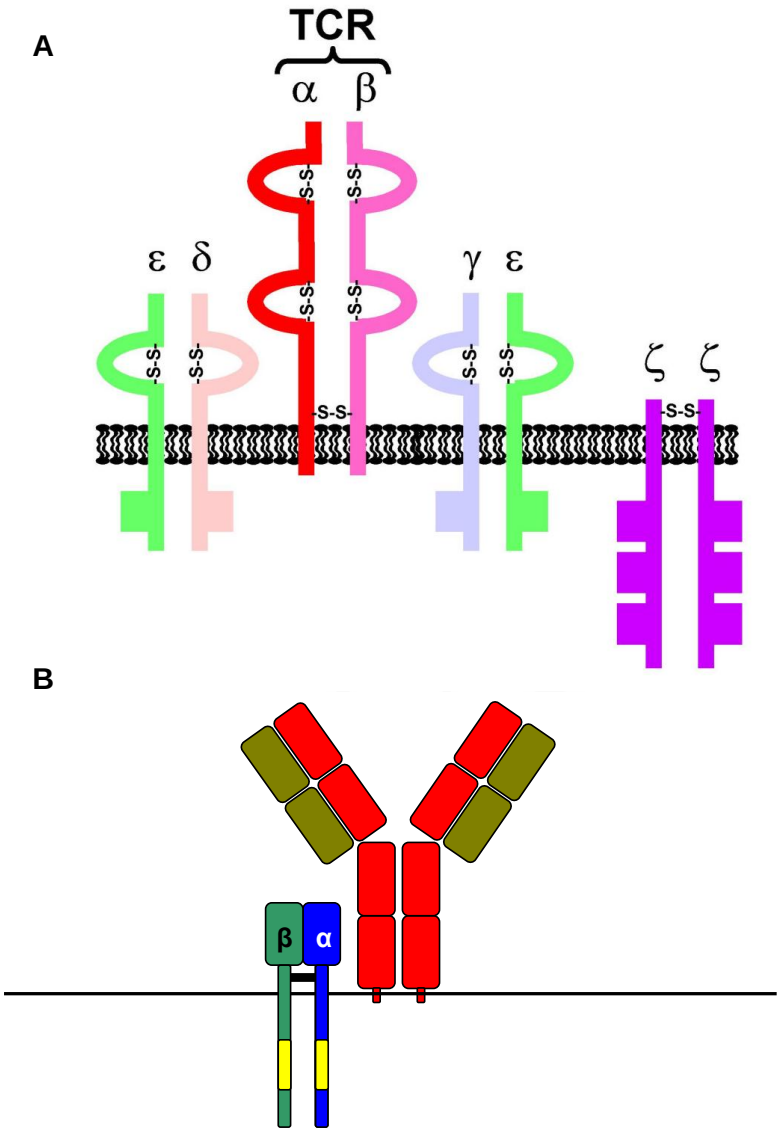


Figure 1.4 Signalling complexes from B and T lymphocytes

The CD3 complex (A) (de Felipe, 2004), consisting of CD3γ, CD3ε, two molecules of CD3δ and a homodimer of a disulphide linked CD3ζ, enables T cell signalling after TCR engagement. Downstream phosphorylation of the immuno-tyrosine activating motif leads to activation of the T cell. The signalling complex found on B cells (B) consists of an Igα chain linked by a disulphide bridge to the Igβ chain. Each of these proteins contains a single immuno-tyrosine activating motif (ITAM, yellow) that enables signalling when the B cell receptor has been ligated with antigen.

tyrosine activation motifs (ITAMs) contained within cytoplasmic tails of the CD3 chains occurs (Samelson & Klausner, 1992), leading to activation of the T cell. The removal of CD45 from the immunological synapse is important as the phosphatase activity of this molecule would prevent the kinase activity of Lck, preventing activation of the T cell.

1.6 The B Cell Receptor and Structure

Immunoglobulin can exist as either membrane bound or secreted protein with the only difference being the presence of a transmembrane and short cytomplasmic region at the C-terminus on the membrane bound form (Wall & Kuehl, 1983). Although the cytoplasmic tail of the Ig consists of only 3 amino acids (lysine-valine-lysine) (Reth et al, 1987), it has been demonstrated that the tail is very important in the signalling of IgM (Parikh et al, 1991). The immunoglobulin is a tetrameric complex formed from four protein chains with two identical heavy chains bound to two identical light chains (Kabat, 1976) (Figure 1.3 B). The heavy chains are formed of 4 distinct domains with variability seen in the domain closest to the amino terminus, termed the variable (V) region, with little variability seen in the 3 domains following it, termed the constant (C) regions. The light chains are formed of two distinct domains with a variable region at the amino terminus and a constant region at the carboxy terminus. The variable regions from both chains combine to produce the antibody binding region. Akin to T cells, the variable regions contain 3 hypervariable regions (CDR1-3) within them. These regions generate the area of the antibody that specifically binds to the antigen by forming protein loops that are extended at the leading edge of the protein. The extreme variability of these regions leads to the diversity seen in the antibody repertoire. The heavy chains can be divided into 5 isotypes depending on their use of

constant regions, Igα (IgA), Igδ (IgD), Igε (IgE), Igγ (IgG) and Igμ (IgM). There are 2 further sub-classes of IgA and 4 further sub-classes of IgG with each of the different sub-classes resulting in different effector functions. The light chains are derived from two different isotypes, either Igκ or Igλ. Mature naïve B cells express both IgM and IgD at the cell surface, however, after activation of the B cell they can undergo a process known as class-switching in the germinal centre (Dong et al, 2001) whereby the antibody expresses a different heavy chain, IgA, IgG or IgE. Activated B cells lose expression of IgD and mature further to plasma cells which can produce large amounts of antibody. Immunoglobulin of the IgA isotype can be found as a dimer linked by the joining (J) chain and immunoglobulin of the IgM isotype can be found as a pentamer also linked by a J chain (Koshland, 1975).

Similar to the TCR, surface-bound immunoglobulin contains a very small cytoplasmic region which cannot mediate signal transduction. The surface Ig is associated with two molecules Igα (CD79a) and Igβ (CD79b) which can transmit the activation signals the cell requires (Torres et al, 1996) (Figure 1.4 B). The Igα and Igβ molecules exist as a disulphide-linked heterodimer and they contain a longer cytoplasmic tail which encodes for ITAMs, similar to those found on CD3 (Samelson & Klausner, 1992). Downstream signalling by phosphorylation of the ITAMs on Igα and Igβ leads to activation of the B cell. Improper activation of B cells, without the co-stimulation of Igα and Igβ, can lead to anergy (a state of unresponsiveness) or apoptosis of the B cell.

1.7 Antigen Receptor Gene Rearrangement

T cell receptors (TCRs) are encoded in germline DNA in gene segments with those encoding the α-chain located on chromosome 14 and those encoding the β-chain located on chromosome 7. These segments encode for many constant (C), variable (V) and joining (J) segments for the α-chain and β-chain and an extra diversity (D) segment within the β-chain (Table 1.1). Similar to the genes encoding the TCRs, the BCRs are encoded in germline DNA within gene segments found on different chromosomes. The immunoglobulin (Ig) heavy chain gene locus is located on chromosome 14 whereas the Ig light chain kappa (κ) locus is found on chromosome 2 and the light chain lambda (λ) locus is found on chromosome 22. There are many gene segments which encode for variable, constant and joining regions for the Ig heavy chain and Ig light chain and, similar to the TCR β-chain, an extra diversity region in the Ig heavy chain (Table 1.2). These gene segments can be utilised to form a full length, functional Ig heavy chain (Early et al, 1980). Although these gene segments are some distance away from each other, they are joined together to form full length antigen receptors (Hozumi & Tonegawa, 1976). It is thought that the arrangement of gene segments for TCRs is at least 60-80 million years old (Klein, J. Immunology, 2nd Ed, 1997).

1.7.1 Lymphocyte Antigen Receptor Re-Arrangement

Formation of a full length antigen receptor occurs by somatic recombination whereby a single gene segment from each region (V, (D), J and C) is joined together with the intervening segments being excised. For example, gene rearrangement for the TCR β-chain begins with the $D_\beta 1$ segment joined to one of 6 $J_\beta 1$ segments or the $D_\beta 2$ segment is joined to one of 7 $D_\beta 2$ segments to form $D_\beta J_\beta$. One of the 48 V_β segments

Locus	Variable	Diversity	Joining	Constant
TCRα	45	0	50	1
TCRβ	48	2	13	2

Table 1.1 Gene segments found in the T cell receptor loci

T cell receptors are generated using gene segments with a single variable, diversity (in the β chain only), joining and constant segments forming a full length TCR. Recombination of gene segments during T cell development produces a functional TCR expressed at the surface of the T cell ready for thymic selection.

Homo sapiens immunoglobulin and T cell receptor genes - Accessed 13 Oct 2010 - IMGT website (http://www.imgt.org/IMGT_GENE-DB/GENElect?Query=4.2+&species=Homo+sapiens)

Locus	Variable	Diversity	Joining	Constant
IgH	50	23	6	9
Igκ	43	0	5	1
Igλ	33	0	5	4-5

Table 1.2 Gene segments found in the immunoglobulin loci

B cell recptors are also generated using gene segments with a single variable, diversity (in IgH only), joining and constant segments forming a full length BCR. Similar to TCR production, recombination of Ig gene segments occurs during B cell development and allows selection of B cells in the bone marrow.

Homo sapiens immunoglobulin and T cell receptor genes - Accessed 13 Oct 2010 - IMGT website (http://www.imgt.org/IMGT_GENE-DB/GENElect?Query=4.2+&species=Homo+sapiens)

is then selected to join the $D_\beta J_\beta$ to form V_β-$D_\beta J_\beta$, with intervening V_β segments excised. The V_β-$D_\beta J_\beta$ is then linked to one of two β-chain constant regions to form the transcript for a full length TCR β-chain (Figure 1.5). The re-arrangement of V, D and J gene segments, and the insertion and deletion of nucleotides that accompanies the re-arrangement, forms the CDR regions within the antigen receptors. Lymphocytes must be able to distinguish between vast numbers of proteins presented to them and therefore it is important that the most variable part of the antigen receptors bind to the antigenic protein/peptide. CDRs 1 & 2, found within the variable region, exhibit a small amount of variability but it is the CDR3 region which has the greatest variability (Gauss & Lieber, 1996). Therefore, antigen receptor diversity is largely generated by the joining of these segments (Weigert et al, 1980). The formation of the full length TCR α-chain and the Ig heavy and light chains occurs in much the same way although the TCR α-chain and Ig light chain do not contain a diversity region.

To produce full length, functional receptors, the gene segments between those selected to form the antigen receptor need to be excised. V, D and J gene segments are flanked by conserved heptamer or nonamer DNA sequences which act as a signal to ensure that gene segments are only joined in a functional manner (Grawunder et al, 1998). The heptamer-spacer-nonamer sequence is known as the recombination signal sequence (RSS) (Sakano et al, 1979) and a gene segment flanked by a 12bp spacer can only recombine with a gene segment flanked by a 23bp spacer (Figure 1.6). The RSS can be recognised by a collection of specific enzymes known as VDJ recombinases, which includes recombination activating genes 1 and 2 (RAG1 and RAG 2). These two enzymes induce DNA cleavage in such a way that all of the

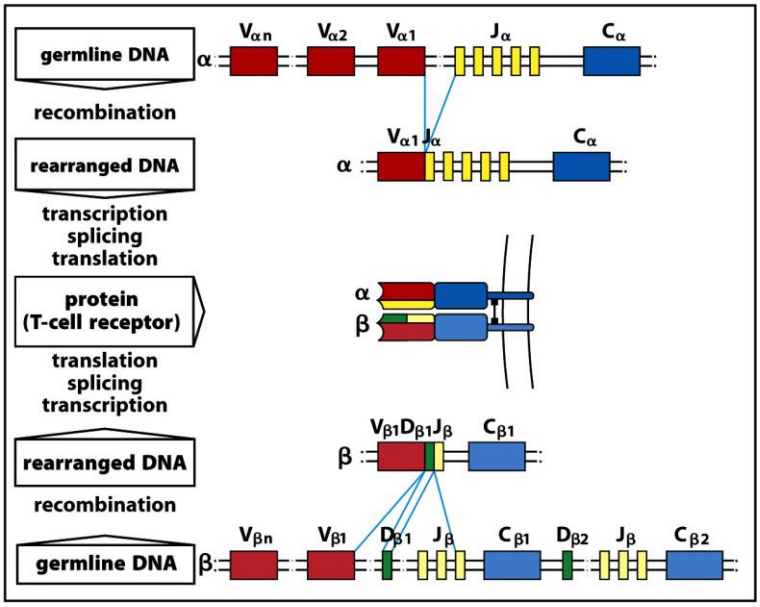


Figure 1.5 Re-arrangement of gene segments to form a full length T cell receptor

One Vα gene segment is joined to a single Jα gene segment with excision of DNA between the two gene segments. The Vα-Jα is then joined to the Cα gene segment to form the full length transcript which is then spliced and translated. The β-chain is very similar with the only difference is the Vβ gene segment is joined to one of the Dβ gene segments before being joined to one of the Jβ gene segments to for the Vβ-Dβ-Jβ recombination. This will then join to one of the two Cβ gene segments to form the full length transcript, which will then be spliced and translated. The two protein chains will then pair together and transport to the surface of the T cell (Janeway et al, 6th Ed).

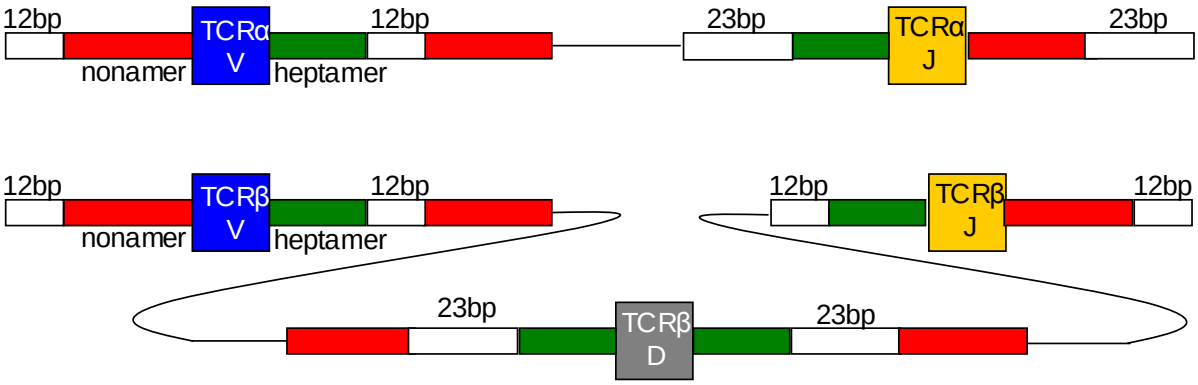


Figure 1.6 12/23 rule in re-arrangement of V, D and J gene segments

The V, D and J gene segments are separated by specific sequences that mean only one single V region, flanked by a recombinant signal sequence (RSS) encoding 12bp can be joined to one single J region, flanked by a RSS encoding for 23bp, for TCRα. For TCRβ, one V region, flanked by a RSS with 12bp, can be joined to one D region, flanked by 23bp, and one J region, flanked by 12bp. This prevents incorrect gene segment re-arrangement such as a V region joining to a J region in TCRβ because both of these segments are flanked by 12bp spacers. A single V region can only join to a single J region after the V region has been joined to a D region (Adapted from Janeway et al, 6th Ed).

intervening segments are removed and the selected segments are brought together (Oettinger et al, 1990). Cleavage of the DNA causes hairpin loops which can be cleaved by RAG1 and 2 (Shockett & Schatz, 1999) generating DNA overhangs on both strands which form palindromic (P) nucleotides. The addition of further nucleotides onto the DNA overhangs (N nucleotide insertion) is facilitated by the enzyme terminal deoxynucleotidyl transferase (TdT) and cells lacking TdT have a lack of N insertions with a corresponding lack of diversity (Komori et al, 1993). Pairing and ligation of the DNA overhangs occurs with removal of unwanted nucleotides and finally, DNA synthesis fills the spaces on both strands to re-form double stranded DNA (Figure 1.7).

1.7.2 Generating the Antigen Receptor Diversity

The gene segments encoded can generate 2200-2300 different α-chains and between 950-1250 different β-chains, allowing for the production of ~$3x10^6$ receptors when the TCRα and β chains are paired. This figure is far smaller than the theoretical maximum of 10^{12} TCR pairings (comprised of 10^4 α-chains and 10^8 β-chains) (IMGT). Regarding the BCR, the gene segments alone could generate 5200-6300 different Ig heavy chains, 155-175 different Ig kappa chains and 116-165 different Ig lambda chains, therefore generating a potential maximum of $2x10^6$ antibodies. Similarly to TCRs, the number of potential antibodies is far below the actual number of 10^{12} antibodies thought to be the theoretical maximum (consisting of 10^7 IgH chains, $6x10^4$ Ig lambda chains and $4x10^4$ Ig kappa chains) (http://imgt.cines.fr).

One of the reasons for the increase in this theoretical number of antigen receptors is N region insertions which slightly alters the amino acid sequence of Ig/TCRs and

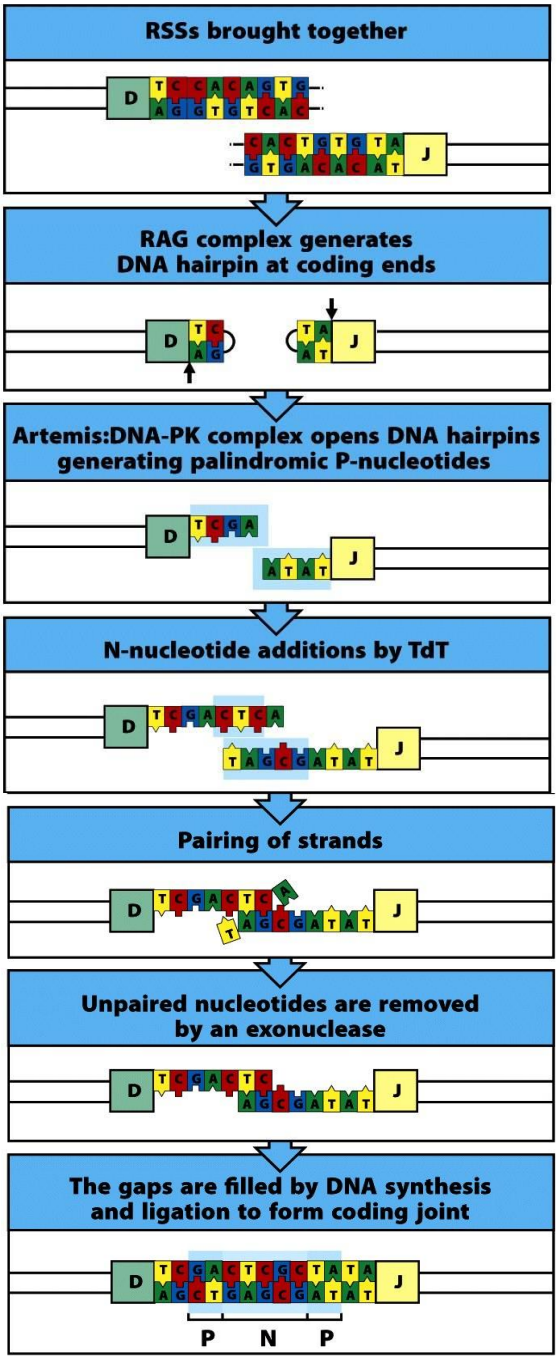


Figure 1.7 Generation of N and P insertions

Recombination signal sequences are brought together and RAG1 and 2 generate hairpins at both ends of the DNA. Cleavage of the hairpin loops generates palindromic (P) nucleotide overhangs with further insertion of N-nucleotides by terminal deoxynucleotidyl transferase (TdT). The additional nucleotides pair together and the DNA double-strand is reformed by synthesis and ligation of nucleotides (Janeway et al, 6th Ed).

therefore alters the specificity as well. The actual number of functional antigen receptors present in the lymphocyte repertoire has not been identified although estimates suggest there are around $25x10^6$ unique, functional TCRs (Arstila et al., 1999) and a similar number of antibodies as well. This is much lower than the theoretical maximum, although both antibodies and TCRs will be lost due to protein misfolding and deletion in the thymus or bone marrow for receptors which recognise self proteins. The insertions and/or deletions mean that the DNA encoding these receptors is non-germline and therefore could potentially be immunogenic.

1.7.3 Other Mechanisms for Generating Diversity

T cells cannot mutate their T cell receptors as the mutations may result in the TCR losing specificity for the MHC molecule or peptide. Therefore, after generation by gene re-arrangement, the sequence of the TCR is fixed and absolute (Davis & Bjorkman, 1988). However, B cells are able to mutate the sequence of their antigen receptor in an attempt to generate antibodies with a higher affinity for the antigen in a process termed somatic hypermutation.

Somatic hypermutation occurs after B cells have become activated and requires T cell help (Lenschow, Walunas, & Bluestone, 1996). Point mutations are introduced amongst the variable region of the heavy and light chains but not amongst the constant region of either chain (Mckean et al., 1984). The mutations cause changes to the interaction between antibody and antigen, allowing the B cell to produce antibodies with a higher affinity for the antigen. This gives rise to the phenomenon known as affinity maturation. Although the end point is an antibody with a higher affinity for antigen, there will be many mutations that cause the antibody to recognise

the antigen with a lower affinity or will abolish recognition completely. These B cells will be deleted whereas those with a higher affinity will differentiate further. The mutations that cause somatic hypermutation are not completely random and are concentrated within "hotspots" throughout the V region, including the CDRs (Betz et al, 1993).

1.8 MHC-Peptide Complexes

1.8.1 MHC Class-I Structure

MHC class-I-peptide complexes consist of a 45kDa glycosylated transmembrane protein heavy chain, which is non-covalently bound to a 12kDa soluble protein, beta-2-microglobulin (β_2m), and a small peptide (usually 8-10 amino acids in length) (Figure 1.8 (A)). The MHC heavy chains are located on chromosome 6 at 3 different loci named human leukocyte antigen (HLA) -A, -B and -C. They are highly polymorphic with more than 300 different heavy chains known, 80 alleles for HLA-A, 180 alleles for HLA-B and 40 alleles for HLA-C (Bodmer et al., 1997). Every individual expresses six HLA alleles, two from each locus. The MHC class-I heavy chain protein is made up of 3 domains (α_1 - α_3) which, when folded produces a groove containing six pockets (A-F), into which a small peptide can bind (Figure 1.9 A). Every MHC class-I allele contains a slightly different binding groove which can accommodate a restricted set of amino acid side chains depending on which allele is expressed (Falk et al, 1991). Therefore, allele specific peptide binding motifs can be designed depending on which amino acid residues bind to the different pockets. HLA-A*0201 is a MHC class-I allele which has been extensively studied and has a peptide binding motif containing a leucine or isoleucine residue at position 2 and a valine or leucine residue at position 9 (Drijfhout et al, 1995). MHC class-I

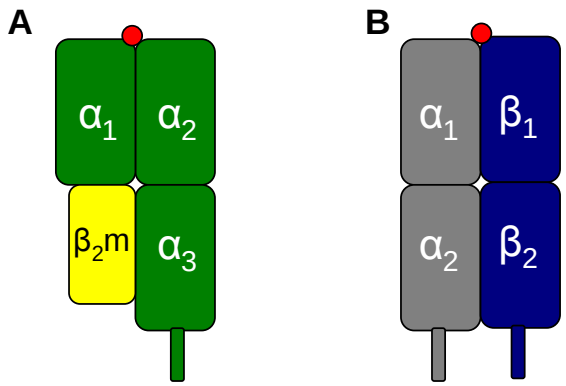


Figure 1.8 Structure of MHC class I and II

MHC class I molecules are composed of 2 disulphide-linked protein chains and a bound peptide (A). The heavy chain consists of 3 domains, α_1-α_3 (Green), with the peptide binding groove made from both α_1 and α_2 domains. The α_3 domain interacts with β_2 microglobulin (β_2m, Yellow) and once completely formed, the β_2m and peptide (Red) stabilise the heavy chain in the correctly folded conformation. MHC class II molecules are also composed of 2 disulphide linked protein chains and a bound peptide (B). Each of the 2 chains consists of two domains α_1-α_2 (Grey) and β_1-β_2 (Blue) with the peptide binding groove formed from the α_1 and β_1 domains containing the peptide (Red).

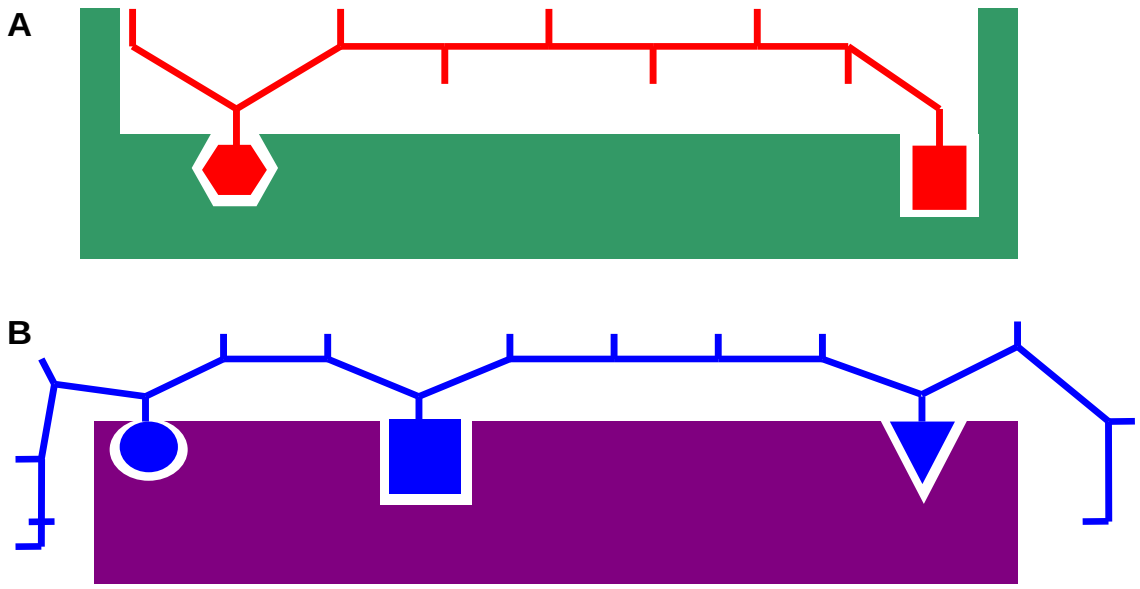


Figure 1.9 Peptide binding to MHC class I and II

Peptides are bound to MHC molecules through interactions of the amino acid side-chain of the peptides with pockets in the peptide-binding cleft in the MHC molecule. The peptide binding cleft in MHC class I molecules (A) is closed at both ends and can only accommodate peptides between 8 and 11 amino acids long. The second amino acid interacts with the first binding pocket and the final amino acid interacts with the last binding pocket. The peptide-binding cleft in MHC class II molecules (B) is open-ended and as a results can bind to peptides which vary in length from 13 amino acids to 25-30 amino acids. The central core of amino acids in each of the peptides binds to the peptide-binding cleft in a similar fashion to the MHC class I binding peptides with the amino acid side-chains interacting with the pockets formed in the MHC class II molecule.

polymorphism results in the differential expression of peptides, from the same antigen, dependent on which MHC class-I alleles are expressed between individuals. Therefore, all protein antigens will contain a large number of antigenic peptides, recognised by the cellular immune response through binding to different MHC class-I alleles. Although the peptide binding groove on MHC class-I molecules is enclosed at both ends, there are peptides longer than 10 amino acids known to bind to MHC class-I alleles (Collins et al, 1995 and Takahashi et al, 1999). Studies using X-ray crystallography have shown that the amino terminal residues still bind to the A-pocket and the carboxy terminal residue still binds to the F-pocket. However, the residues in the middle of the peptide are forced to bulge out of the peptide binding groove due to the limited space available (Guo et al., 1992).

1.8.2 Assembly of MHC Class-I

The MHC heavy chain protein (α_1-α_3) is synthesised in the endoplasmic reticulum (ER) where it is inserted into the membrane. The newly synthesised protein binds to calnexin to aid in the folding process and to prevent the protein from mis-folding and aggregating (Degen & Williams, 1991). Calnexin is not an absolute requirement at this stage of assembly as calnexin-deficient cell lines continue to express MHC class-I at the cell surface (Balow et al, 1995 and Scott & Dawson, 1995). Once folded, the heavy chain protein binds to β_2m and this heterodimer binds to calreticulin, which aids in stabilising and folding of the heavy chain:β_2m complex (Sadasivan et al, 1996). However, MHC class-I molecules can fold correctly in the absence of calreticulin (J. Neefjes & H. Ploegh, 1992) suggesting that this chaperone is also non-essential but may improve efficiency of peptide loading. The heavy chain:β_2m heterodimer interacts with another chaperone protein, tapasin, which cross-links the

heavy chain:β_2m protein complex with the transporter associated with antigen processing (TAP) complex (Ortmann et al, 1994 and Suh et al., 1994). Contrary to the other chaperones in the MHC class-I assembly pathway, tapasin appears to be essential for the correct folding of MHC class-I complexes but not for the folding of any other protein within the ER (Sadasivan et al, 1996). Tapasin is also important in loading peptides with a lower affinity onto MHC class-I (Zarling et al, 2003). By stabilising the peptide-free MHC class-I complex, it allows lower binding peptides more time to bind to the peptide binding groove. Cells deficient in tapasin demonstrate a decreased expression of MHC class-I molecules at the cell surface probably due to the inability of the MHC class-I molecule binding to TAP (Grandea III et al, 1995) with those that are expressed containing a peptide with a high affinity for MHC class-I (Zarling et al, 2003). Therefore, it is thought that tapasin has a similar function in HLA class-I assembly as HLA-DM has in MHC class-II assembly (Brocke et al, 2002). Another chaperone protein required in the assembly of HLA class-I molecules is endoplasmic reticulum protein of 57kDa (ERp57). It is thought that this chaperone is required for assembly and although its function is unknown, it is thought that it either aids in the MHC class-I complex refolding or it may trim longer peptides, which have been transported into the ER via TAP, for loading onto MHC class-I (Hughes & Cresswell, 1998, Lindquist et al, 1998 and Morrice & Powis, 1998). Once bound to TAP, the MHC class-I heterodimer waits to bind to a peptide which has been transported into the ER via the TAP complex. Once loaded, the newly formed MHC class-I heterotrimeric complex is released from TAP (Ortmann et al, 1994 and Suh et al., 1994) and is transported from the ER via the golgi complex to the cell surface. MHC molecules rely on the peptide to stabilise the whole complex and only MHC molecules which contain a peptide and are stable will be transported to

the cell surface. To prevent build up of peptides within the ER, those which are not loaded onto MHC class-I molecules are transported back across the ER membrane back into the cytosol where they are broken down, although this transport is not mediated by TAP (Roelse & Neefjes, 1994). All nucleated cells express MHC class-I molecules on their surface, meaning that an array of endogenous proteins can be expressed at the surface of every one of these cells for surveillance by $CD8^+$ T cells. Under normal circumstances, $CD8^+$ T cells do not recognise any "self" peptides which are expressed by MHC class-I molecules.

Continual expression of MHC class-I molecules, presenting peptides from self proteins, is essential for cells to survive as it has been previously demonstrated that cells lacking MHC class-I molecules on the surface are a target for NK cell mediated lysis (Kaufman et al, 1993). MHC class-I molecules also act as a key component of an immune response as it allows all cells to present non-self (antigenic) peptides on the surface of the cell. This is one of very few mechanisms by which an infected cell can alert the immune response that it is harbouring intracellular pathogens or has become malignant and that it should be targeted for $CD8^+$ T cell mediated lysis to prevent further spread. However, MHC class-I expression is such a vital component of the anti-viral immune response, viruses (and tumours) have developed sophisticated techniques, at every stage of the antigen processing and presentation pathway, to try to down-modulate the expression of viral (antigenic) peptides in complex with MHC class-I at the cell surface. Epstein Barr virus encodes for Epstein Barr nuclear antigen-1 (EBNA-1) which contains a glycine-alanine repeat which interferes with the processing of antigenic peptide and subsequently the expression of peptides (Levitskaya et al., 1995). Cytomegalovirus (CMV) encodes for the

glycoprotein US3 which retains the MHC class-I complex in the ER (Ahn et al., 1996 and Jones et al., 1996), thus preventing expression at the cell surface.

1.8.3 MHC Class-II Structure

There are 3 major families of MHC class-II molecules; HLA-DP, -DQ and -DR which are encoded within the MHC II loci on chromosome 6 (alongside the MHC class-I genes) (Monaco, 1993). MHC class-II molecules are heterodimers of two transmembrane glycoprotein heavy chains, the α-chain of approximately 33-35kDa and the β-chain of approximately 25-30kDa (Brown et al, 1993). The two chains are both divided into four domains, two extracellular domains (α_1-α_2 and β_1-β_2) of 90-100 amino acids each, a transmembrane domain of 20-25 amino acids and a short intracellular domain of 10-15 amino acids (Cresswell, 1994) (Figure 1.8 B). The α_1 and β_1 domains form the peptide binding groove similar to that found in MHC class-I molecules except the groove in MHC class-II molecules is open ended allowing longer peptides to bind to the MHC class-II complex. The longer peptides, 13-25 residues in length (Hunt et al., 1992, Newcomb & Cresswel, 1993 and Rudensky et al, 1991), fall over the edge of the peptide binding groove and hang down the side of the molecule (Figure 1.9 B). The α_2 and β_2 domains each contain an internal disulphide bond which links the two heavy chains (Kappes & Strominger, 1988). The structure of the peptide binding groove of MHC class-II was predicted on the basis of sequence similarities between the α_1- α_2 domains of MHC class-I and the α_1 and β_1 domains of MHC class-II (Brown et al., 1988) with x-ray crystallography confirming the prediction (Brown et al., 1993).

The invariant chain (Ii) is a transmembrane protein 216 amino acids in length with a molecular weight ~31-33kDa. In the absence of MHC class-II molecules, the Ii exists as a trimer although it has been suggested that the Ii exists as a hexamer (Marks et al, 1990). The Ii is thought to bind to the MHC class-II peptide binding groove of free MHC class-II heterodimers, stabilising the protein complex and preventing premature binding of peptide.

1.8.4 MHC Class-II Assembly

The α and β chains of MHC class-II are synthesised in the ER and integrate into the membrane of the ER where the Ii is also integrated after its synthesis. Within hours, three newly synthesised α-chains and three β-chains associate with the Ii trimer to form a nonamer (Lamb & Cresswell, 1992). To prevent peptides from binding to the MHC class-II complex, the peptide binding cleft is filled with a region of the Ii (Riberdy et al, 1992 and Sette et al., 1992). The Ii also appears to have a role in stabilising the MHC class-II complex. In mice, a deficiency in the Ii leads to poor expression of MHC class-II molecules at the surface of the cell and large accumulation of MHC class-II within the ER (Bikoff et al., 1993, Lamb et al, 1991 and Schaiff et al, 1992). The MHC class-II complex is stabilised with calnexin in a mechanism similar to MHC class-I assembly. It is thought that calnexin is important in the association of MHC class-II heavy chains with the Ii trimer (Anderson & Cresswell, 1994). Only when the nonamer has been fully formed is the complex exported from the ER through the golgi complex (Anderson & Cresswell, 1994), where they can then be found in endosomal pathway (Arunachalam et al, 1994, Lamb et al, 1991, Lotteau et al., 1990 and Romagnoli et al, 1993) within the MHC class-II containing compartment (MIIC) (Peters et al, 1991 & Neefjes et al, 1990). In order to

load peptides, first the Ii must be removed from the peptide binding groove. It is thought that this is done by proteolysis of the Ii as addition of chloroquine (a lysomotropic reagent) inhibits Ii dissociation (Nowell & Quaranta, 1985) and prevents MHC class-II expression at the cell surface. The degradation of Ii chain appears to utilise different proteases including cathepsins B, L and S. Cells treated with leupeptin (an inhibitor of cathepsins) caused accumulation of Ii intermediates suggesting that cathepsins are important and that Ii is degraded in stages (Neefjes & Ploegh, 1992, Roche & Cresswell, 1991 and Stebbins et al, 1995). The last piece of the Ii to be removed is the class-II associated invariant chain peptide (CLIP) which occupies the peptide binding groove and is removed by cathepsin S (Riese et al., 1996, Riese et al., 1998 and Villadangos et al, 1997). HLA-DM is a molecule which has a vital role in the exchange of peptides in the peptide binding groove. HLA-DM deficient lines express MHC class-II at the surface of the cell but all of the class-II complexes contain CLIP (Riberdy et al, 1992, Sette et al., 1992 and Mellins et al., 1994). It is thought that HLA-DM stabilises the MHC class-II complex during the removal of CLIP and the subsequent time it takes to load another peptide into the groove (Kropshofer et al, 1997 and Vogt et al, 1996). HLA-DM therefore appears to function as a peptide exchanger of MHC class-II molecules. Once loaded with an exogenous peptide, the heterotrimeric complex can then be transported and expressed at the surface of the cell for recognition by $CD4^+$ T cells.

Whereas MHC class-I molecules are expressed on all nucleated cells, the expression of MHC class-II molecules is much more tightly regulated and only specialised antigen presenting cells (APCs) express MHC class-II molecules. This means that only a select few cell types can initiate a $CD4^+$ T cell immune response to a pathogen.

MHC class-II is constitutively expressed on professional APCs but can be induced on other cell types using IFN-γ (Loughlin et al, 1993 and Mach et al, 1996).

1.9 Antigen Processing

Antigen processing is classically defined in two separate pathways processing either endogenous or exogenous protein.

1.9.1 Endogenous Processing - MHC Class-I

Endogenous proteins are degraded and digested into smaller peptide fragments by the 20S proteasome, a 700kDa protein complex composed of two rings of outer α subunits and two rings of inner β subunits (Groll et al, 1997). The rings form a channel, into which proteins are fed and are then digested into shorter peptide fragments ready for further processing (Löwe et al., 1995). The 20S proteasome is specific and cannot cleave proteins at every residue. It is restricted to cleaving proteins at the carboxy terminus of acidic, basic, hydrophobic, branched chain and small, neutral amino acids (Orlowski et al, 1993 and Cardozo et al, 1994). The length of peptides produced by proteasomal cleavage is very important (Ehring et al, 1996) and can have an effect on whether the peptides are further degraded or not (Dolenc et al, 1998) with peptides constituting 14 amino acids or greater in length are degraded very quick after proteasomal cleavage. Peptides closer to 9 amino acids in length are degraded much slower allowing for the build up of peptides, of an optimum length, for MHC class-I loading. The importance of peptide length after proteasomal cleavage suggests that there is a "molecular ruler", due to the space within the proteasome, which aids in the cleavage of peptides of correct length (Wenzel et al, 1994). During infection, IFN-γ inducible components LMP2 and LMP7 are produced

forming the immunoproteasome (Brown et al, 1991, Glynne et al., 1991, Kelly et al., 1991 and Ortiz-Navarette et al., 1991). The inclusion of the LMP2 and LMP7 subunits slightly alters the peptides generated by cleavage of the proteasome (Stohwasser et al, 1996) and it is thought that there is an increased generation of peptides which contain a basic or hydrophobic carboxy terminal residue (Heemels & Ploegh, 1994, Momburg et al., 1994 & Schumacher et al., 1994). Many peptidases including aminopeptidases (Craiu et al, 1997) and tripeptidyl protease II (TPPII) (Geier et al., 1999) have also been shown to be involved in antigen processing, "nibbling" the amino residues to reduce the length of peptides to around 8-11 amino acids. A large percentage of newly synthesised proteins are thought to be directed towards the proteasome for degradation although there is some debate as to the actual figure with reports suggesting anywhere between 30% (Schubert et al., 2000) and 80% (Reits et al, 2000). Peptides are then transported into the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP) (Monaco et al, 2010 and Spies et al., 1990). TAP is a heterodimer (TAP1 and TAP2), made up of a total of 15 transmembrane subunits, 8 subunits from TAP1 and 7 subunits from TAP2 (Vos et al, 1999). TAP binds to peptides in the cytosol and transports them across the membrane in an ATP-dependant manner (Higgins, 1992), where they can interact with newly synthesised MHC molecules. MHC molecules which do not contain peptides are unstable and as a result are retained within the ER (Demars et al, 1985). There have been previous reports demonstrating that an inherited mutation in one of the two TAP genes results in low MHC class-I expression at the surface (de La Salle et al., 1994), leading to a lower frequency of $CD8^+$ T cells.

1.9.2 Exogenous Processing - MHC Class-II

MHC class-II molecules bind peptides predominantly derived from extracellular or membrane bound proteins internalized by the class-II-bearing cell (Unanue, 1984). The internalised proteins are contained within an endosome/lysosome which is then acidified to break down the proteins. Disruption of the acidification using chloroquine can inhibit the antigen processing and presentation functions within the cells (Ziegler & Unanue, 1982). The acidification of the endosome/lysosome is also thought to facilitate the removal of CLIP (Riberdy et al, 1992), allowing a newly produced peptide to bind to the binding groove. As well as acidification, there are endosomal proteinases, such as cathepsins, which further degrade the protein in the acidic environment. Previous studies have shown that proteinase inhibitors such as leupeptin can also inhibit the antigen processing and presentation pathway (Buus & Werdelin, 1986 and Takahashi et al, 1989). It appears that cathepsins S and L are essential for antigen processing as mice lacking these proteinases demonstrated a restriction in the $CD4^+$ T cell repertoire (Nakagawa et al., 1998, Nakagawa et al., 1999 and Riese et al., 1998) suggesting that there is presentation of peptides in complex with MHC class-II but there is a reduction in the peptides presented. However, mice lacking cathepsins B and D demonstrated a normal $CD4^+$ T cell repertoire (Deussing et al., 1998) suggesting that the activity of these proteinases was not as important. Once the proteins have been fully processed, the peptides are ready to be loaded onto an MHC class-II molecule. Antigens can increase the efficiency by which they are internalised by binding to specific cell surface receptors such as Fc receptors and surface immunoglobulin on B cells or by engaging with complement or pathogen receptors such as TLRs. Internalisation of antigen bound to surface Ig is

seen to increase the efficiency of presentation of the antigen by up to 1000-fold (Lanzavecchia, 1985).

1.9.3 Cross Presentation

The two antigen processing pathways may appear distinct but there is considerable overlap between them. Cross presentation was first discovered in 1976 when allogenic cells cultured together developed CTLs specific for minor antigens. This could only have occurred if cellular material had been "passed" between the cells cultured together (Bevan, MJ, 1976a and Bevan, MJ, 1976b). Further studies managed to determine that cross-presentation occurred only in the professional antigen presenting cells (APCs) dendritic cells and macrophages (Kovacsovics-Bankowski et al, 1993 and Shen et al, 1997) but not B cells (Rock et al, 1993). Later studies attempted to determine how cross presentation occurred, whether it was due to passage of whole protein, peptides or nucleic acids. Immunisation with whole protein antigens fail to initiate a $CD8^+$ immune response (Kovacsovics-Bankowski et al, 1993 and Rock & Clark, 1996 & Falo Jr et al, 1995) whereas peptides from heat-shock proteins appeared to stimulate $CD8^+$ T cells (Udono & Srivastava, 1993). However, it was further determined that whole protein which had been made particulate by adsorption to innate particles (1-5nm) could cause cross-presentation using much lower protein concentrations (Harding & Song, 1994 and Kovacsovics-Bankowski et al, 1993). Therefore, it is now generally accepted that cellular proteins are the predominant form of antigen which is cross-presented. Once the antigen has been internalised by the APC, there appears to be more than one pathway in which the exogenous antigen could be loaded onto MHC class-I molecules. The phagosome-to-cytosol pathway was first described when it was found that APCs lacking

proteasomes or those which were TAP-deficient could not cross present antigen (Kovacsovics-Bankowski & Rock, 1995). This would suggest that the protein had been passed across the phagosomal membrane into the cytosol and would therefore join the MHC class-I classical processing pathway. Another pathway, termed the vacuolar pathway, was discovered when there was cross presentation which did not involve TAP and was insensitive to proteasome inhibitors (Rock, 1996). This second pathway was blocked by the addition of leupeptin (a protease inhibitor) suggesting that the activity of proteases is essential (Shen et al, 2004). The vacuolar pathway involves the presence of molecules involved in the MHC class-I processing pathway in the membrane of a large vacuole within the cells and the fusion of the phagosome with the vacuole (Rock & Shen, 2005). The fusion of these two compartments would bring together the MHC class-I molecules with the protein and peptides required for antigen presentation. Cross presentation would appear to be, potentially, a very dangerous concept as it would allow healthy APCs to present antigen to cytotoxic $CD8^+$ T cells. These cells could therefore be seen as infected and there could be the potential for killing healthy APCs. However, it is now accepted that cross presentation, mediated by $CD8^+$ conventional DCs and precursor $CD8^-$ $CD24^+$ conventional DCs (Bedoui et al, 2009), is an important mechanism for surveillance by the immune system.

1.9.4 Antigen Presentation

Peptides which bind to MHC class-I are usually very specific in their length due to the closed structure of the peptide binding groove with peptides of 8-11 amino acids being able to fit into the restricted space. MHC Class-I molecules are very important in viral and intracellular bacterial infections. It is one of few signals that the cell can

use to highlight itself as infected and therefore should be killed to try to prevent the spread of the pathogen. Cells constitutively express MHC class-I molecules and a failure to do this could result in the cell being seen as infected or transformed and recognised and killed by NK cells. Some viruses including CMV have been shown to down-regulate the expression of MHC class-I on the surface as an evasion mechanism to prevent the cell from being seen as infected.

1.10 Peptide Binding Motifs and Computer Algorithms

MHC molecules are expressed at the cell surface presenting a peptide for T cells to survey. Pockets within the peptide binding groove mean that the MHC molecule selects peptides that can fit the most stably into the groove based on the structure and hydrophobicity of the amino acid side chains on the peptide (Garrett et al, 1989 and Saper et al, 1991) (Figure 1.9). Peptide binding motifs can be determined by isolating peptides from MHC class-I complexes and analysing the peptides that are expressed (Falk et al, 1991 and Jardetzky et al, 1991) or by analysing the sequences that are supposed to bind to MHC molecules (Romero et al, 1991). There are amino acids within almost every peptide which are invariant for each MHC allele, termed anchor residues. The amino acid side chains are inserted into pockets contained in the peptide binding groove which stretch deep into the MHC protein. The anchor residue at position 2 becomes buried deep in pocket A and the residue at position 9 binds to pocket F (Saper et al, 1991). There are other positions within the peptide sequence which are important in MHC-binding and although they are not as important as anchor residues, they are still important in binding and are termed auxiliary anchors (Falk et al, 1991). However, the peptide-binding motifs are not absolute and there are peptides which bind to MHC molecules that do not conform to the peptide-binding

motif (Clerici et al., 1991 and Dadaglio et al., 1991). There are many computer algorithms designed to predict the ability of any peptide to bind to MHC molecules, two of which (SYFPEITHI and BIMAS) are discussed further.

1.10.1 SYFPEITHI

SYFPEITHI (Rammensee et al, 1999) uses a scoring system designed to give each amino acid residue a score between 0 and 10 with optimal anchors given the highest value, sub-optimal amino acids given a low score and amino acids which are disadvantageous can be given a negative score. Using the CMV-derived peptide NLVPMVATV, which binds strongly to HLA-A2 as an example, the leucine residue at position 2 (an optimal anchor) would give the value 10 whereas the sub-optimal residue at position 6, valine, produces a value of 1. The sum score of amino acids at each position gives an overall peptide-binding score, 28 in the case of NLVPMVATV. The algorithm can predict peptide binding for many HLA class-I alleles but not as many HLA class-II alleles because of the more variable peptide binding groove.

1.10.2 BIMAS

The results from BIMAS (Parker et al, 1994) predict the half-time of dissociation of HLA class-I complexes expressing the peptide, at 37°C and pH 6.5 for HLA-A2, and based on the anchor residues expected for other HLA class-I alleles. Initially, the score for each epitope is set to 1 and this is multiplied by the co-efficient of that amino acid at each position along the whole of the epitope. The algorithm is slightly different to SYFPEITHI in the fact that it places more emphasis on the anchor residues with highly favourable amino acids given a co-efficient markedly higher than

1 and the slightly less favourable anchors given a co-efficient of slightly greater than 1. Amino acids at anchor positions which are not well tolerated are given a co-efficient markedly less than 1 and so on. Using the CMV-derived peptide NLVPMVATV as an example again, the optimal anchor residue leucine at position 2 has a co-efficient of 72 whereas the sub-optimal valine at position 6 has a co-efficient of 1. This results in the half-time for dissociation of this peptide on HLA-A2 being 159.97 minutes. Although SYFPEITHI attempts to determine peptide binding for HLA class-II molecules, BIMAS can only predict peptide binding for a limited number of HLA class-I alleles.

One of the major problems using computer algorithms is the reliance on how accurate any of them are. Previous studies have attempted to determine the accuracy of SYFPEITHI and BIMAS by predicting peptides using the computer algorithms and then employing the T2 binding assay to determine peptide binding affinities (Gomez-Nunez et al, 2006). Using SYFPEITHI, the study calculated false negatives at 14% and the false positives at 12% whereas the rate of true positives was measured at 42% and the true negatives measured at 32%. Therefore, the sensitivity and specificity was demonstrated as 75% and 73% respectively. Using BIMAS the study calculated false negatives at 8% and false positives at 8% as well whereas the rate of true positives was measured at 48% and the true negatives measured at 36%. As a result of this, the sensitivity and specificity was demonstrated as 86% and 82% respectively. These results suggest that the BIMAS algorithm is the better of the two algorithms investigated but SYFPEITHI produces similar results as well.

Although the study demonstrates the usefulness of both computer algorithms, it is important to remember the fallibility of both algorithms when analysing any data sets. On average, the programs are 80% accurate and therefore 4 out of 5 peptides will be predicted correctly and therefore this is important to consider when analysing the results and formulating conclusions.

1.11 Idiotype

The idiotype is the collection of unique antigenic determinants expressed within the variable regions of an Ig or TCR molecule. The idiotype was the first known tumour associated antigen (Janeway et al, 1975) and it was suggested that it could act as a tumour-associated antigen for the potential therapy for B and T cell lymphomas (Stevenson & Stevenson, 1983). There have been many studies using the idiotype as tumour associated antigen for a variety of lymphomas with varying degrees of success. However, it was "The Network Theory of the Immune System" published by Niels Jerne, who later received the Nobel Peace Prize for his work, in 1974 that brought the idiotype into the immunotherapy world. Although this theory is not directly relevant to the work shown in this thesis, the idiotype network theory is very important in the history of idiotypic studies and therefore important for this thesis.

1.11.1 Idiotype Network Theory

The network theory of the immune system was first postulated by Niels Jerne in 1974 (Jerne, 1974) which included the presence of an idiotype network theory. This theory suggests that every idiotope, a unique set of antigenic determinants contained within an Ig, can be recognised by a set of paratopes, the antigen binding region of an Ig, and that every paratope can be recognised by a set of idiotopes. Therefore, the expansion

of the B cell repertoire is regulated by anti-idiotypic antibodies which recognise idiotopes from other antibodies. It also encompasses the suppression of B cells by T cells which recognise the idiotopes within the B cell receptor, suggesting that B cells remain functional as long as their idiotype has not been recognised by a T cell. Therefore, B cells and T cells regulate the functional repertoire of B cells. This theory is "contradictory" to the well known and accepted clonal theory whereby the B cell repertoire is edited of self-reactive clones by recognition of self proteins during selection processes. The clonal theory dictates that each lymphocyte clone arises completely independently of all other B cell clones whereas with Jerne's "Idiotype Network Theory", all B cell clones share interactions and these can have an influence on the development of other clones (Bona & Bonilla, 1996).

The network theory is based on the principle that antigen elicits an antibody response which results in production of antibodies against the antigen. The idiotypic regions of the antibody elicit an anti-idiotypic antibody response. This anti-idiotypic antibody contains idiotypic determinants that will then elicit another anti-idiotypic antibody response which will then be continually repeated (Figure 1.10). The immune system is said to be in a dynamic state, even in the absence of immunogenic antigen, with lymphocytes interacting with one another to generate homeostasis and Jerne postulated that this was due to the interaction of idiotopes and paratopes. The network theory was ground-breaking in 1974 and led to further idiotype-based studies and on to its use in immunotherapy.

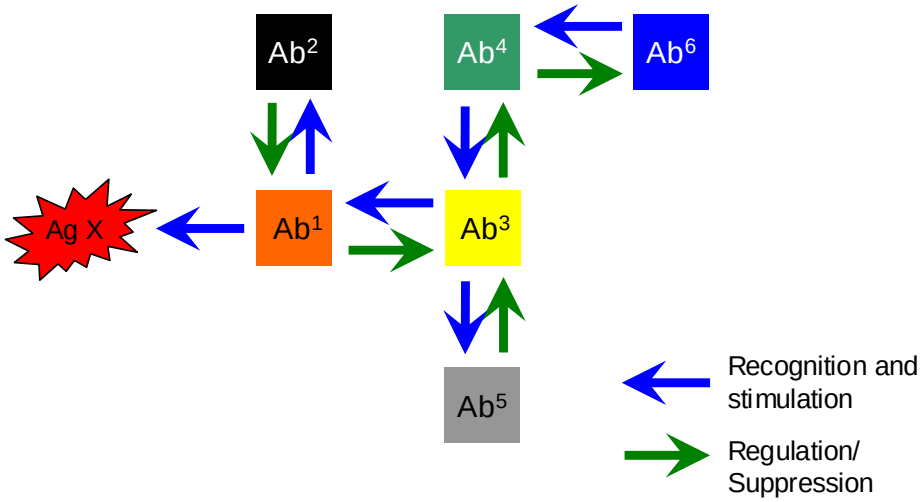


Figure 1.10 B cell idiotype network theory

The network theory of the immune system involves the regulation of B cells by antibodies that recognise idiotopes on different antibodies. Ab^1 recognises antigen X and an immune response is generated. Ab^2 contains an idiotope which is an exact replica of the antigen, recognised by Ag^1, on antigen X and is therefore recognised by Ab^1. The idiotype of Ab^1 is recognised by Ab^3, resulting in regulation of Ab^1 and stimulation of Ab^3. The idiotype of Ab^5 is also recognised by Ab^3 resulting in further stimulation of Ab^5. However, the idiotype of Ab3 is recognised by Ab^4 and this results in regulation of Ab^3 and stimulation of Ab^4. Meanwhile, Ab^4 is recognised by Ab^6, resulting in regulation of Ab^4 and stimulation of Ab^6. (Adapted from Jerne, 1974).

1.12 Immune Responses to Idiotype-Derived Epitopes

1.12.1 T Cell Responses to B Cell Lymphomas

Previous studies have attempted to determine the possibility of raising a T cell response against peptides from the idiotype and also from the BCR as a whole. Generating an immune response against idiotype-derived peptides, being tumour cell specific, was hoped to result in the clearance of tumour cells without targeting healthy non-tumour B cells. However, every B cell tumour will contain a different idiotypic sequence and also may not contain an idiotype-derived epitope suitable for a T cell response. This means that the T cells generated for therapeutic use will be different for every patient and there is the possibility that many patients couldn't be treated. Therefore, in order to broaden the possible applicability of the idiotype-derived peptide therapy, studies have focussed on epitopes from within the framework region of the tumour BCR (Tobin et al., 2003, Ghiotto et al., 2004 and Widhopf et al., 2004). The use of framework region derived epitopes would remove the need for patient-specific T cell responses to be generated. However, the use of these epitopes would then target healthy B cells from the patient B cell repertoire for T cell mediated effects. This may not be as bad as first thought as Rituximab, a monoclonal antibody therapy used in treatment of CLL patients, targets surface-bound CD20 which is found on all B cells, including malignant B cells.

Many studies have used a bioinformatic approach to analyse idiotype sequences for potential HLA-binding epitopes that could potentially be recognised by T cells. Computer algorithms including SYFPEITHI and BIMAS are used to predict HLA-binding epitopes from the idiotype sequence (Harig et al., 2001, Hansson et al, 2003 and Molinier-Frenkel et al., 2005). Many of the studies attempt to alter the peptide

sequence (heteroclitic peptides) to increase the affinity of the peptide for HLA (Harig et al., 2001) and have shown that T cells generated against the heteroclitic peptide can also recognise the native peptide as well as the tumour cell that the sequence was derived from.

1.12.2 Immunotherapy Towards the Idiotype

Targeting the idiotype could lead to potential therapies against many B cell malignancies including B cell chronic lymphocytic leukaemia (CLL), multiple myeloma (MM) and follicular lymphoma (FL). After the idiotype was suggested to be a tumour specific antigen with the potential to treat these patients, the problem of immunological tolerance was raised. Peripheral tolerance could be caused by cells expressing the tumour antigen without the secondary signals required for T cell activation, leading to anergy (Matzinger, 1994).

The goal of immunotherapy is to break tolerance and generate both cellular and humoral responses against the idiotype and for these responses to be long-lasting. The vaccine used and the method of vaccination will have a major effect on the type of response that is generated. It will also have a bearing on whether there is immunological memory to the idiotype or whether the immune response is transient. $CD4^+$ T cells are required to generate long lasting humoral and cellular immunity and as a result, the idiotype protein, which is not particularly immunogenic by itself, was conjugated to a carrier protein that was immunogenic. During the initial studies, the carrier protein was usually keyhole limpet haemocyanin (KLH), a large immunogenic protein composed of many subunits from the keyhole limpet. The KLH would initiate

an inflammatory milieu which would allow generation of an immune response against the idiotype protein.

1.12.3 Early Antibody Immunotherapy

Soon after the idiotype was shown to be a potential therapeutic target, attempts were made to target the idiotype using antibody therapies. Passive immunisation with either polyclonal (Hamblin et al, 1980) or monoclonal (Miller et al, 1982 & Lowder et al., 1987) antibodies was shown to be effective. However, generation of antibodies, either mono- or polyclonal, was costly and laborious as the therapy had to be tailored for each patient. Thus, although the diversity of the idiotype made it a very useful target for therapy, it was also proving to be a significant problem. Ideally, therapy would target a conserved region of the idiotype that was expressed in all patients so as to generate a single therapeutic antibody that could be used to treat a large number of patients. However, the diversity generated in the idiotype region suggests that a broad specificity antibody therapy against the idiotype may not be possible.

1.12.4 Idiotype Protein Vaccination

After the success of the antibody therapies, the immunotherapy studies moved on to active immunisation using idiotype protein in follicular lymphoma (Table 1.3) (Baskar et al, 2004, Weng et al, 2004 and Inogès et al., 2006). Early B cell lymphoma studies in mice using idiotype protein, alone or coupled to KLH, as a vaccine demonstrated its ability to suppress B cell tumours and generate protective immunity (Kaminski et al, 1987 and George et al, 1988). However, it appeared that it was anti-idiotypic antibodies that were induced, and not a cellular response. Furthermore, a study in multiple myeloma demonstrated that immunisation of a healthy donor with

Author (Ref)	Year	Vaccine	Adjuvant	No. of patients	Neoplasms	Anti-Id/tumor immune response (%)		Tumor response
						Ab	T cells	
Massaia et al	1999	Id-KLH	IL-2 or GM-CSF	12	MM	0	22	Progression free range 9 - 36 months
Hsu et al	1997	Id-KLH	various immunologic adjuvants	41	FL	41	17	Two CR patients with residual disease; Progression Free = 7.9 yr in patients with anti-Id response *vs.* 1.3 yr in non-immune
Bendandi et al	1999	Id-KLH	GM-CSF	20	FL	75	95	Molecular remission in 8/11 patients
Barrios et al	2002	Id-KLH	SAF or GM-CSF	9	FL	89	N/A	One CR and one transient PR from four patients with residual disease; molecular remission in 3/5 patients
Baskar et al	2004	Id-KLH	GM-CSF		FL	N/A	48	9/13 patients with FL and 2/10 MM patients produced T cell responses
Weng et al	2004	Id-KLH	GM-CSF	136	FL	35	20	FCγR polymorphisms correlated with clinical outcome
Neelapu et al	2005	Id-KLH	GM-CSF	26	MCL	30	87	N/A
Inoges et al	2006	Id-KLH	GM-CSF	25	FL	52	72	N/A
Redfern et al	2006	Id-KLH	GM-CSF	31	SLL/CLL, FL	20	67	One CR and three PR/31 patients

Id: idiotype; KLH: keyhole limpet hemocyanin; GM-CSF: granulocyte–macrophage colony stimulating factor; DC: dendritic cell; FL: follicular lymphoma; MCL: mantle cell lymphoma; MM: multiple myeloma; DLBCL: diffuse large B cell lymphoma; CLL: chronic lymphocytic leukaemia; SLL: small lymphocytic leukaemia; LPL: lymphoplasmacytic lymphoma; CR: complete response; PR: partial response; N/A: not assessable. Information for table taken from Ruffini et al, 2002.

Table 1.3 Published phase I/II clinical trials of idiotype protein vaccination in B cell neoplasms

Early phase clinical trials using idiotype protein vaccination demonstrates humoral and cellular anti-idiotype responses in the majority of patients treated. Data taken from Ruffini et al, 2002.

myeloma IgG coupled to KLH could generate a cellular Id-specific response, which was then taken from the donor and transplanted into the patient. $CD4^+$ idiotype-specific responses were detected in the patient up to day 60. This study showed that transfer of immunity from an immunised donor to a patient could prove to be an alternative method of generating an idiotype-specific response (Kwak et al., 1995). However, one of the major issues with this therapeutic strategy is the need for an HLA-matched donor and the potential for post-transplant complications. Later studies in MM using the more conventional vaccination with idiotype protein coupled to KLH were completed but were not as successful as the FL (Massaia et al., 1999). One of the potential issues with vaccinating MM patients is the high-dose chemotherapy and stem cell transplant they receive prior to the vaccination. However, the presence of T-cell responses to idiotype and KLH proteins suggested that the timing of vaccination was not a problem.

The actively induced antibody in follicular lymphoma (FL) studies produced protection similar to that achieved by passive immunisation with anti-idiotype antibodies (Brown et al., 1989). Although the outcome of protein vaccination appeared to be the same as passive immunisation with antibody, it was demonstrated that, in the presence of the whole idiotype protein, the antibody response prevented the tumour cell from immune evasion by altering the expression of the idiotype (Maloney et al., 1994). It was further demonstrated that immunisation with idiotype protein could potentially generate $CD4^+$ T cells which have subsequently been shown to be important in the anti-tumour response (King et al., 1998).

The pre-clinical studies in humans and mice, which had yielded encouraging results, led to a clinical trial using idiotype protein coupled to KLH in patients with follicular lymphoma. The results of the clinical trial were encouraging with 20 of 41 patients generating an immune response, predominantly humoral, against the idiotype protein contained in the vaccine (Hsu et al., 1997). Patients generating an immune response demonstrated a significantly longer progression-free disease and overall survival compared with those patients who failed to mount an anti-idiotype immune response.

After further success vaccinating FL patients with idiotype protein coupled to KLH (Bendandi et al., 1999), where 8 of 11 patients converted to molecular remission, three multi-centre phase III clinical trials began (Table 1.4). The first phase III trial to begin was instigated by the National Cancer Institute, National Institute for Health (USA) and is sponsored by Biovest (BiovaxId). This began in February 2000 using idiotype protein, taken from a biopsy sample of the lymphoma, coupled to KLH as a vaccine and KLH alone as control. Results reported at the 2009 American Society of Clinical Oncology suggested that there may be a positive outcome after vaccination with the idiotype vaccine. The median time to relapse was 44 months in patients administered with the vaccine compared with 30 months in patients given placebo ($p=0.045$) (Schuster et al, 2009). The other phase III clinical trials used similar vaccines sponsored by Favrille (FavId; started in 2004) and Genitope (MyVax®; started in November 2000) with the main difference between the trials being the patients recruited. The Biovest trial included patients with stage III or IV FL, Genitope recruited patients with stage II - IV FL and the Favrille trial included patients with stage I - IV FL (All clinical trial information taken from http://.clinicaltrials.gov). The results from the Genitope study have not yet been

	NCI/Biovest	Genitope	Favrille
Patients	177	287	349
Disease	Untreated	Untreated	Untreated/Relapse
Prior Therapy	CHOP	CVP	Rituximab
Patient Eligibility	CR	CR, PR	CR, PR, SD
Vaccine Type	Id-KLH	Id-KLH	Id-KLH
Adjuvant	GM-CSF	GM-CSF	GM-CSF
Primary Endpoint	PFS	PFS	PFS

Id: idiotype; KLH: keyhole limpet hemocyanin; GM-CSF: granulocyte-macrophage colony stimulating factor; CR: complete response; PR: partial response; SD: stable disease; PACE: prednisone, doxorubicin, cyclophosphamide, etoposide; R-CHOP: rituximab, cyclophosphamide, doxorubicin, vincristine, prednisone; CVP: cyclo- phosphamide, vincristine, prednisone; PFS: progression-free survival

Table 1.4 Phase III clinical trials of idiotype vaccination in follicular lymphoma

After successful early phase clinical trials, three phase III clinical trials began using idiotype protein vaccination in patients with follicular lymphoma. Table shows details from each of the three trials with only two of the three trials ongoing following the cessation of the Favrille sponsored trial. Results from the Genitope study are expected soon but results from the Biovest sponsored study demonstrated a slightly longer median time to relapse in patients treated with idiotype vaccination compared with untreated control patients. Information taken from http://clinicaltrials.gov

released but are expected in the near future. However, the clinical trial using FavId (Renamed to SpecifId) was stopped in May 2008, after recruiting 349 patients, after it failed to show a statistically significant increase in the time to progression (Freedman et al, 2009).

1.12.5 Later Protein Vaccination Studies Towards Follicular Lymphoma

During the course of these phase III clinical trials, further FL patient studies have demonstrated similar clinical benefits of idiotype protein vaccination. The logical progression of development of a new treatment is to initiate clinical trials leading from phase I to II and finally phase III. However, there has been doubts raised as to whether this is the correct course for the idiotype vaccine trials because every patient receives a slightly different vaccine due to their idiotype being different from the next patient. It has been known since the earliest studies that idiotypes vary in their ability to stimulate an immune response (Lynch et al, 1972). Therefore, to address this issue in the efficacy of idiotype vaccinations, one study used an innovative method for determining clinical efficacy. It has previously been shown that antibody therapy, using Rituximab, with or without chemotherapy in FL patients can induce complete responses in patients (Czuczman et al., 1999 & Coiffier et al., 2002). However, the 2nd complete response, with a median duration of 13 months, is almost always shorter than the 1st complete response and subsequent responses tend to get shorter and shorter (Rohatiner & Lister, 2005). The study by Inogès et al., 2006 demonstrated a longer 2nd complete response by patients who generated a humoral and/or cellular immune response against the vaccine compared with those who didn't generate an immune response. Interestingly, the study by Inogès et al., 2006 also demonstrated that all patients with an immune response to the idiotype protein had a longer 2nd

response than the median duration (13 months) and it was also longer than the 1st complete response as well. Every patient that failed to generate an idiotype-specific immune response produced similar results to those seen before with a shorter 2nd clinical response. Therefore, these results demonstrate the potential clinical benefit associated with idiotype protein vaccination in B cell lymphoma. The experimental outcome of this study suggests that the endpoint of future clinical trials may be better suited to looking at duration of complete response in patients treated with the idiotype vaccines. The studies described investigate the vaccination of patients after the treatment with chemotherapy generally resulting in minimal residual disease. This would suggest that idiotype specific vaccination is best suited in conjunction with chemotherapy regimens.

The results from vaccination studies using idiotype protein suggest that there is a possibility in producing a humoral and a $CD4^+$ T cell response, which suggests that the problem of tolerance is either not present or can be overcome (Hsu et al., 1997 and Bendandi et al., 1999). It also suggests that there was an immune repertoire present in patients which had failed to prevent disease progression, but could be stimulated using the idiotype protein. The results from the phase III clinical trials are eagerly awaited and may have a large influence on any further work on idiotype protein vaccination.

1.12.6 Dendritic Cell Vaccines

Inducing a primary T cell immune response requires the presentation of antigen by dendritic cells (DCs). There have been many studies determining usefulness of DCs, loaded with idiotype protein, processing and presenting idiotype peptides to T cells.

However, there are a few drawbacks to using DCs as therapy to treat B cell lymphoma. Although DCs are potent stimulators of immune responses, they can also be potent inhibitors as well (Banchereau & Steinman, 1998). They require maturation signals to allow them to stimulate the immune system and it was shown that immature DCs pulsed with antigen resulted in tolerance (Dhodapkar et al, 2001). This may be a major issue in MM where recovery of idiotype protein in multiple myeloma is made easier by the fact that there is a large amount of idiotype protein in patient serum but the presence of idiotype protein, at the time of vaccination, could prove to be tolerogenic (Bogen et al, 2000). Another issue with DCs is the cost and time involved in generating them to a suitable clinical grade to allow infusion into patients. Despite these issues, there have been studies investigating the immune response to idiotype-derived epitopes after vaccination of patients with DCs pulsed with idiotype protein and/or KLH in follicular lymphoma (Table 1.5) (Timmerman et al., 2002) and multiple myeloma (Reichardt et al., 2003). The results from both of the pre-clinical studies demonstrated the generation of anti-idiotype T cells after vaccination using DCs suggesting that there is not a tolerance barrier to overcome. As a result of this, phase I/II clinical trials have begun in an attempt to further study the clinical efficacy of DC vaccines in both follicular lymphoma and multiple myeloma. The phase I/II clinical trial in MM patients (Curti et al., 2007) demonstrated the safety of the idiotype-pulsed DC vaccine and induced idiotype-specific T cell responses in 8 of 15 patients vaccinated. There is another phase I/II clinical trial ongoing to determine the safety and potential efficacy of DC vaccines in B cell lymphomas with the trial predicted to finish later this year (http://clinicaltrials.gov). The results of the pre-clinical studies along with the initial results from the multiple myeloma trial suggest

Author (Ref)	Year	Vaccine	Adjuvant	No. of patients	Neoplasms	Anti-Id/tumor immune response (%)		Tumor response
						Ab	T cells	
Timmerman et al	2002	Id or Id-KLH	N/A	35	FL	26	49	Tumour regression in 10/28 patients
Reichardt et al	2003	Id-pulsed DC followed by Id-KLH Booster	N/A	12	MM	N/A	20	2 patients in clinical partial response after 25 and 29 months; 6 patients died from disease progression or complications
Curti et al	2007	Whole Ig-KLH or Id Peptide-KLH	N/A	15	MM	27	53	6/15 patients received whole Ig and 9/15 patients received Id peptides; 7/15 patients have stable disease at 26 months
Id: idiotype; KLH: keyhole limpet hemocyanin; DC: dendritic cell; FL: follicular lymphoma; MM: multiple myeloma; N/A: not assessable								

Table 1.5 Published phase I/II clinical trials of idiotype dendritic cell vaccination in B cell neoplasms

Early phase clinical trials using dendritic cells pulsed with idiotype peptides as a vaccine demonstrated both humoral and cellular anti-idiotype immune responses in all trials undertaken in patients with multiple myeloma and follicular lymphoma. Information taken from Ruffini et al, 2002.

Author (Ref)	Year	Vaccine	Adjuvant	No. of patients	Neoplasms	Anti-Id/tumor immune response (%)		Tumor response
						Ab	T cells	
Timmerman et al	2002	Heavy and Light Chain	N/A	12	FL	33	33	
Rice et al	2008	scFV-FrC	N/A	25	FL	38		6/16 patients generated cellular and/or humoral response
King et al	1998	scFv-FrC	N/A	15	MM	27	53	6/15 patients received whole Ig and 9/15 patients received Id peptides; 7/15 patients have stable disease at 26 months
Id: idiotype; KLH: keyhole limpet hemocyanin; Ig: immunoglobulinDC: dendritic cell; FL: follicular lymphoma; MM: multiple myeloma; scFV: single chain fragment variable; FrC: Fragment C from tetanus toxin: N/A: not assessable								

Table 1.6 Published phase I/II clinical trials of idiotype DNA vaccination in B cell neoplasms

Early phase clinical trials using DNA vaccination encoding for idiotype protein conjugated to a fragment of tetanus toxin acting as an adjuvant. Humoral and cellular anti-idiotype immune responses are seen in all trials undertaken. Information taken from Ruffini et al, 2002.

that there is a possibility that DC vaccines could be clinically relevant although much will depend on any future data from clinical trials.

1.12.7 DNA Vaccines

DNA vaccines are known to induce both cellular and humoral immune responses against various cancers (Stevenson & Anderson, 1999). The use of DNA vaccines in infectious disease clinical trials have shown encouraging results with vaccination generating few side effects and inducing antigen-specific immune responses (MacGregor et al., 1998 and Wang et al., 1998). The DNA sequence for the idiotype is incorporated into a DNA plasmid backbone which contains a promoter, usually the CMV immediate-early promoter, to drive high level gene expression. Following introduction into the host, the gene of interest is synthesised in the host cells. Also incorporated within the backbone is bacterial DNA, which encodes for pathogen-associated molecular patterns (CpG motifs), which can stimulate the innate immune system, leading to the stimulation of the adaptive immune system (Hoebe et al., 2006).

DNA vaccines can be introduced via 3 routes; intramuscularly, intradermally or into skin cells using a gene gun. Once administered, the DNA is transfected into cells and the synthesis of the gene of interest is initiated. DNA vaccines administered via the intradermal route are thought to transfect skin-derived dendritic cells which then process and present antigen to T cells (Akbari et al., 1999 and Porgador et al., 1998). DNA vaccines administered by intramuscular route are thought to transfect muscle cells. However, muscle cells do not express the MHC class-II molecules and co-stimulatory molecules, required to present antigen to and activate $CD4^+$ T cells. It is

thought that muscle cells "pass" the newly synthesised protein to professional APCs, such as dendritic cells, in a process known as "cross-presentation". The result of this process means DCs can present the antigen to T cells with the required co-stimulation so that they can activate T cells (Corr et al, 1996 and Corr et al, 1999). Although DNA vaccination attempts to induce every arm of the immune system, it predominantly induces $CD4^+$ T helper cells as well as $CD8^+$ cytotoxic T cells (Gurunathan et al, 2000).

DNA vaccines allow optimisation of the protein of interest by addition of sequences that could target the protein to the required cellular location. Leader sequences to target the idiotype protein to the endoplasmic reticulum where it could be secreted to generate a specific antibody response. Otherwise, the protein could be targeted to the cytosol to allow processing in the classical MHC class-I processing pathway (Rice et al, 2008). This targeting of the idiotype protein could prove to be very useful in DNA vaccination but could not be used in protein vaccination.

1.12.8 DNA Vaccines Against Idiotype

The earliest DNA vaccination was developed quickly due to the need to overcome difficulties in generating individual, patient-specific protein vaccines (Hawkins et al., 1994). It is easier to identify the sequence of the idiotype region of the BCR than it is to recover the BCR protein from tumour cells. One of the first DNA constructs to be generated for this work was to encode both the full length IgH and IgL chains under a single promoter. Results from the initial pre-clinical studies showed that this was an effective approach, leading to a clinical trial using this method. However, the results of the clinical trial demonstrated only 1 of 12 patients developed a cellular response

and no patients developed a humoral response after a single vaccination (Table 1.6) (Timmerman, et al., 2002). There was an increased response after a second round of vaccinations with 2 patients producing an antibody response and 4 patients producing a cellular response. However, the responses seen at this stage were not specific and were cross-reactive with Id proteins from the other patient tumours. Despite the modest induction of cellular and humoral immune responses, the clinical trial demonstrated the potential that idiotype-based DNA vaccination could have in the future.

Another construct for DNA vaccination that was developed was the linking of the IgH variable region to the IgL variable region, forming the single chain variable fragment (scFv) (Hawkins et al., 1994). The idiotype protein produced by the DNA construct retained all of the idiotypic determinants as it could still be recognised by monoclonal antibodies. However, studies investigating the immunogenicity of scFv demonstrated that scFv alone was not particularly immunogenic. Therefore, similar to protein vaccination requiring a carrier protein to stimulate a stronger immune response, DNA vaccines were engineered using similar technologies. One of the first proteins used to stimulate a stronger immune response to the scFv DNA vaccine was the Fragment C (FrC) component from tetanus toxin. The results of the fusion protein vaccination in mice demonstrated a marked increase in the induction of antibodies against the idiotype (Spellerberg et al., 1997). It is thought that the immune response against FrC aids in the amplification of the idiotype-specific immune response. The induction of an antibody response requires CD4 T_H cell help and because the B cells have been transfected with both FrC and the idiotype proteins, the idiotype-specific B cells will express peptides from the FrC. Therefore, the FrC-specific T_H cells can recognise and

activate the idiotype-specific B cells, indirectly augmenting the idiotype-specific antibody response (King et al., 1998).

A clinical trial (UK-007) set up as a pilot study of idiotype-based DNA vaccination of follicular lymphoma was completed in 2007. The study involved the intramuscular vaccination of 25 patients with naked DNA encoding for the idiotype scFv coupled to FrC (Rice et al, 2008). The vaccination was well tolerated and an anti-FrC response was generated in 18 of 25 patients, albeit slower than conventional protein vaccines (Böcher et al, 1999 and Rahman et al., 2000). Idiotype-specific responses were investigated in 16 of the vaccinated patients with 6 demonstrating either cellular and/or antibody responses. However, the responses generated by those patients were relatively low and there is a need to improve these responses. It has been shown that the method of DNA introduction could be very important with microparticles (Otten et al., 2003) and electroporation (Tollefsen et al., 2002) demonstrating increased generation of immune responses.

After the low idiotype-specific responses seen in the clinical trial involving the scFv-FrC DNA vaccine of FL patients, another potential adjuvant, an agent which can be added to a vaccine to stimulate the immune response is the pentameric B subunit from the heat labile enterotoxin found in E. coli (Chen et al., 2009). Enterotoxin B, in its pentameric form, binds to the toxin receptor ganglioside GM1. The murine study demonstrated the scFV vaccine coupled to the toxin subunit could still bind to the toxin receptor after synthesis of the protein from the vaccinated plasmid. Generation of idiotype-specific antibody was also demonstrated suggesting that the toxin, used to couple the scFV to, has potential in future therapy.

Similar to follicular lymphoma, although smaller in scale, DNA vaccines have also been tested against multiple myeloma. Mouse models demonstrated protective immunity after scFv DNA vaccination but vaccination with the idiotype protein failed to induce the same protective immunity (King et al., 1998). However, the majority of studies into idiotype vaccination in multiple myeloma centred around protein vaccination or dendritic cell vaccines. The myeloma idiotype protein is easier to recover from myeloma patients and this may have a bearing on the direction of therapy for myeloma in the future.

1.12.9 Generation of Immune Responses to T Cell Lymphoma

Compared with the volume of studies completed on responses towards the B cell idiotype and immunotherapy using this idiotype, there has been very few studies into the idiotype contained within the TCR. This is mainly due to the small number of patients who present with T cell lymphoma compared with those who present with B cell lymphomas. However, there has been investigation into T cell idiotypes where studies have used various methods to generate T cell responses against the T cell idiotype from T cell lymphomas including mycosis fungoides (MF) and cutaneous T cell lymphoma (CTCL). In one study, the determination of synthetic peptide T cell epitopes, using combinatorial peptide libraries, which mimics a natural TAA have been used to generate a T cell response (Linnemann et al, 2000). The peptides could be used to generate T cell responses in many patients suggesting that there is a natural epitope presented by the T cell lymphoma which is mimicked by the synthetic peptide which was used. Other studies have used sequence analysis of the CDR3 region and peptide prediction algorithms to discover potential CDR3 epitopes (Berger et al., 1998

and Berger et al, 2001). The generation of T cell responses to synthetic peptides led them to believe that there was the possibility that tumour cells could present CDR3-derived epitopes on their surface. They went on to elute peptides from the surface of CTCL cells and found a novel peptide, which could not be identified using a protein database, which was very similar to the sequence of the V region of the T cell lymphoma. Further work studying the sequence of the eluted peptide and T cell responses against it demonstrated that there was a possibility that the T cell lymphoma may present peptides from the TCR on the cell surface (Berger et al., 1998).

1.12.10 Immunotherapy Using the TCR Idiotype

Most of the studies into T cell lymphomas and the TCR idiotype have used adenoviral transduction (Wong & Levy, 2000) and, similarly to the B cell work, dendritic cells pulsed with tumour cell lysate and used as a vaccine (Gatza & Okada, 2002). However, one study has demonstrated this targeted approach against pathogenic T cells reactive against idiotype peptides derived from myelin basic protein (MBP) reactive T cells (Zang et al, 2000). A panel of epitopes from the CDR2 and 3 regions from MBP-reactive T cells were used to generate anti-idiotypic T cells ex vivo and used as a vaccine in patients with MS. The results demonstrated that T cells reactive against the CDR2-derived epitopes were less immunogenic than those generated against CDR3-derived epitopes and that these T cells, $CD8^+$, could specifically lyse autologous MBP-reactive T cells.

Despite apparent progress in the study of the T cell idiotype and the potential for future immunotherapy, the problems that apply to the B cell idiotype apply to the T

cell idiotype as well. The vast majority of patients who present with T cell lymphoma will contain a unique idiotype region and as a result, any therapy produced would be patient specific, and therefore could only be used to treat a single patient. Consequently, therapy for T cell lymphomas would be more attractive if it wasn't patient specific. However, this may mean that the target would not be tumour cell specific and could result in the targeting of otherwise healthy T cells. Although, there has been a suggestion that T cell lymphomas express a peptide which is present in many patients (Linnemann et al, 2000) and this could prove to be a useful immunotherapeutic target.

Immunotherapy targeting the idiotype has moved on dramatically in recent decades with many potential vaccines trialled in many common lymphoid malignancies. The results of these trials are, to date, disappointing and this may partly be due to tolerance against these antigens. However, one fundamental issue remains, the specificity of each vaccine for every single patient. This will continue to prove a difficult problem to overcome due to the hugely diverse nature of the idiotype region.

1.13 The Paradox of the Adaptive Immune System

The immune system must be able to recognise every conceivable antigen that could potentially cause harm and for this there needs to be an extraordinary number of receptors specific for each of these antigens. However, the amount of DNA needed to encode for this number of receptors would constitute a large part of the genome, and yet the loci for the B cell and T cell antigen receptors are relatively small. As discussed previously, the generation of each antigen receptor occurs from recombination of gene segments with deliberate mutations or "mistakes" between the segments which can alter the specificity immeasurably. These apparently random mutations give rise to short sequences of DNA which are considered "non-germline". Considering that there are thought to be ~$25x10^6$ TCRs (Arstila et al., 1999) and a similar number of BCRs, this would imply there is vast antigenic diversity within the lymphocyte compartment which could potentially be recognised as non-self by the immune response and have the potential to trigger autoimmune reactions. Moreover, because antigen receptor diversity is critical for robust adaptive immunity, this vast incurred antigenic diversity becomes a functional prerequisite.

This presents a curious paradox in that if the immune response is tolerant not only to germline-encoded self antigens, but also the vast antigenic universe created by antigen receptors, then it could become non-functional. Such a nihilistic state would be self-defeating and would counter any selective advantage of evolving such an adaptive immune response. Conversely, maintaining the functional integrity of the adaptive immune cell repertoire by avoiding tolerance to antigen receptors, particularly circulating antibodies, would likely lead to an autoimmune state whereby a small number of antibody peptide fragments would be identical to pathogenic epitopes.

To circumvent this problem, it could be argued that three possible mechanisms could prevent the potential autoimmunity whilst maintaining a useful repertoire of T and B cells. Firstly, lymphocyte antigen receptors may be actively excluded from antigen processing thus preventing their presentation at the cell surface. In this manner, these antigen receptors would be treated as exceptional antigens, perhaps through possession of signal motifs excluding antigen processing - tolerance through ignorance.

Secondly, the immune system could be functionally tolerant towards CDR3-derived peptides resulting in prevention of activation of T cells after recognition of CDR3-derived peptides - tolerance though peripheral mechanism.

Lastly, tolerance could be achieved through immunoediting of the lymphocyte antigen receptor repertoire, whereby T or B cell bearing HLA-binding peptides within their CDR3 regions would be deleted - tolerant through immuno-editing of the antigen receptor repertoire. Consequently, lymphocytes expressing receptors which do not contain CDR3-derived HLA-binding peptides would populate the periphery. This third mechanism is analogous to thymic central tolerance.

1.14 Mathematical Model of B Cell Receptor Diversity

In order to demonstrate the potentially dangerous nature of the immune system, due to its diversity, a mathematical model of B cells has been created (Figure 1.11). In a typical donor, there have been estimates of ~$25x10^6$ unique B cells (Arstila et al, 1999) with an average CDR3 heavy chain consisting of 25 amino acids and an average CDR3 light chain consisting of 22 amino acids. Using this conservative estimate of the number of unique B cells, every person has ~47 CDR3-derived 9mers per B cell and potentially $1.2x10^9$ CDR3-derived peptides from their B cell repertoire alone. There is the potential to produce $5.12x10^{11}$ different nonamer peptides (20 amino acids at 9 different positions). Therefore, the chance of any given nonamer peptide sequence (e.g. AAAAAAAAA) being found in any CDR3 in the entire B cell repertoire would be 0.23% (Figure 1.11 A). One caveat to these calculations, would be any observed bias toward the random nature of the B cell CDR3. In reality, there will always be some bias introduced as a consequence of the conserved regions in the joining region of the CDR3 meaning that the number of unique peptides in the CDR3 is possibly as low as 10-12 peptide nonamers in each Ig chain. Taking this into account, there is still 0.11% chance that any given nonamer peptide sequence would be found in the B cell CDR3 proteome.

However, it is conceivable to suggest that there are a larger number of unique B cells considering there are ~$1x10^9$ B cells in the blood alone (~10% B cells in PBMCs; $2x10^9$ PBMCs per litre producing $0.2x10^9$ B cells per litre and assuming there are ~5 litres blood per person, there would be $1x10^9$ B cells in the blood alone). If this is true, there could be a larger number of unique B cells in humans. Using a less conservative

estimate of the number of unique B cells in the body, $2x10^8$, there would be a 1.84% chance that any given nonamer peptide sequence would be found in the B cell CDR3 proteome.

From the perspective of focussing on a given HLA allele such as HLA-A2, every possible peptide is thought to have approximately 0.5% chance of binding to HLA-A2 (Yewdell and Bennink, 1999). Consequently, there is approximately a 99.5% chance that any given peptide will not bind to HLA-A2 (Figure 1.11 B). If it is assumed that there are 47 CDR3-derived peptides per B cell, there is ~79% chance that none of the CDR3-derived peptides will bind to HLA-A2 (0.995^{47}x100%). Therefore, the chance that any peptide from the B cell CDR3 will bind to HLA-A2 is ~21%. Again, this is assuming that every peptide within the CDR3 is unique which is unlikely to be true. Taking this concern into account, a more conservative estimate might be 10-12 unique CDR3-derived peptides in each Ig chain, there is still 11% chance that any peptide within the CDR3 will bind to HLA-A2 (or indeed any given HLA Class-I allele). If we now consider that any individual has 6 Class-I alleles then the chances that any given peptide within the CDR3 will bind to any of the 6 HLA Class-I alleles will be approximately $1-(1-0.11)^6$ = ~51% using the more conservative estimates.

Although the figures described here are purely theoretical, it is perhaps surprising how potentially dangerous the antigen receptor diversity could be. Considering these figures, it is reasonable to postulate that there would be a survival advantage to evolve a mechanism, as yet undefined, which prevents the inclusion of self-HLA-binding peptides within the B cell and/or T cell CDR3, thus limiting the risk of developing autoimmune disease.

A Number of B cells: 25,000,000

Length of CDR3: Heavy Chain - 25 amino acids

Light Chain - 22 amino acids

Total - 47 amino acids ➔ 47 CDR3-derived peptides

Total number of CDR3-derived peptides: = 25,000,000 x 47

= 1.18×10^9 peptides

Total number of possible peptides = 20^9

= 5.12×10^{11}

Chance of any peptide being found in the CDR3 = $1.18 \times 10^9 / 5.12 \times 10^{11}$ x100 %

= 0.23%

There is 0.23% chance than an immunogenic or viral peptide could be found in the entire BCR CDR3 proteome.

B Every peptide is thought to have ~1% chance of binding to an HLA-A2 molecule

Chances of a CDR3-derived peptide not binding to HLA-A2 = $(1 - 0.01)^{47}$ x 100 %

= 62%

There is a 62% chance that no CDR3-peptides will bind to an HLA-A2

Therefore, the chance that a CDR3-peptide will bind to MHC = 1 - 62%

= 38%

There is ~38% chance that a B cell will contain an MHC-binding CDR3-derived peptide binding to HLA-A2.

Figure 1.11 Mathematical model of B cell receptor diversity

The B cell receptor is an extremely diverse protein formed from re-arrangement of gene segments with extreme variability between segments which can potentially be non-self protein sequence. This re-arrangement is responsible for most of the diversity seen in the repertoire. However, it is also the diversity coupled with the potentially non-self sequence that can be very dangerous. A mathematical model was developed in order to demonstrate the potential danger of the CDR3 by determining the potential for an immunogenic peptide to be contained within the CDR3. The first model (A) uses a conservative estimate of the number of unique BCRs present in the immune system and estimates the probability that an immunogenic peptide (e.g.viral) could be found within the CDR3 of any B cell in a single person. The second model (B) estimates the probability of a peptide from the CDR3 binding specifically to a single HLA allele, in this case HLA-A2.

1.15 Hypothesis, Aims and Objectives

B and T cell receptors undergo germline gene segment rearrangement to produce full length functional receptors. However, these insertions and deletions between gene segments generate a sequence which is non-germline and could potentially be a source of immunogenic peptides. Current understanding suggests lymphocyte antigen receptors undergo two methods of selection in the thymus or bone marrow.

However, in order to prevent an autoimmune reaction, there must be mechanisms in place to prevent T cell recognition of these non-self peptides (see section 1.13). In this study it is hypothesised that there is no peripheral tolerance towards non-self peptides contained within the CDR3. Therefore, to prevent the initiation of an autoimmune reaction there is a third selection process whereby antigen receptors with a potentially immunogenic CDR3-derived peptide are deleted from the lymphocyte repertoire. If this hypothesis is true, the observed frequency of HLA-A2 binding peptides found within the CDR3 region of an HLA-$A2^{+}$ donor will be decreased compared with an HLA-$A2^{-}$ donor.

If the hypothesis is not proven and there are HLA-binding peptides present in the CDR3 of HLA-positive donors, a second hypothesis can be tested; HLA-binding peptides within the CDR3 of antigen receptors are excluded from Class-I antigen processing and not presented at the cell surface. This second hypothesis could help to define the absence of lymphocyte interaction as either tolerance or the prevention of antigen processing of CDR3-derived peptides.

The aims of this project are:

Investigate the lymphocyte antigen receptor CDR3 sequences and determine the presence of immunoediting within the lymphocyte antigen receptor repertoire

Ascertain the presence or absence of naturally processed and presented CDR3-derived peptides in B and/or T cells through the development of models of B and T cell CDR3 antigen processing

These aims would be pursued by sequencing the CDR3 sequences of BCRs and TCRs to determine any differences in the frequency of HLA-binding peptides in HLA-positive and negative donors. Presentation of CDR3-derived peptides would be determined by recognition of target cells using peptide-specific T cells and attempts made to investigate the antigen processing pathway involved.

Chapter 2 - Materials and Methods

2.1 Molecular Biology

2.1.1 RNA Extraction from PBMCs

RNA was extracted from healthy laboratory donors with known HLA type (Table 2.1) so as to give 5 HLA-$A2^+$ and 5 HLA-$A2^-$ donors. RNA extraction was completed using the RNeasy RNA extraction kit (Qiagen, West Sussex UK). Briefly, 1-2x10^6 PBMCs were washed to remove residual serum from the cells. 350µl of buffer RLT + 1% 2-mercaptoethanol was added to the cells and mixed thoroughly. The cells were then homogenised by passing the lysate 5-10 times through a 21G needle fitted to a syringe. 350µl of 70% ethanol was added to the homogenised lysate and pipetted onto the RNA spin column. This was then spun at 8,000g for 15 seconds and the supernatant discarded. 700µl of buffer RW1 was added onto the column, spun again and the supernatant discarded. The column was then washed twice with 500µl buffer RPE + ethanol with the supernatant discarded each time. The RNA was eluted from the column using 30µl of RNAse-free water. This was added to the column and spun at 13,000rpm for 1 minute. The RNA yield was then determined by spectrophotometry and stored at -20°C until needed.

2.1.2 cDNA Synthesis

cDNA synthesis was done using the Superscript II First-Strand Synthesis for RT-PCR Kit (Invitrogen, Paisley, UK). Briefly, 3µl of RNA was incubated with 1µl of 10mM dNTP mix and 1µl of 0.5µg.ml oligo dT primer at 65°C for 5 mins. The RNA/primer mix was used for reverse transcription in a 20µl reaction incubated at 42°C for 50mins

Table 4.1 HLA Type of Laboratory Donors	
Donor ID	HLA Type
Id 001	A1, B8
Id 002	A2, B7
Id 003	A1, B7, B8
Id 004	A1, B8
Id 005	B7
Id 006	A1, B7
Id 007	A1, A2, B8
Id 008	A2, B8
Id 009	A2
Id 010	A2, B7

Table 2.1 HLA Type of laboratory donors for used for sequencing

The donors were chosen so each of the HLA-types investigated contained 5 positive donors and 5 negative donors.

containing 2µl 10X RT buffer, 4µl 25mM MgCl2, 2µl 0.1M DTT and 1µl RNAse inhibitor. After 2 mins, 50units of Superscript II RT was added to each sample. Reactions were terminated by incubation at 70°C for 15 mins and RNAse H was added to remove the residual RNA and incubated at 37°C for 20 mins. The presence of cDNA was verified by amplification using primers specific for the TCR/BCR.

2.1.3 Amplification of BCR/TCR Transcripts from cDNA

cDNA was amplified with a panel of primers specific for TCRVB families 1-24 (Table 2.2) (Invitrogen, Paisley, UK) or primers specific for IgH isotypes IgA, IgG and IgM (Table 2.3) (Invitrogen, Paisley, UK). BCRs were amplified from within the framework region 1 (FR1) of the variable region to the constant region and the TCRs were amplified from the beginning of the variable region to the constant region. Amplification of the DNA for both TCR and BCR was 35 cycles of 95°C for 30sec, 65°C for 30 sec and 72°C for 1 min in reactions containing 10mM dNTPs, 1uM each primer and 1 unit of Expand High Fidelity DNA polymerase (Roche, West Sussex UK). 50µl of each reaction was loaded onto a 1% TBE (Boric Acid, Tris.HCl and EDTA) agarose gel and run for 50 mins at 120V. Bands were visualised by SYBR safe gel stain (Invitrogen, Paisley, UK) and the size was determined by comparison to a 100bp molecular weight marker (Invitrogen, Paisley UK) (Figure 2.1). This method may lead to variation in amplification as the annealing temperature used may be optimal for some of the primers but not for the others. The extent of this has been minimised and this method has been used previously on many occasions (Kou et al., 2000 & Yao et al., 2006).

Vα	Sequence	Vβ	Sequence
1	TTGCCCTGAGAGATGCCAGAG	1	ACAACAGTTCCCTGACTTGCA
2	GTGTTCCAGAGGGAGCCATTGCC	2	TCAACCATGCAAGCCTGACCT
3	GGTGAACAGTCAACAGGGAGA	3	TCTCTAGAGAGAAGAAGGAGCGC
4	ACAAGCATTACTGTACTCCTA	4	ACATATGAGAGTGGATTTGTCATT
5	GGCCCTGAACATTCAGGA	5.1	ACTTCAGTGAGACACAGAGAAAC
6	GTCACTTTCTAGCCTGCTGA	5.2	CCCTAACTATAGCTCTGAGCT
7	AGGAGCCATTGTCCAGATAAA	6	AGGCCTGAGGGATCCGTCT
8	GGAGAGAATGTGGAGCAGCATC	7	ACCTGAATGCCCCAACAGCTCT
9	ATCTCAGTGCTTGTGATAATA	8	ATTTACTTTAACAACAACGTTCCG
10	ACCCAGCTGCTGGAGCAGAGCCCT	9	TAAATCTCCAGACAAAGCTCACT
11	AGAAAGCAAGGACCAAGTGTT	10	TCCAAAAACTCATCCTGTACCTT
12	CAGAAGGTAACTCAAGCGCAGACT	11	AACAGTCTCCAGAATAAGGACG
13	GCTTATGAGAACACTGCGT	12	AAAGGAGAAGTCTCAGAT
14	GCAGCTTCCCTTCCAGCAAT	13.1	TCCTGAAGACAGGACAGAGCA
15	AGAACCTGACTGCCCAGGAA	13.2	TTGGTGAGGGTACAACTGCCA
16	CATCTCCATGGACTCATATGA	14	TCTCTCGAAAAGAGAAGAGGAAT
17	GACTATACTAACAGCATGT	15	GTGTCTCTCGACAGGCACAG
18	TGTCAGGCAATGACAAGG	16	AAGAGTCTAAACAGGATGAGTC
23	TGACACAGATTCCTGCAGCT	17	ACAGATAGTAAATGACTTTCAGAAA
24	TGAGCCCCTTCAGCAACTTAAGG	18	AGATGAGTCAGGAATGCCAAAG
25	ATATAAGGCTGGTGAATTGACCTCAAAT	19	TCTCAATGCCCCAAGAACGCA
26	TAAACTGCACGTCATCAAAGACG	20	GCTCTGAGGTGCCCCAGAATCTC
27	ACCACTTCAGACAGACTGTATT	21	CAAAGGAGTAGACTCCACTC
28	GTAGCATATTAGATAAGAAAGAACTT	22	CAGTGACTATCATTCTGAACTG
29	ACAACCAGTGCAGAGTCCTC	23	ATCTCAGAGAAGTCTGAAATAT
		24	CCGAACACTTCTTTCTGCTTTC
Cα	CTTGTCACTGGATTTAGAGTCTC	Cβ	TGCTTCTGATGGCTCAAACAC

Vα: TCR alpha chain variable region; Cα: TCR alpha chain constant region; Vβ: TCR beta chain variable region; Cβ: TCR beta chain constant region

Table 2.2. Oligonucleotide primer sequences to amplify the TCR variable region and CDR3

Table of all oligonucleotide primers used to amplify the TCR variable region for both the α and ß chains including the CDR3 region. Taken from Okamoto, Y et al. 1994.

2.1.4 Molecular Cloning of TCR/BCR transcripts

The PCR products were purified from the gel by Gel Extraction kit (Qiagen, West Sussex, UK) and cloned into pCR TOPO 4 TA vector (Invitrogen, Paisley UK). The vector containing the amplified DNA was transformed into TOP10 chemically competent E.coli (Invitrogen, Paisley UK) and grown overnight on agar under ampicillin selection (100µg/ml). Following overnight incubation, colonies were picked, suspended in 50µl LB broth + 100µg/ml ampicillin and grown for 4 hours ready for colony PCR.

2.1.5 Colony PCR

PCR reactions were set up (as previously described) containing 10µM dNTPs, 1µM of each primer (M13 forward and M13 reverse) and 1 unit Expand High Fidelity enzyme (Roche, UK) and 5µl of bacterial culture. An initial incubation at 95°C for 5mins was used to lyse the bacteria and make the plasmid DNA available for amplification. After this, the DNA was subject to 35 cycles of 95°C 30sec, 65°C for 30sec and 72°C for 1 min. A final elongation step of 72°C for 10mins was added to ensure all DNA strands were fully elongated. 10µl of each reaction was loaded onto a 1% TBE agarose gel and run for 50 mins at 120V. Bands were visualised by SYBR safe gel staining (Invitrogen, UK) and the size was determined by comparison to a 100bp molecular weight marker (Invitrogen, Paisley UK) (Figure 2.2). Bacterial cultures resulting in positive PCR reactions were added to 5mls LB broth supplemented with 100µg/ml ampicillin and grown overnight at 37°C in a shaking incubator. Cultures were pelleted and double stranded plasmid DNA was extracted from cell pellets using a Qiaprep

Isotype	Primer Sequence
IgA	GAGGCTCAGCGGGAAGACCTT
IgG	GGGGAAGTAGTCCTTGACCAG
IgM	CAGGAGAAAGTGATGGAGTCG
FR1	SAGGTRCAGCTGBWGSAGTCNG

FR1: Framework region 1; S: C/G; R: A/G; B: T/C/G; W: A/T; N: A/C/G/T

Table 2.3. Oligonucleotide primer sequences to amplify the BCR variable region and CDR3

Table of oligonucleotide primers used to amplify the BCR from within framework 1 of the Ig heavy chain variable region including the CDR3 region. Taken from Fais et al, 1998.

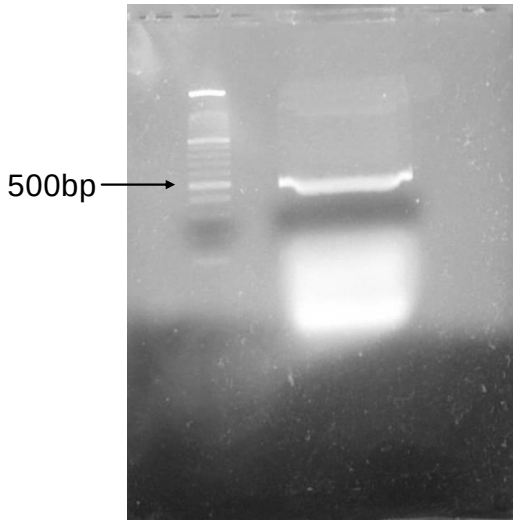


Figure 2.1. Amplification of TCRβ-chain CDR3

cDNA was synthesised from RNA extracted from unfractionated PBMCs. The TCR chain was amplified using a mixture of V region primers and a single constant region primer. PCR products run on a 1% agarose gel and visualised under an ultraviolet light. Amplicons of TCR variable regions were expected ~500-550bp.

spin miniprep kit (Qiagen, West Sussex UK) following the manufacturer's protocol. DNA was stored at -20°C until ready for sequencing.

2.1.6 HLA Typing of Patient DNA Samples

PCR reactions were set up (as previously described) containing 10μM dNTPs, 1μM of each primer (Table 2.4), 1 unit Expand High Fidelity enzyme (Roche, UK) and 1μl genomic DNA. DNA was subject to 35 cycles of 95°C 30sec, 65°C for 60sec and 72°C for 1 min. A final elongation step of 72°C for 10mins was added to ensure all DNA strands were fully elongated as described (Tonks et al, 1999). PCR products were run on 1% agarose gel and visualised under UV light (Figure 2.3).

2.2 Automated DNA Sequencing

2.2.1 Functional Genomics

DNA sequencing was performed using an M13 primer (Invitrogen, UK) and a Bigdye Terminator cycle sequencing kit (Applied Biosystems, UK). 200-500ng of double stranded plasmid DNA was used as template in the reactions. Cycle sequencing reactions consisted of 25 cycles of 96°C for 10 secs, 50°C for 5 secs and 60°C for 4 mins. Sequencing products are purified by precipitating DNA in an automated system. Purified products were run on ABI3700 DNA sequencer.

2.2.2 Polymorphic DNA Technologies

DNA sequencing was performed using an M13 primer. 600ng of double stranded plasmid DNA was used as a template in the reactions. Samples were run in fully

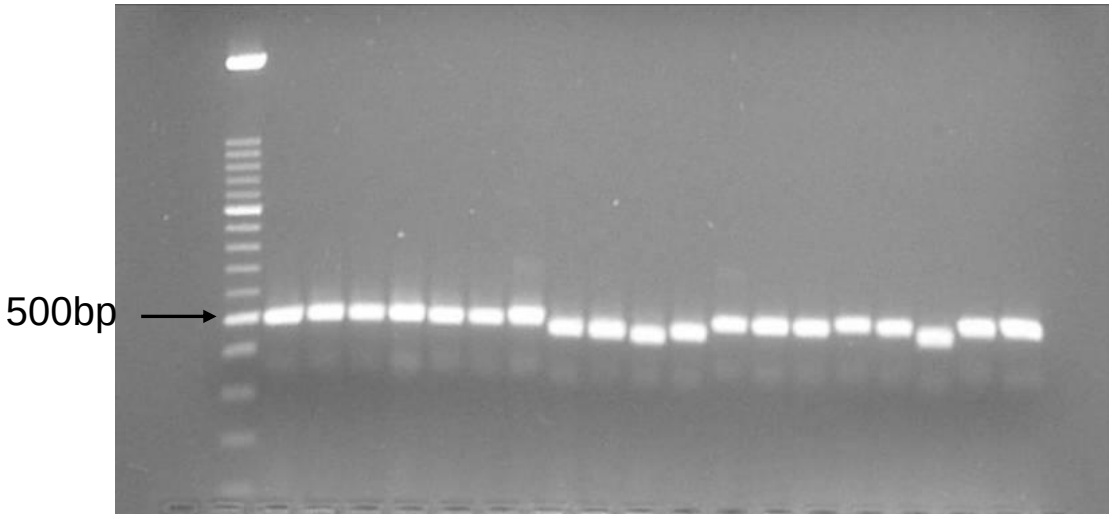


Figure 2.2. PCR to amplify the TCRV β chains from individual colonies of bacteria.

The TCR chain β chain CDR3 regions amplified fom the cDNA were cloned into the TA vector and transformed into bacterial cells. With each colony containing a single TCR β chain, bacterial colonies were picked and grown in 50µl LB + ampicillin. Bacterial cells were used as template for the colony PCR to test which colonies had been transfected with the TA vector containing a TCR β chain within it. PCR products were run on a 1% agarose gel and visualised under ultraviolet light. Bands ~500-550bp were used for sequencing and further work.

Primer Name	Sequence
A2 Forward	TCC TCG TCC CCA GGC TCT
A2 Reverse	GTG GCC CCT GGT ACC CGT

Table 2.4 PCR primers to determine HLA-A2 type of patients

Primers, taken from Tonks et al, 1999, were used to amplify genomic DNA from paitents with various B cell lymphomas to determine which patients were HLA-$A2^{+.}$ Dna sent from University of Southampton (kind gift from Prof. C Ottensmeier).

automated sequencing systems. Sequencing returned was analysed using ChromasPro and translated into amino acid sequence using Translate (www.expasy.ch).

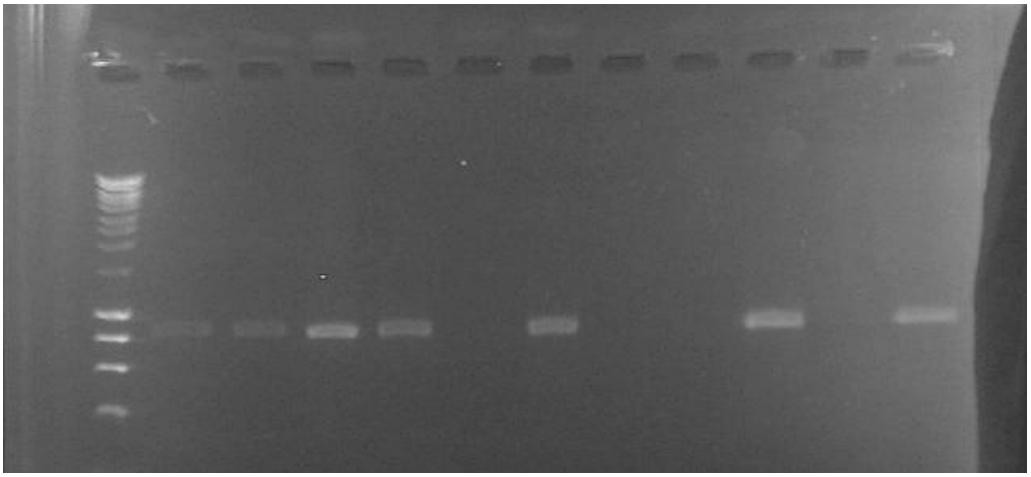
2.3 Peptide Prediction Studies Using Syfpeithi and BIMAS

Sequences of TCR beta chains and Ig heavy chains were determined by reference to previously published known TCR and Ig families. CDR3 region sequences were determined and used to predict potentially immunogenic epitopes within the CDR3 using the computer algorithms Syfpeithi (www.syfpeithi.de) and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/)

2.4 Mutagenesis of CDR3 Region of TCR Beta Chain

A full length T cell receptor beta chain (kindly donated by Lee Machado), within the TA vector (Invitrogen, Paisley, UK), was used as a starting point for mutagenesis studies. Phosphorylated primers were designed (Table 2.5) to mutate the CDR3 region of the TCR β-chain to encode for CMV-derived CD8 T cell epitopes. The PCR was performed using Phusion enzyme (New England Biolabs, USA) with the PCR product for each set of primers (containing different CDR3-peptides) being run on a 1% agarose gel (Figure 2.4). The DNA was re-circularised using DNA ligase (Roche, West Sussex, UK), and used for transfection studies. The gene was sequenced to demonstrate insertion of the new sequence in the correct orientation (Table 2.6).

A



B

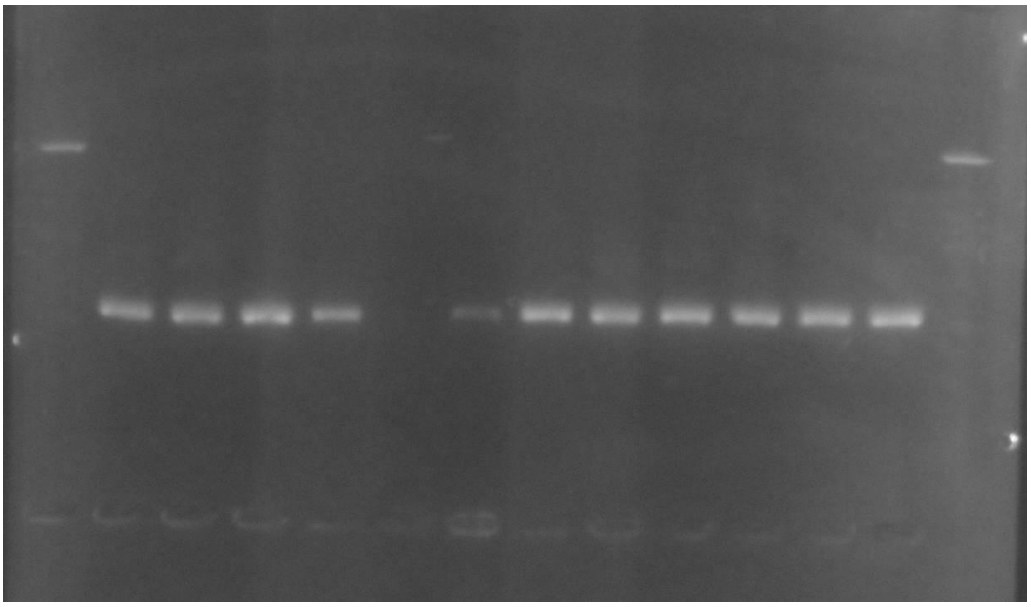


Figure 2.3 PCR to amplify HLA-A2 to determine HLA-A2 type of patients

DNA from patients with B cell lymphomas was amplified using oligonucleotide primers specific for HLA-A2 to determine which of the patients were HLA-$A2^+$ (A). The primers used are known to produce a PCR product of ~900bp in HLA-$A2^+$ individuals. Actin was amplified as control (B) to demonstrate that the DNA was of good enough quality for PCR amplification.

2.5 Tissue Culture and Cellular Experiments

2.5.1 Peptides

Peptides were synthesized by AltaBioscience (Birmingham UK) or Genscript (New Jersey, USA), reconstituted in DMSO at a final concentration of 10mg/ml and stored at -20°C. Reference to each peptide is made by the first three amino acids e.g. ALCDTGRRAL is designated as ALC and so on.

2.5.2 Isolation of mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by Ficoll density gradient centrifugation. Whole blood was diluted 1:1 with RPMI 1640 (Gibco, Paisley UK or made in-house from powder ordered from Sigma (R6504)) and then layered onto Ficoll (Lymphoprep, Nycomed, Sweden) and spun for 25min at 1800rpm with the brake off. The lymphocyte layer was then removed with a transfer pipette to a fresh sterile tube and resuspended in RPMI. Cells were spun for 10min at 1600rpm and the supernatant discarded. Cell pellets were resuspended once more in RPMI and spun again (this time at 1200rpm). The supernatant was discarded and cells were resuspended in RPMI supplemented with 10% foetal calf serum (FCS), 2mM L-glutamine and 100U/ml penicillin-streptomycin (Sigma, UK). The lymphocyte yield was determined using a haemocytometer and a light microscope. Cells were then used immediately or cryopreserved. Freezing media contained 90% FCS and 10% DMSO (Sigma, UK).

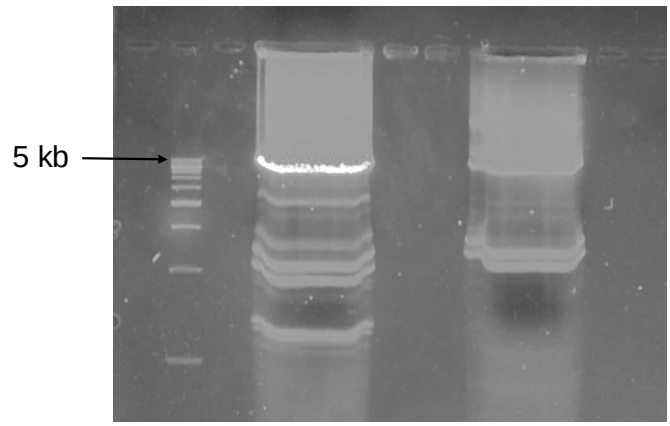


Figure 2.4. PCR to amplify the complete TCRβ chain using two different sets of primers.

DNA encoding the full length TCR β-chain was used as a template for mutation PCR to introduce CMV viral epitopes into the CDR3. Amplified DNA was run on a 1% agarose gel and visualised using SYBR safe gel stain under a UV box. The full length TCR β chain was 5.5kb in size and the corresponding band cut-out of the gel, purified and used for further experimentation.

Primer Name	Primer sequence
NLV forward	P-GTGGCTACGGTTGGGCCGGGCACCAGGC
NLV reverse	P-CATGGGCACCAGGTTGCTGCTGGCGCAG
RNLV forward	P-GTTGCTACGGTTCAGGGGCCGGGCACCA
RNLV reverse	P-CATGGGCACCAGGTTGCGGGTGCTGGCG
RPH forward	P-AACGGCTTTACGGTGTTGGGGCCGGGCA
RPH reverse	P-GCGCTCGTGGGGGCGGCTGCTGGCGCAG
TPR forward	P-GGCGGCGGCGCCATGGGGCCGGGCACCA
TPR reverse	P-GGTAACGCGGGGCGTGCTGCTGGCGCAG
DYSN forward	P-AGTACCCGTTACGTGGGGCCGGGCACCA
DYSN reverse	P-GTGGGTGTTGGAATAATCGCTGCTGGCG

Table 2.5. Oligonucleotide primer sequences to introduce viral epitopes in the CDR3

Oligonucleotide primers were designed and synthesised to introduce CMV viral epitopes into the CDR3 region. Amplification of the TCRβ chain using the primer sets would mutate the CDR3 to incorporate the viral epitopes.

2.5.3 Cell staining with antibodies and tetramers

$5x10^5$ PBMC were incubated with 1µl of antibody at room temperature for 30 minutes and washed in MACS buffer (PBS (made in-house from tablets, Oxoid (Hampshire, UK) + 2% FCS + 2mM EDTA). Cells were analysed on a Coulter EPICS-XL flow cytometer or Becton Dickinson Facs Canto II. Acquired data was analysed using WinMDIv2.8 software (Scripps Institute, California, USA) or FlowJo7 (Celeza GmbH, Switzerland). T cell receptor beta chain variable region 7 (TCRVβ7) usage by transfected cells was determined by addition of 1µl of TCRVβ7 phycoerythrin-conjugated antibody (Abcam, Cambridge UK) to $5x10^5$ transfected cells at room temperature for 20 mins. Cells were washed in MACS buffer and analysed on a Coulter EPICS-XL flow cytometer.

2.5.4 Magnetic selection of antibody-bound PBMCs

5-10 $x10^6$ PBMC were stained with antibody as described earlier, washed and then incubated with anti-PE microbeads (Miltenyi Biotech, Surrey UK) for 30min on ice. Cells were washed again twice in MACS buffer (PBS (made in-house from tablets, Oxoid (Hampshire, UK) containing 0.5% BSA and 2mM EDTA) and resuspended in 0.5ml cold MACS buffer. A miniMACS MS (Miltenyi Biotec, Germany) separation column was placed onto the column holder attached to a magnet. The column was washed with 1ml of cold MACS buffer. The cell suspension was added to the column and the eluted cells (negatively selected fraction) collected in a tube. The column was washed extensively with 3x0.5ml of cold MACS buffer. More washes were performed if greater purity was desired (at the expense of yield). The column was then removed from the magnet and cells still bound were eluted out with buffer using

T Cell Clone	Sequence
NLV	PEDSALYLCASS**NLVPMVATV**GPGTRLTVTEDLKNVFP
RNLV	PEDSALYLCASS**RNLVPMVATVQ**GPGTRLTVTEDLKNVFP
RPH	PEDSALYLCASS**RPHERNGFTVL**GPGTRLTVTEDLKNVFP
TPR	PEDSALYLCASS**TPRVTGGGAM**GPGTRLTVTEDLKNVFP
No Mutation	PEDSALYLCASSPGRWYEQYFGPGTRLTVTEDLKNVFP

Table 2.6. Sequencing of the mutated CDR3 of the full length TCR β-chain

The TCR β-chain was mutated using PCR to incorporate CMV-derived, immunogenic peptides within the CDR3 region. The sequencing results shown here confirm the presence of the immunogenic peptides within the CDR3 region.

a sterile plunger. A small aliquot of cells from each fraction could be stained with further antibodies (if required) and analysed by flow cytometry.

2.5.5 IFNγ ELISA

IFNγ release by T cells was determined by using the Human IFNγ ELISA Kit – Ready-SET-Go! (Ebiosciences, SanDiego CA, USA). T cell clones or lines were washed to remove any residual IFNγ from the culture supernatant and resuspended in LCL media (RPMI + 10% FCS + 2mM L-Glutamine) at 10^5/ml. Target cells were pulsed with peptide (10μg/ml) in serum-free media at 37°C for 2hrs and then washed to remove excess peptide. Where no peptide was added, the cells were washed and all target cells were resuspended in LCL media at $5x10^5$/ml. Normally, 10,000 effector T cells and 50,000 target cells per well were used in the stimulation step (E:T ratio 1:5). Cell numbers could be increased or decreased depending on available cell numbers. 100μl of effector cells and 100μl of target cells were added to wells of a 96-well U-bottomed plate. The plate was incubated at 37°C for 16 hours in 5% CO_2.

A 96-well flat bottomed plate was coated with 100μl of IFNγ capture antibody, sealed and incubated at 4°C for 12 hours. The plate was washed 5 times (PBS (made in-house from tablets, Oxoid (Hampshire, UK) + 0.05% Tween 20 (Melford Labs, Suffolk, UK)), blocked using 200μl of assay diluent per well and stored at room temperature for 1 hour. The plate was washed again as above and 100μl of culture supernatant added to the wells from the overnight culture plate. Wells containing known amounts of IFNγ were also set up to allow quantitative analysis of the ELISA. The plate was sealed and stored at room temperature for 2 hours. After washing (as above), 100μl/well of IFNγ detection antibody was added and the plate incubated at

room temperature for 1 hour. After washing (as above), 100µl/well avidin-HRP was added and the plate incubated at room temperature for 30 mins. The plate was washed 7 times using wash buffer and 100µl/well TMB added before storing at room temperature in the dark for 15 mins to allow for colour development. Colour change from colourless to blue was indicative of IFNγ production. To stop the reaction, 50µl/well 1M phosphoric acid was added and the plate was taken to be read in a spectrophotometer at A_{450}.

2.5.6 IFN-γ ELISpot

The ELISpot wells (Mabtech, France) came pre-coated with anti-IFN-γ antibody and were washed once in 200µl RPMI + 10% FCS before use. PBMCs and PBMCs depleted of B cells were washed in RPMI + 10% FCS and resuspended at $5x10^5$/ml in fresh RPMI + FCS. 200µl of cells were added to each well and 50µl of immune complex antibody or 5µl peptide (500µg/ml) was added to individual wells. For the positive control, 1µl anti-CD3 (Mabtech, France) was used and 10µl PBS (made in-house from tablets, Oxoid (Hampshire, UK) was used for the negative control. The plate was covered and incubated at 37°C for 16-18 hours. The cells were flicked off and the plated was washed four times with PBS and 200µl PBS + 0.5% FCS was added to each well. The plate was incubated for 30 minutes at room temperature before being washed four times in PBS. The secondary antibody (alkaline phosphatase conjugated) (Mabtech, France) was diluted 1:200 in PBS and 100µl added to each well. The plate was incubated at room temperature for 2 hours and washed four times in PBS. To each well, 100µl substrate BCIP/NBT (Mabtech, France) was added and left to develop for 10-12 minutes at room temperature. The wells were extensively washed in tap water to stop the reaction and the wells were left

to dry in a cool, dark place. The spots in each well were counted using an ELISpot reader.

2.5.7 Generation of Polyclonal Cytotoxic T Lymphocyte (CTL) lines

PBMC were isolated in the normal manner from fresh blood. After the second wash with RPMI, cells were resuspended in 100µl of RPMI containing 5µg/ml of CMV peptide (such as NLV) and incubated for 1-2hrs at 37°C. Cells were then resuspended in fresh media at $1x10^6$ cells/ml in 2ml cultures. After 24 hours, the used media was removed and fresh media added containing 20IU/ml recombinant IL-2 (Peprotech UK). After a week, fresh T cell media (5ng/ml IL-7 and 5ng/ml IL-15) (Peprotech, UK) was added and cultures were re-fed every 3-4 days. These cultures were stained with tetramer to determine the expansion and purity of the peptide-specific cytotoxic T cells. They were either used within 10-14 days or could be cryopreserved at -80°C for future assays.

2.5.8 Generation of Naïve T Cell Response

2.5.8.1 Production of Dendritic Cells

Generation of naïve T cell responses followed a previously published protocol (Ho et al, 2006). Briefly, unfractionated PBMCs were washed in RPMI + 10% heat inactivated AB human serum (Biosera, East Sussex, UK) and resuspended at $5x10^6$ cells/ml. $1x10^7$ cells were added to each well of a 6 well plate and incubated at 37°C for 2 hours. Non-adherent cells were washed off and 3mls of fresh media containing 800IU/ml GM-CSF (Peprotech, UK) and 1000IU/ml IL-4 (Peprotech, UK) was added to the adherent cells in each well and the plate incubated at 37°C overnight. The following day, 300µl maturation media (RPMI + 10% AB human serum + 100ng/ml

TNF-α (Peprotech, UK) + 100ng/ml IL-1β (Peprotech, UK) + 10,000IU/m IL-6 (Peprotech, UK) and 10μg/ml prostaglandin-E_2 (Peprotech, UK)) was added to each well and incubated at 37°C for a further 24 hours. Dendritic cells were harvested in wash buffer (PBS (made in-house from tablets, Oxoid (Hampshire, UK) + 0.5% BSA (Sigma, UK) + 2mM EDTA (Sigma, UK)) and washed twice in wash buffer. Cells were resuspended in RPMI with 10μg/ml peptide of interest and incubated at 37°C for 2 hours. The peptide loaded DCs were washed twice in RPMI + 10% AB human serum and resuspended at $1x10^6$ cells/ml.

2.5.8.2 $CD8^+$ T Cells

Non-adherent cells (described above) were washed in wash buffer and CD8 T cells isolated using magnetic selection with anti-CD8 PE antibody (BD Bioscience, UK) and anti-PE beads (Miltenyi Biotec, Germany). Isolated $CD8^+$ T cells were then cryopreserved at -80°C until needed. Cells were thawed and washed in RPMI + 10% AB human serum, counted and resuspended at $5x10^6$/ml.

2.5.8.3 Generation of Naïve T Cell Response

Isolated $CD8^+$ T cells were plated out in 1ml at $5x10^6$ cells/ml in a 6 well plate and dendritic cells were plated out in 1ml at $1x10^6$ cells/ml. Cells were re-fed after 48 and 96 hours by removing 1ml of used media and replacing it with 1ml of fresh media containing 10ng/ml IL-7 (Peprotech, UK) and 10ng/ml IL-15 (Peprotech, UK).

After 7 days culture, T cell lines were re-stimulated using fresh autologous PBMCs. PBMCs were washed in RPMI + 10% AB human serum and resuspended at $1x10^7$/ml and peptide-pulsed using 50μl of 500μg/ml peptide. After 2 hours incubation at 37°C,

the cells were irradiated using 35Gy. The cells were washed 3 times in RPMI and resuspended in RPMI + 10% AB human serum at $1x10^7$ cells/ml. 1ml of used media was removed from the T cell lines and 1ml of fresh media plus peptide-pulsed feeder cells was added. Cells were re-fed every 2 days with media + cytokines as described previously. After 14 days, IFN-γ cytokine secretion assay was performed to determine whether there was any peptide-specific response.

2.5.9 IFNγ Cytokine secretion assay (CSA)

PBMC were stimulated for 3 hours at 37°C with 1μg/ml of the appropriate peptide, in 2ml cultures at $1x10^6$ PBMC/ml of T cell cloning media (RPMI plus 10% FCS, 1% human serum, 2mM L-Glutamine and 100U/ml penicillin-streptomycin solution). After the incubation, cells were washed three times with cold MACS Buffer and then resuspended in a volume of approximately 80μl. 20μl of IFNγ capture reagent (Miltenyi Biotec, Germany) was added to the cells, which were then incubated for 5min on ice before diluting in warm T cell cloning media to between 0.5 and $1x10^6$ cells/ml. The cells were incubated at 37°C for 45 min with agitation every 5min. The tube was placed in a horizontal position to minimize capture reagent binding in a non-specific manner. After this, cells were washed twice with cold MACS buffer and resuspended in a volume of approximately 80μl. 20μl of PE-conjugated IFN-γ detection antibody (Miltenyi Biotech, Germany) was added and cells were incubated on ice for 10-15min. Cells were also counter-stained with anti-CD8 at this stage. After 2 washes with cold PBS, cells were resuspended in 0.5ml MACS buffer and were used for cell sorting using the MS columns (Miltenyi Biotec, Germany). Cells collected in the positive fraction were counted using a haemocytometer and either analysed on the FACS machine or cloned using limiting dilution assay methods.

2.5.10 Single Cell Cloning

2.5.10.1 FACSorting

PBMC were stained with tetramers and anti-CD8 FITC and then washed with sterile MACS buffer prior to sorting using a FACS Vantage cell sorter (Becton Dickinson). Cells were sorted using the TurboSort® setup allowing 5,000 events per second to be sorted. T cell cloning mix was prepared: 10^6 irradiated allogeneic PBMC and 10^5 irradiated EBV-transformed autologous LCLs/ml., in T cell clining media containing 50U/ml recombinant IL-2 (Peprotech EC, London UK) and 5ng/ml IL-7. Single $CD8^{pos}$ $tetramer^{pos}$ cells were sorted into 96-well plates containing 200µl of cloning mix. A 96-well plate containing only cloning mix and no T cells added was used as a control.

2.5.10.2 Cloning by Limiting Dilution

Cells (EBV-transformed LCLs and other B cell types) were counted and seeded into LCL growth media (RPMI + 10% FCS + 2mM L-Glutamine + 100U/ml Penicillin + 100U/ml Streptomycin) at 0 cells per well (1 plate), 0.3 cells per well (12 plates), 3 cells per well (3 plates) and 30 cells per well (1 plates). After 14-21 days of culture at 37°C in 5% CO_2, growing cultures were expanded to 2ml and seeded in 24-well plates. These cultures were re-fed every 3-4 days and expanded when needed.

2.5.11 Rapid Expansion Protocol (REP)

For expansion of cell numbers, the desired clone was subjected to a REP. On day 1 of the REP, 1-2x10^5 T cells were added to 25ml of REP media (RPMI plus 10% FCS, 1% Human serum, 2mM L-Glutamine, 25µM 2-mercaptoethanol (Sigma, UK),

12.5mM Hepes (Sigma, UK), 30ng/ml OKT3 and 100u/ml penicillin-streptomycin solution) containing $25x10^6$ irradiated allogeneic PBMC and $5x10^6$ irradiated autologous peptide pulsed LCLs. After 24hr (day 2) IL-2 was added to each REP to a final concentration of 50U/ml. On day 4, the cells were resuspended and pelleted by a 5min spin at 1200rpm. Cells were then resuspended in 25ml of fresh REP media containing 50U/ml IL-2 (and no OKT3). Every three to four days afterwards, half the media was replaced with fresh media and IL-2. Cytotoxicity assays were performed at least four days after stimulation with fresh IL-2. After 15 days the cells were frozen in multiple aliquots containing $2x10^6$ cells per vial and stored long-term in liquid nitrogen.

2.6 Transfection and transduction of cells

2.6.1 Mirus Bio – TransIT Jurkat Transfection Reagent

$1.5x10^6$ Jurkat T cells (an immortalised human leukaemic T cell line) were pelleted by centrifuging at 1400rpm for 10 mins and resuspended in 2 mls complete media and seeded into a single well of a 6-well plate. In an eppendorf, 1-5µl of TransIT reagent (MirusBio, Wisconsin, USA) was added dropwise into 200µl OptiMEM serum free media, mixed thoroughly and incubated at room temperature for 20 mins. 2µg of DNA was added to the diluted TransIT reagent and incubated at room temperature for 15 mins. The DNA Suspension was then added dropwise into the cells and the plate was rocked gently to distribute the complexes evenly. The cells were then incubated at 37°C under 5% CO_2.

2.6.2 Invitrogen – Lipofectamine LTX and PLUS Reagent

$2x10^5$ Jurkat T cells were pelleted and resuspended in 2mls complete media and seeded into a single well of a 6-well plate. In an eppendorf, 1-2µg of DNA was diluted in 500µl Opti-MEM (Gibco, Paisley, UK) serum free media and mixed thoroughly. 1-2µl of PLUS reagent (Invitrogen, Paisley, UK) was added to the diluted DNA, mixed thoroughly and incubated at room temperature for 5 mins. 1.5-5µl Lipofectamine LTX reagent (Invitrogen, Paisley, UK) was then added to the diluted DNA, mixed thoroughly and incubated at room temperature for 30 mins. The DNA:Lipofectamine complexes were then added dropwise into the cells in the 6-well plate and mixed gently by rocking the plate back and forth. The cells were then incubated at 37°C under 5% CO_2. The cells were cultured for at least 48 hours before testing for transfected protein production.

2.6.3 Lentiviral Transfection and Transduction

2.6.3.1 Lentiviral vectors

Bicistronic lentiviral vectors (Clontech, Saint-Germain-en-Laye, France) pLVX-IRES-puromycin and pLVX-IRES-neomycin (Figure 2.5) were used as well as pTRIP (kind gift from Dr P. Balfe).

2.6.3.2 Transfection of Cells to Produce Lentivirus Particles

Human embryonic kidney-293T cells (HEK-293T) were grown in monolayers in T25 flasks overnight in 293 media (Dulbecco Modified Eagle Medium (Gibco, UK) supplemented with 10% FCS and penicillin/streptomycin). Lipofectamine LTX transfection reagent (Invitrogen, Paisley, UK) was used to transfect the HEK-293T

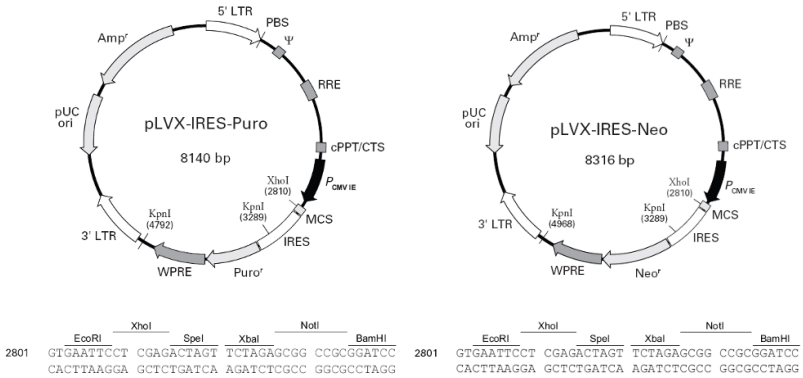


Figure 2.5 Lentiviral Expression Vectors Used for Transduction

The Rituximab-NLV antibody, containing the mutated CDR3 region to include the CMV-derived peptide, was originally in the pLVX-IRES vector with the heavy chain upstream of the IRES sequence and the light chain downstream of the IRES sequence. Soon after, the heavy and light chains were split up and ligated into two different vectors to allow for antibiotic selection. The heavy chain was placed into the pLVX-IRES-Puromycin vector and the light chain was placed into the pLVX-IRES-Neomycin vector.

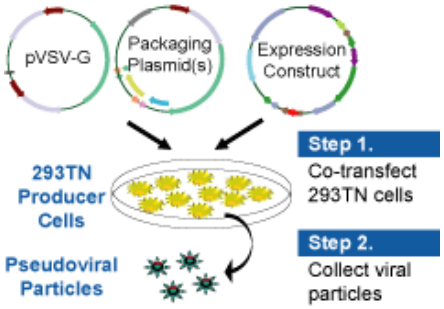


Figure 2.6 Production of lentiviral particles used for transductions

The lentivirus vectors (VSV-G, Gag-Pol and expression construct) are transfected into the 293T or Lenti-X 293T cells. The 293T cells produce the lentiviral particles after 24-48 hours. The virus particles are harvested ready for transduction studies.

cells. Briefly, dilute 8µg total DNA (4µg Gene of Interest, 1.5µg VSV-G & 2.5µg Gag-Pol) in 1000µl OptiMem (Gibco, UK) and incubate at room temperature for 5 mins (Figure 2.6). Add 10µl PLUS reagent to diluted DNA and incubate at room temperature for 10 mins. Add 15µl of Lipofectamine LTX reagent to mixture and incubate at room temperature for 30 mins. The cells are prepared for transfection by removing the media from the overnight incubation and washing once gently with OptiMem. The DNA-reagent mixture is then gently pipetted onto the HEK-293T cells and the flask laid flat at 37°C for 4 hours before adding 2mls of fresh HEK-293T cell media. The cells are then incubated overnight at 37°C.

After 24hrs, the media is removed and 3mls fresh HEK-293T cell media is gently pipetted onto the cells. The cells are then left at 37°C overnight and the media is harvested as this will contain the lentivirus particles. Fresh media is again pipetted onto the cells and incubated overnight at 37°C. The media, containing more lentivirus particles, is then harvested and added to the initial harvest. The media is then spun at 2000rpm for 10 mins to remove any cell debris from the harvest before being aliquoted into 1ml tubes and frozen at -80°C until required.

2.6.3.3 Alternative Method to Transduce Cells Using Lentivirus

After transfecting the packaging cell and prior to production of lentivirus particles, $5x10^6$ target cells were washed in RPMI and re-suspended in 3mls RPMI + 10% FCS. After removing the spent media from the packaging cell line, the washed target cells were added to the packaging cell line and co-cultured for 48 hours at 37°C to allow transduction to occur. After 48 hours, transduced target cells were removed form the

flask and washed in RPMI and re-suspended in 5mls RPMI + 10% FCS and cultured in a 25cm^2 flask. After a further 48 hours, the cells were tested for protein production.

2.6.3.4 Production of Rituximab-NLV

DNA encoding the heavy and light chain of Rituximab was taken from DrugBank (http://www.drugbank.ca/drugs/DB00073, v2.5, accessed 19 November 2009) and the heavy chain CDR3 region mutated to incorporate the CMV epitope NLVPMVATV. DNA encoding the Rituximab sequence was ordered from GenScript (New Jersey, USA).

In order to produce lentiviral particles containing the DNA encoding mutated Rituximab, 3 different methods of transfection were employed. Firstly, DNA encoding the heavy chain (2µg) and light chain (2µg) was mixed together before adding the transfection reagent LTX to it and incubated for 20 minutes at room temperature (A). Lenti-X HEK-293T cells (Clontech, Saint-Germain-en Laye, France) were transfected with the DNA-LTX mixture for 30 minutes. 5mls of fresh DMEM + 10% FCS was added and the cells were incubated overnight at 37°C. The second method added the LTX to the heavy chain in one eppendorf and LTX to the light chain in another eppendorf. The two mixtures were incubated at room temperature for 20 minutes and would be mixed in the flask during transfection (B). Lenti-X HEK-293T cells were transfected with the two DNA-LTX mixtures for 30 minutes. 5mls of fresh DMEM + 10% FCS was added and the cells were incubated overnight at 37°C. The third method again contained LTX-Heavy chain in one eppendorf and LTX-light chain in another eppendorf, again incubated for 20 mintues (C). Two flasks of cells were used for transfection, one was transfected with the LTX-heavy chain and the

other was transfected with the LTX-light chain. Lenti-X HEK-293T cells were transfected with the DNA-LTX mixture for 30 minutes. 5mls of fresh DMEM + 10% FCS was added and the cells were incubated overnight at 37°C. After 24 hours, media was removed from flasks A and B and 5mls of fresh media was added. The media in the two flasks for C were swapped so that the media in the heavy chain flask was placed in the light chain flask and the media from the light chain flask was placed in the heavy chain flask. This was to ensure that the cells would be infected with DNA encoding for both the heavy and light chain. All of the flasks were incubated for a further 48 hours at 37°C. The media was removed from all flasks and replaced with fresh media containing 2µg/ml puromycin and 250µg/ml G418. Transfected cells were incubated for 4 days at 37°C until confluent and the media taken to assay for production of Rituximab-NLV by ELISA. Cells were fed with fresh media containing antibiotics and IgG depleted FCS and split once per week and the supernatant harvested and stored for purification.

2.6.3.5 Purification of Rituximab-NLV

Media containing Rituximab-NLV was spun at 3000rpm for 30-60 minutes to concentrate it to 1ml using 10kDa centrifugal filtration (Sartorius, Germany). This was then purified using either Protein A or Protein G columns (GE Healthcare, UK). After washing the column in PBS (made in-house from tablets, Oxoid (Hampshire, UK), the media was added to the beads and incubated at room temperature for 5 minutes. The column was spun at 13,000rpm for 30 seconds and the flow through discarded. The column was washed in 500µl PBS, spun again at 13,000rpm for 30 seconds, the flow through discarded and repeated two further times. The antibody was eluted from the column by adding 125µl 0.1M acetic acid (Sigma, UK) and

incubating for 3 minutes at room temperature. The column was spun for 30 seconds at 13,000rpm and the elution process repeated using another 125µl 0.1M acetic acid. The eluate was then buffered using 250µl 0.2M Na_2CO_3 (Sigma, UK) and stored at 4°C.

The purification of Rituximab-NLV was tested by ELISA using a mouse anti-human IgG antibody and an anti-mouse-HRP conjugated antibody (eBiosciences, UK). Purified Rituximab-NLV was coated on a 96 well flat bottomed plate (Nunc, Germany) at 37°C for 60 minutes. The plate was washed in PBS tween/PBS and the wells were blocked with 2% BSA (eBiosciences, UK) for 60 minutes at room temperature. After washing, the mouse anti-human IgG antibody (Sigma, UK) was diluted 1:500 in PBS and added to each well. The plate was incubated at room temperature for 60 minutes and washed. The second antibody anti-mouse HRP was diluted 1:500, added to each well and incubated at room temperature for 60 minutes. The plate was washed extensively before addition of TMB and the plate was left to allow blue colour to develop for 10-15 minutes. After colour had developed, 10% HCl was added to each well to stop the reaction and the wells were read using the GloMax 96 well plate luminometer (Promega, UK).

2.6.3.6 Formation of an Immune Complex

Heating

Rituximab-NLV was heated to 65°C for 10-30 minutes to induce unfolding and aggregation of the protein. The antibody was then incubated at 37°C for 15 minutes before being used for further work.

Anti-Kappa Antibody

In order to form a complex which is closer to physiological conditions, an anti-kappa antibody (Generated in-house and kind gift from Dr Margaret Goodall) was used to cross-link the kappa light chain used by Rituximab. Equal amounts of Rituximab-NLV and anti-kappa antibody were mixed and incubated for 30 minutes at room temperature. The antibody complex was then ready to use for further work.

2.6.3.7 Concentration of Lentivirus Particles

Lentiviral particles were concentrated using Lenti-X concentrator (Clontech, Saint-Germain en Laye, France). After spinning the lentiviral supernatant to remove cellular debris, the supernatant is diluted 3 parts supernatant to 1 part Lenti-X concentrator and mixed gently. The mixture is incubated at 4°C for 1 hour before being spun at 1500g for 45 mins. The supernatant is removed and the pellet is resuspended in fresh DMEM + 10% FCS and aliquoted before being stored at -80°C.

2.6.3.8 Transduction of PBMCs and Cell Lines with Lentivirus

$1x10^6$ PBMCs or various cell lines per virus transduction were spun down and re-suspended in RPMI (Prepared in-house from powder ordered from Sigma (R6504)) + 10% FCS + penicillin/streptomycin (Sigma, UK) at 100µl per virus transduction. 100µl of cells were added to individual wells of a 24-well plate. 500-1000µl of virus cell supernatant (stored as described previously) was added to the cells and incubated at 37°C for 4-6 hours. 1.5mls of fresh RPMI media was added on top of the transductions and the cells were incubated at 37°C for at least 48 hours and they could then be tested for protein expression.

2.7 Conjugation of Peptide to Antibody

2.7.1 Peptide Tagging Antibodies

Antibody clone BU69 (Generated in-house, kind gift from Dr Margaret Goodall, 500µg/ml), anti-CD70, was used to attach the peptides ordered from GenScript, UK (Table 5.1). BU69 (500µg) was mixed with 1.2mg Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate (SMCC (Sigma; Dorset, UK)) previously dissolved in 400µl PBS and incubated at room temperature for 1 hour. Excess SMCC was removed from the antibody using a protein G column (GE Healthcare, UK). Briefly, the protein G column was washed in PBS and the antibody allowed to bind to the protein G beads for 5 minutes. The column was washed 3 times with PBS to remove the excess SMCC and the antibody eluted with 2x 125µl 0.1M acetic acid. The eluted antibody-peptide complex was buffered with 250µl 0.2M Na_2CO_3. Peptides were dissolved in DMSO to 10mg/ml and 2µl of peptide was added to 1µl TCEP to reduce the cysteinylated peptides and incubated at room temperature for 10 mins. 100µl of SMCC-antibody was added to each of the peptides and incubated at room temperature for 2 hours. To remove excess peptide from the antibody complex, the complexes were purified using the protein G column again. The complexes were eluted in acetic acid and buffered with Na_2CO_3 again before being stored at 4°C.

2.7.2 Staining Cells with Peptide-Tagged Antibodies

Target cells were stained with 10µl biotinylated peptide conjugated antibody for 30 minutes at room temperature, washed and 1µl streptavidin-PE added (Invitrogen. Paisley, UK). After incubating at room temperature for 20 minutes, the cells were washed and analysed on the flow cytometer to ensure that the conjugated antibody

could bind to cells (Figure 2.7). For T cell recognition, $1x10^5$ target cells were seeded into 96 well V-bottom plates, spun down and washed before being re-suspended in 100µl media. 25-50µl peptide tagged antibody was added to the cells and incubated at room temperature for 30 mins. The cells were then washed in RPMI and spun down with the supernatant discarded. The antibody-coated cells were re-suspended in 200µl RPMI + FCS and incubated overnight (~14hrs) before the cells were used for T cell recognition using IFN-gamma ELISA and/or FACS Killing Assay.

2.7.3 FACS-Based Cell Targeting Assay

$1x10^5$ target cells were separated into two tubes with one population stained with 10µl anti-CD45 APC-Cy7 (Becton Dickinson, UK) and the second population stained with 10µl anti-CD45 FITC (Becton Dickinson, UK). The cells were incubated at room temperature for 30 minutes before being washed twice in RPMI. The cells previously stained with anti-CD45 APC-Cy7 were stained with the peptide-tagged antibody and incubated at room temperature for 30 minutes before being washed twice in RPMI. The two cell populations were mixed and seeded into a single well of a 96 well V-bottom plate in 100µl media. $1x10^4$ peptide specific T cells were washed in RPMI and resuspended in 100µl media before being added to the same well. The cells were left for 6 hours at 37°C before being washed in RPMI and moved into FACS tubes. The cultured cells were stained with anti-CD4 APC to discriminate the effectors ($CD4^+$) from the targets ($CD4^-$). The cells were then washed and run on the flow cytometer.

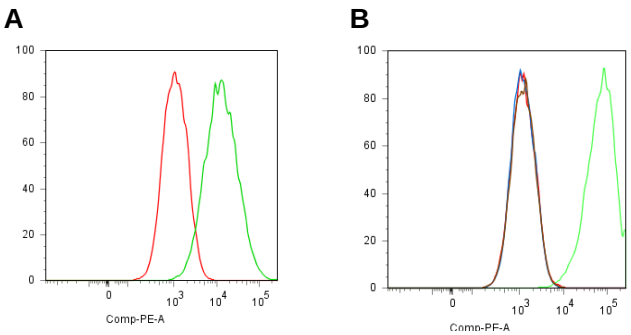


Figure 2.7 Staining cells using peptide conjugated CD20

Cells were stained with either commercially available anti-CD20 (A) or peptide-conjugated rituximab (B). After 30 mins, the antibodies were washed off and streptavidin-PE added for 20 mins. The cells were washed and analysed using the flow cytometer. Results demonstrate the ability of the peptide-conjugated Rituximab to bind to the B cells showing that the conjugation of the peptides does not interfere with the antibody binding domain.

2.7.4 Staining Target Cells with Peptide-Conjugated Secondary Antibody

In order to test a number of antibodies easily, an anti-mouse IgG secondary antibody conjugated with the viral peptide (as previously discussed) was used. Briefly, target cells were stained with a primary antibody, not previously conjugated with a peptide, for 30 mins at room temperature. After washing once in PBS (made in-house from tablets, Oxoid (Hampshire, UK), the target cells were stained with the secondary antibody conjugated with the viral peptide. After incubation for 30 mins at room temperature, the cells were washed in PBS (made in-house from tablets, Oxoid (Hampshire, UK) and antibody-coated cells were re-suspended in 200µl RPMI + FCS and incubated overnight (~14hrs) before the cells were used for T cell recognition using IFN-gamma ELISA (eBiosciences, UK) and/or FACS Cell Targeting Assay.

2.7.5 Quantification of Protein Concentration

Protein concentration was quantified using the Qubit assay (Invitrogen, Paisley, UK). To make the test buffer for each sample, 199µl buffer was added to 1µl of protein assay reagent. Firstly, 3 standard protein solutions were used to calibrate the machine. 10µl of each standard solution was added to 190µl test buffer and incubated at room temperature for 15 minutes. The samples were then read using the Qubit fluorimeter to calibrate the machine. Five microlitres of the protein solution to test was added to 195µl of test buffer and incubated for 15 minutes. The samples were then read on the Qubit fluorimeter which could then produce the concentration of protein in each sample.

2.8 Statistical Analysis

Results were analysed and presented using GraphPad Prism 5 (version 5.01). For comparisons between donors in HLA-positive and HLA-negative cohorts, chi-squared test was used. For comparisons between unpaired data sets, an unpaired t-test was performed. All *p* values were considered significant if less than 0.05. Apart from discussions with supervisors, no further statistical support was sought.

3. Immunoediting of the B Cell Receptor Repertoire

3.1 Introduction

B cells can express B cell receptors on the cell surface, which may contain potentially immunogenic peptides within the "non-self" CDR3. These peptides could potentially initiate an immune response if they were to be presented at the surface of the B cell in complex with MHC molecules. Unlike T cells, B cells can express membrane-bound receptors and secrete soluble B cell receptors (immunoglobulin). Therefore, if the receptor contained potentially immunogenic peptides within the CDR3, secreted antibodies containing these peptides could be taken up by many cells (dendritic cells for instance) and initiate immune responses against them by priming the immune system to the CDR3 of a B cell receptor leading to autoimmune responses against B cells.

I set out to test the hypothesis that B cells bearing BCRs containing HLA-binding peptides within their CDR3s are not subject to peripheral immunological tolerance and are deleted in what could possibly be a further round of B cell selection after selection in the bone marrow. If there is no peripheral immunological tolerance towards non-germline encoded CDR3-derived peptides, in an HLA-A*0201^{+} individual (henceforth denoted HLA-A2), any B cells bearing BCRs containing potential HLA-A2 binding peptides would be deleted. However, B cells bearing BCRs containing peptides binding non-self HLA alleles would survive because the host simply would not and could not target them (Figure 3.1). Such a model would predict for a decreased frequency of HLA-A2 binding peptides within the CDR3s of B cell receptors taken from HLA-A2^{+} donors compared with the frequency from A2^{-}

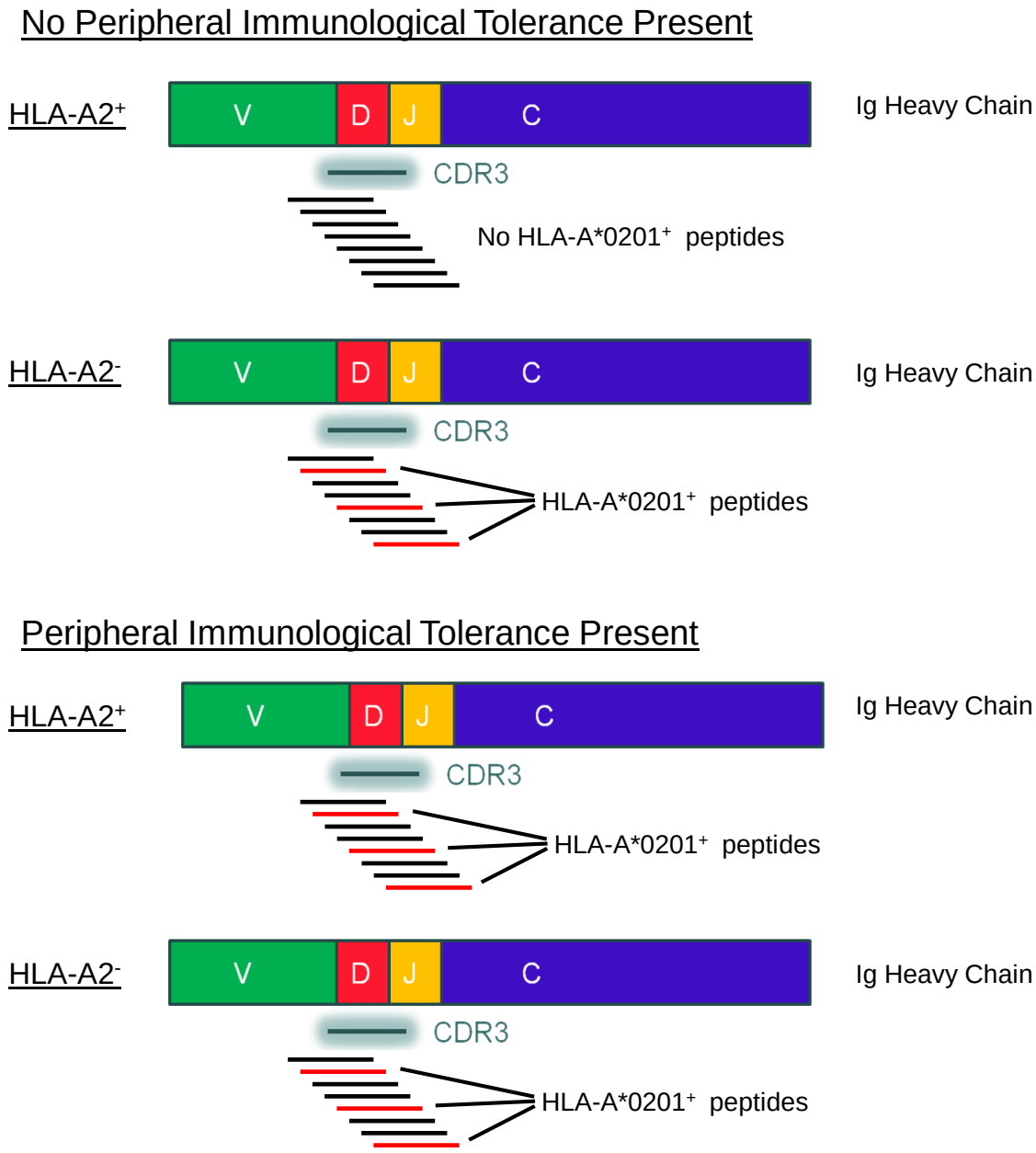


Figure 3.1 Molecular hypothesis for deletion of B cells containing predicted HLA-A*0201 peptides within their CDR3

If there is no immunological peripheral tolerance, a B cell from an HLA-A*0201$^+$ individual containing potential A2-binding peptides derived from the CDR3 region would undergo deletion as the peptide could be presented on the surface of the B cell in complex with HLA-A2. This would result in a decreased frequency of HLA-A2 binding peptides within the CDR3 region of HLA-A2$^+$ donors compared with HLA-A2$^-$ donors. However, if there is immunological tolerance towards CDR3-derived peptides, the frequency of HLA-A2 binding peptides in HLA-A2$^+$ donors would be similar to that from HLA-A2$^-$ donors.

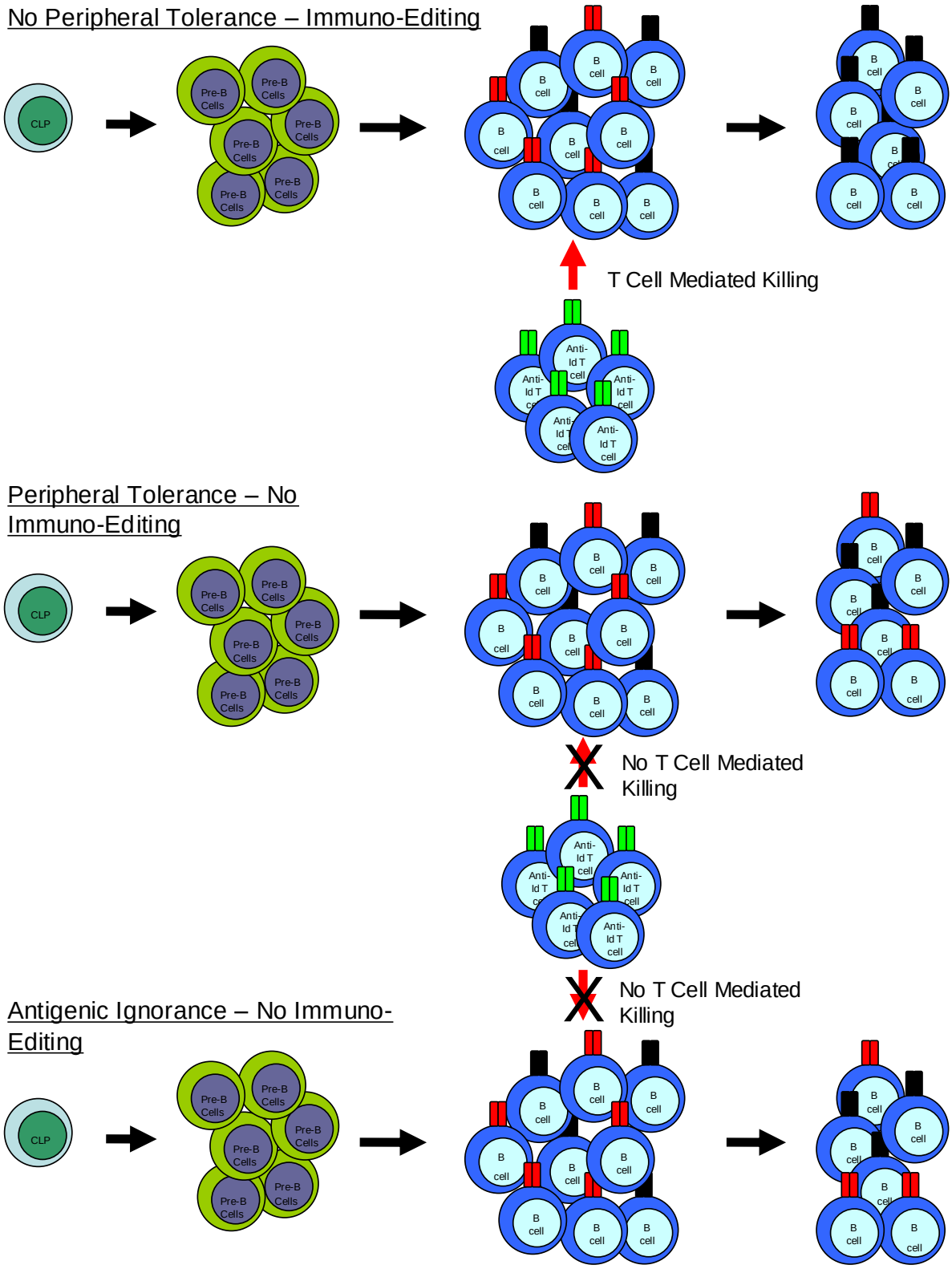
donors. There are two alternative competing hypotheses which would satisfy the lack of any immunoediting of the peripheral BCR repertoire. Firstly, tolerance against BCRs akin to any other self antigen would be subject to peripheral immunological tolerance. Secondly, it is conceivable that BCRs do not access the antigen processing machinery of B cells and are thus privileged antigens resulting in antigenic ignorance. Therefore, there would be no need for B cells containing potential HLA-A2 binding peptides within the CDR3 to be edited from the B cell repertoire, meaning CDR3-derived peptides could not be presented at the surface of the B cell in complex with HLA. Consequently, B cells which contain potential A2-binding peptides could populate the periphery along with B cells which do not (Figure 3.2). Such models would therefore predict the frequency of HLA-A2 binding peptides within the CDR3s of BCRs from an HLA-$A2^+$ donor to be similar to the frequency seen in HLA-$A2^-$ donors as a result.

3.2 Cloning the CDR3 of B Cell Receptors

For the purpose of this work, the Ig heavy chain was used as this has an extra region within the CDR3, providing increased variability. Therefore, the presence of HLA-binding peptides may be increased in the Ig heavy chain when compared to the Ig light chain but the study could possibly be extended to include the Ig light chain. Previous studies suggest that the majority of predicted peptides found within the Ig heavy chain are found within the CDR3 (Hansson et al, 2003). After amplifying the DNA using an oligonucleotide primer specific for the BCR framework region 1 and a second primer specific for the constant region, positive bands were seen at ~550bp (Figure 3.3). The band, containing many different Ig heavy chains from the framework region 1 to the constant region, was cut out and the DNA was purified

Figure 3.2 Cellular hypothesis for deletion of B Cells containing predicted HLA-A*0201 peptides within their CDR3

If there is no peripheral immunological tolerance, a B cell from an HLA-A*0201^{+} individual containing potential A2-binding peptides derived from the CDR3 region (Red) would undergo deletion, by anti-idiotype-specific T cells (Green), as the peptide could be presented on the surface of the B cell in complex with HLA-A2. BCRs which do not contain a potential HLA-A2 binding peptide within the CDR3 region (Black) would populate the periphery. Therefore, peripheral tolerance towards CDR3-derived peptides would result in a decreased frequency of HLA-A2 binding peptides within the CDR3 region of HLA-A2^{+} donors. However, if there is immunological tolerance, or antigenic ignorance, towards CDR3-derived peptides, the B cells containing a BCR with a CDR3-derived peptide would be allowed to populate the periphery and the anti-idiotype-specific T cells would not recognise the B cells. Therefore, the frequency of HLA-A2 binding peptides, within the CDR3 region, in HLA-A2^{+} donors would be similar to that from HLA-A2^{-} donors.


No Peripheral Tolerance – Immuno-Editing
CLP
Pre-B Cells
B cell
T Cell Mediated Killing
Anti-Id T cell
Peripheral Tolerance – No Immuno-Editing
No T Cell Mediated Killing
Antigenic Ignorance – No Immuno-Editing

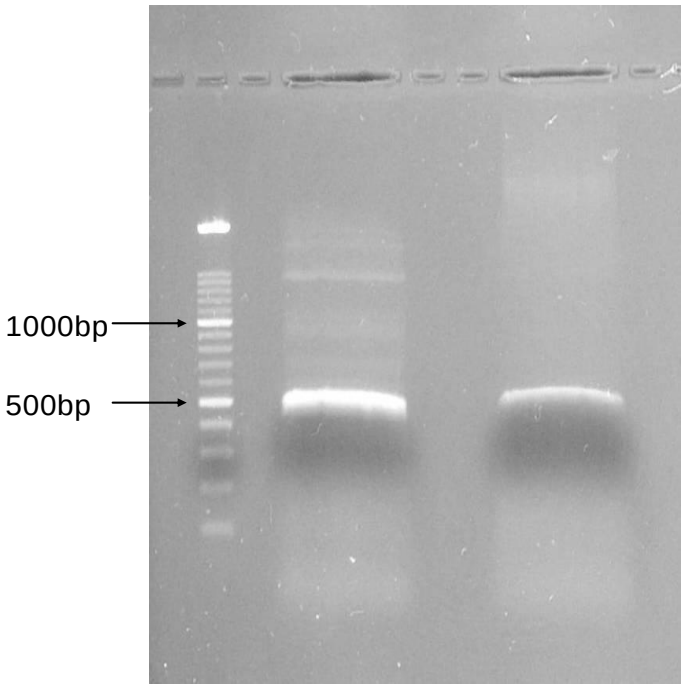


Figure 3.3 PCR to amplify all BCRs using cDNA generated from PBMCs

cDNA was synthesised from RNA, extracted from unfractionated PBMCs, and used as template DNA with one primer corresponding to the Ig framework region 1 within the variable region and a second set of primers which corresponded to the constant regions of IgA, IgG and IgM. After amplification, the PCR was run on a 1% gel and bands visualised using a transilluminator. Amplicons which were visualised at 500-550bp were excised and used for further experimentation.

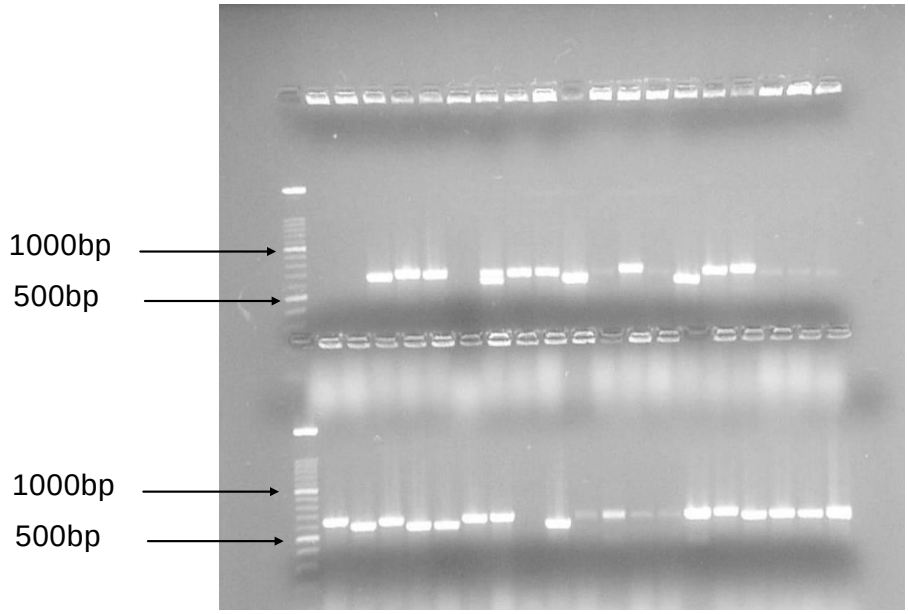


Figure 3.4 Colony PCR to determine BCR amplification

After ligating amplified BCR DNA into the TA cloning vector and transforming E. coli, colonies which had grown were tested for transformation of plasmid DNA encoding BCR DNA. Bacterial transformation produced >100 colonies, 20-40 of which were grown in 50µl LB + ampicillin for 4 hours. Bacterial cells were used as template DNA for PCR amplification using the M13 primer set found within the TA cloning vector. After amplification, PCR reactions were run on a 1% agarose gel and visualised using a transilluminator. Positive colonies contain a band of DNA ~550-650bp.

from the agarose gel. After TA cloning, colonies containing DNA encoding for BCRs were screened using colony PCR with amplicons running to a size of ~600-650bp (Figure 3.4) deemed positive and the DNA used for sequencing. The nucleotide base sequence was checked against known B cell receptor variable region sequences to determine the CDR3 sequence and to prevent analysis of repeated sequences.

3.3 HLA Peptide Prediction Algorithms

The extracted DNA was sequenced and the CDR3 determined for 109 BCRs from HLA-$A2^+$ donors and 125 BCRs from HLA-$A2^-$ donors. CDR3 sequences were analysed for the presence or absence of potential HLA-binding peptides using two different peptide binding matrix-based algorithms, SYFPEITHI (Rammensee et al, 1999) and BIMAS (Parker et al, 1994). Peptide prediction using SYFPEITHI depends on motif-based matrices relying on the presence of specific amino acid residues at the anchor positions and therefore these results may not be indicative of peptide affinity for HLA. However, peptide prediction using BIMAS uses the time taken for dissociation of the peptide from HLA and is therefore indicative of peptide affinity. These algorithms have been used in previous studies investigating the B cell and T cell CDRs (Trojan et al., 2000 & Hansson et al, 2003) and are generally regarded as the most reliable programs for this process. In order to determine the thresholds used to separate peptides that bind to HLA from those which do not, receiver operator curve (ROC) analysis was used. The accuracy of a test such, as the computer algorithms used in this study, can be evaluated using ROC analysis, known HLA-binding peptides and peptides expected not to bind to HLA.

3.4 Performance Analysis of HLA-A2 predictive algorithms

Peptides known to bind to HLA-A2 were sourced from the immune peptide database (IEDB; www.immunpeptide.org) and their binding scores determined using both SYFPEITHI and BIMAS. Random peptide sequences were generated, as non-binding HLA-A2 peptides, and their scores determined using both computer algorithms as well. These figures were plotted together to attempt to determine what the thresholds should be for both of the computer algorithms used in the analysis of my data set. The ROC analysis depends on both sensitivity and specificity and in this case, sensitivity can be defined as the proportion of true positive results of the total number of positive results (true positive + false negative). Specificity can be defined as the proportion of true negative results of the total number of negative results (true negative + false positive). Increasing the criterion value of the test will increase the specificity by decreasing the false positives but the sensitivity will decrease as well as there will be an increase in false negative results. Decreasing the criterion value of the test will increase the sensitivity as the number of true positives will increase but decrease the specificity by increasing the number of false positive results (Figure 3.5, taken from www.medcalc.be/manual/roc.php (Version 11.4)). It is important to select a threshold which provides the highest incidence of true positive and true negative results whilst maintaining the lowest incidence of false positive and false negative results.

3.4.1 SYFPEITHI - HLA-A2-Binding peptides

Analysing the data set (HLA-A2-binding peptides from IEDB) using SYFPEITHI, the cross-over of the curve for peptides that do bind and the curve for peptides that do not bind is ~18 (Figure 3.6). This score is lower than the threshold used in previous publications and may include too many false positive results. The ROC curve

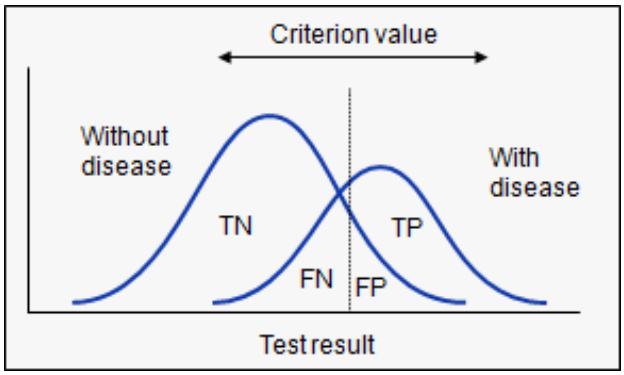


Figure 3.5 Generation and interpretation of receiver operator characteristic

The use of receiver operator characteristic is to try and determine the thresholds which should be set for the analysis of my data set. The graph shows the display of test results from patients without the disease and those with the disease and the possible outcomes of a test to determine whether the patient has the disease. By increasing the criterion value, the number of false positive results is reduced although there is a corresponding increase in the number of false negatives. This means there is an increase in specificity, due to the decrease in false positives, but there is a decrease in the sensitivity, due to the increase in false negatives. By decreasing the criterion value, there is an increase in sensitivity as there is an increase in the true positive results but there is also an increase in false positive results meaning there is a decrease in specificity. There is a need to balance the sensitivity of the test with the specificity.

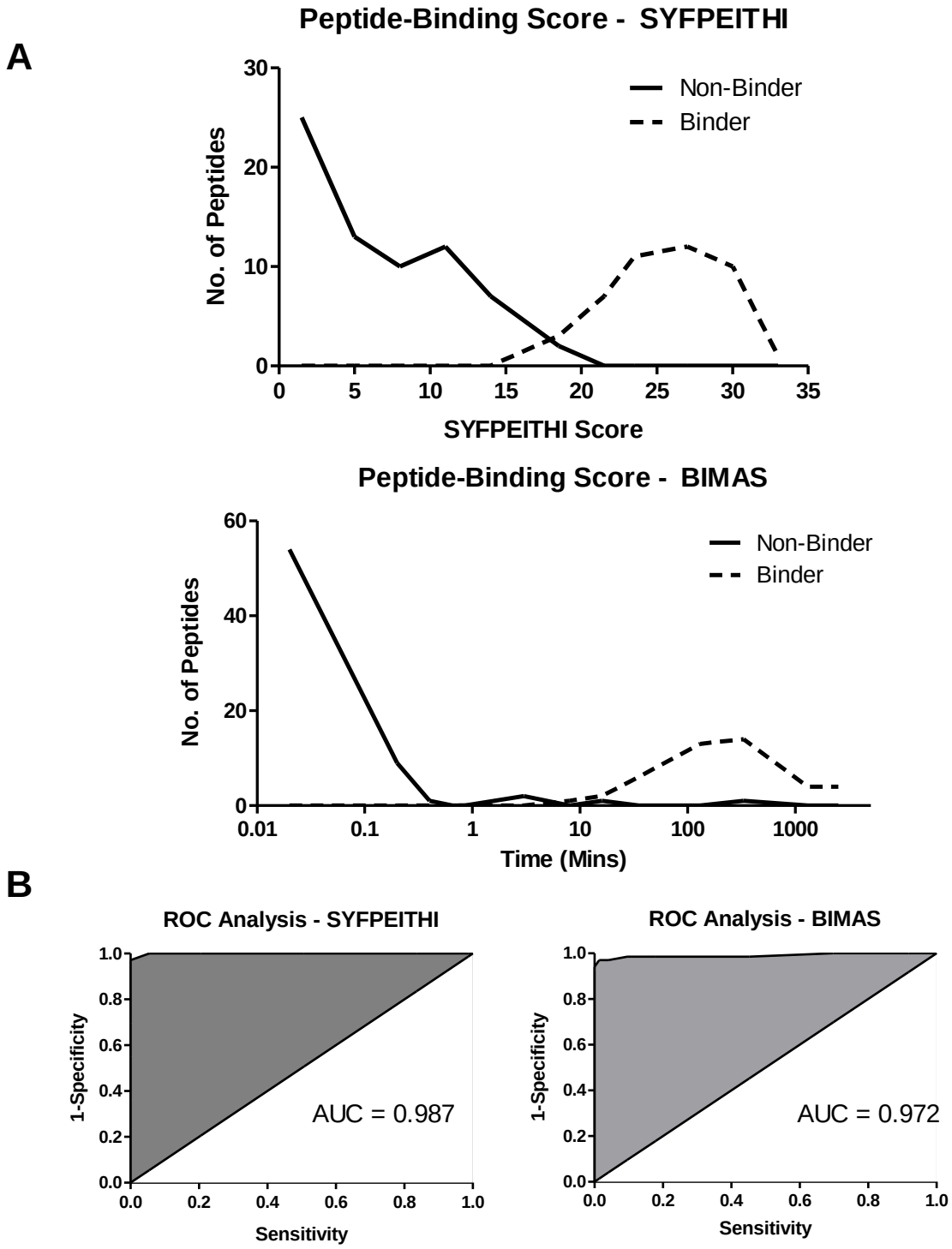


Figure 3.6 Receiver operator characteristic analyses of HLA-A2 using SYFPEITHI and BIMAS

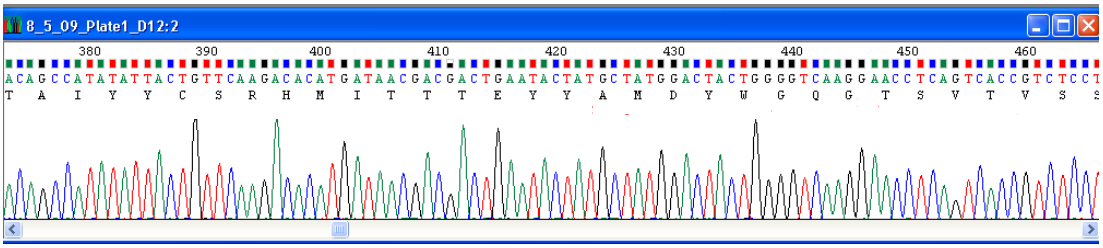
Two peptide data sets, A2-binders and A2-non binders, were analysed using both computer algorithms BIMAS and SYFPEITHI. The scores generated from the two data sets were plotted to determine the thresholds to be set for analysis of the TCR and BCR data sets (A) and the peptide prediction algorithms tested for their specificity and sensitivity using receiver operator curve (ROC) analysis (B).

produced an area under the curve (AUC) of 0.987 using GraphPad Prism 5 (version 5.01). The data set was analysed using a peptide binding threshold of 18 but this may be set too low and may not be used in all analysis. The presence of false positive peptides present could alter the results from the data set and I decided to also determine the SYFPEITHI score that would provide the maximum specificity. Although this would have an impact on the sensitivity, the peptides returned would almost definitely bind to HLA molecules. The curve for non-binding peptides reaches zero at 22, therefore in order to look at 100% specificity the SYFPEITHI score of 22 was also used for analysis.

3.4.2 BIMAS - HLA-A2 Binding peptides

The same data set from IEDB was analysed using BIMAS and the cross-over of the curve for the peptides that do bind and the curve for the peptides that do not bind is ~5 minutes (Figure 3.6). Although this is lower than previous publications, the BIMAS score that provides the maximal sensitivity as well as the maximal specificity would be greater than 5. The ROC curve produced an AUC of 0.972 using calculations by GraphPad Prism 5 (version 5.01). The data set was therefore analysed using a BIMAS binding threshold of greater than 5 to accommodate peptides with a lower affinity for HLA molecules. Similar to the SYFPEITHI analysis, I also wanted to determine the BIMAS score which would give the maximum specificity regardless of the sensitivity so that there would be little chance of any peptides returned being non-binders. Greater than 20 minutes, there is only 1 non-binding peptide (269 mins) and therefore, the BIMAS score with the maximum specificity would be greater than 20 minutes. The data set was analysed using a threshold of 20 minutes to accommodate peptides with a higher affinity for HLA molecules.

A



B

EDTAVYYCARGLPGDIVVVPAAIGGYYFDYWGQGTLVTVSSGSASAPT

Figure 3.7 Sequence analysis of the BCR

PCR products which contained a band ~600bp (Figure 3.4) were sequenced and analysed for the CDR3 sequence. The data returned for the sequencing is processed using the ChromasPro sequencing program which turns the coloured peaks (A: Green, C: Blue, G: Black and T: Red) into the nucleotide base sequence (A). The base sequence is then translated into the amino acid sequence encompassing the variable region (blue) leading into the CDR3 regions (red) which incorporates the diversity and joining regions (green). The 3' oligonucleotide primer is situated within the constant region of the Ig heavy chain and this results in the truncated sequence of the β-chain (pink) (B).

3.5 Frequency of Predicted HLA-A2-Binding Peptides from BCR CDR3

BCRs were sequenced from within the first framework region (FR1) of the variable region to the beginning of the constant region, incorporating the CDR3 (Figure 3.7). The sequence shows the end of the variable region moving into the diversity region followed by the joining region and finishes with the beginning of the constant region. Peptides were considered CDR3-derived when at least 1 amino acid was found within the CDR3 similar to previous published work (Hansson et al, 2003). Initially, the donor sequences were analysed for the presence or absence of HLA-A2 binding peptides within the CDR3s. A total of 109 BCRs from HLA-$A2^+$ donors and 125 BCRs from HLA-$A2^-$ donors, resulting in a total of over 8000 potential 9 amino acid peptides (3700 for HLA-$A2^+$ donors and 4600 for HLA-$A2^-$ donors), were analysed. The CDR3 sequences were analysed using two different HLA binding prediction algorithms, SYFPEITHI and BIMAS and the same thresholds discussed previously.

3.5.1 SYFPEITHI - High Scoring Peptides

The SYFPEITHI algorithm (version 1.0) was employed with a binding threshold of 22 in an attempt to analyse peptides that would almost definitely bind to HLA-A2. There was a statistically significant increase in the frequency of potential HLA-A2 binding peptides contained within the CDR3s of HLA-$A2^-$ donors (0.24%) in comparison with those from the HLA-$A2^+$ cohort (0.03%) (χ^2=6.43, p=0.01) (Figure 3.8). This result suggests that there is potentially no peripheral tolerance towards B cells expressing HLA-A2 binding peptides within the CDR3 in an HLA-$A2^+$ donor.

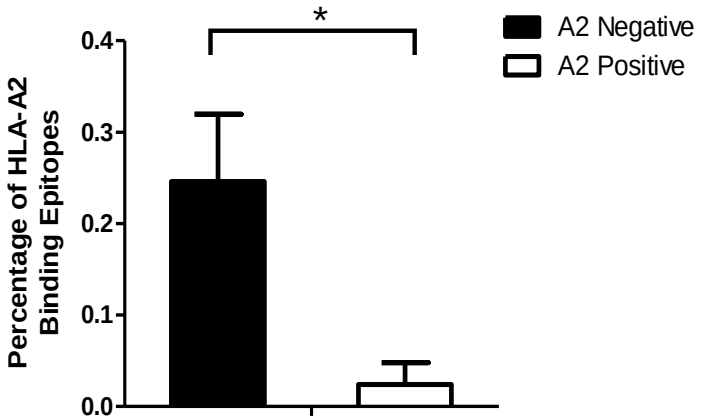


Figure 3.8 Frequency of HLA-A2 peptides present in the CDR3 of BCRs in HLA-A2$^+$ and A2$^-$ healthy donors

Amplified BCRs from healthy donors (separated into A2$^+$ and A2$^-$ cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A2-binding peptides using the SYFPEITHI algorithm. Data is represented as a percentage of the peptides from both donor cohorts analysed where peptide binding was defined by a score of 22 or above. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance, * $P<0.05$.

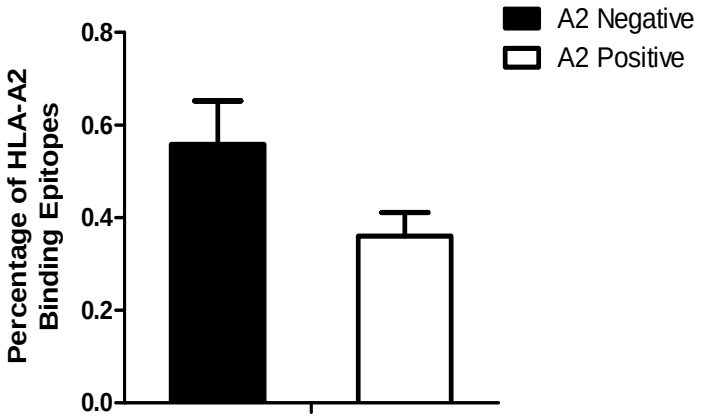


Figure 3.9 Frequency of high affinity HLA-A2 peptides present in the CDR3 of BCRs in HLA-A2$^+$ and A2$^-$ Healthy Donors

Amplified BCRs from healthy donors (separated into A2+ and A2- cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A2-binding peptides using the BIMAS algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 20 or above. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance.

Therefore, the data shown suggests that there may be editing of the BCR repertoire to remove potentially dangerous BCRs containing peptides predicted to bind to HLA-A2 from within the CDR3. Consequently, there may not be peripheral tolerance towards these peptides. The peptides were then analysed using the BIMAS algorithm to determine whether similar results were seen.

3.5.2 BIMAS - High Scoring Peptides

The BIMAS algorithm (originally accessed 10 August 2006 and not updated since) was then used with a peptide binding threshold of 20 in an attempt to include peptides that would almost definitely bind to HLA-A2 only. There is also a decrease in the frequency of peptides predicted to bind to HLA-A2 present in the CDR3 of BCRs in HLA-$A2^+$ (0.35%) when compared with the HLA-$A2^-$ cohort (0.54%) (χ^2=1.70, p=0.19) (Figure 3.9). Despite these results showing no statistical significance, they demonstrate a similar trend to those found when using the SYFPEITHI algorithm. Therefore, these results may show the presence of an immunoediting process in B cells although further studies would be needed to confirm this.

3.5.3 SYFPEITHI & BIMAS - Lower Scoring Peptides

After determining that there was a decrease in the percentage of peptides predicted to bind to HLA-A2 using the higher threshold from both computer algorithms, peptides predicted to bind to HLA-A2 at the lower threshold (using a threshold of 18) were investigated as well. Using SYFPEITHI with a peptide binding threshold of 18, there was still a decrease in the percentage of peptides predicted to bind to HLA-A2 from HLA-$A2^+$ donors (4.84%) compared with those from the $A2^-$ donors (5.16%) although this result was not statistically significant (χ^2=0.43, p=0.40) (Figure 3.10).

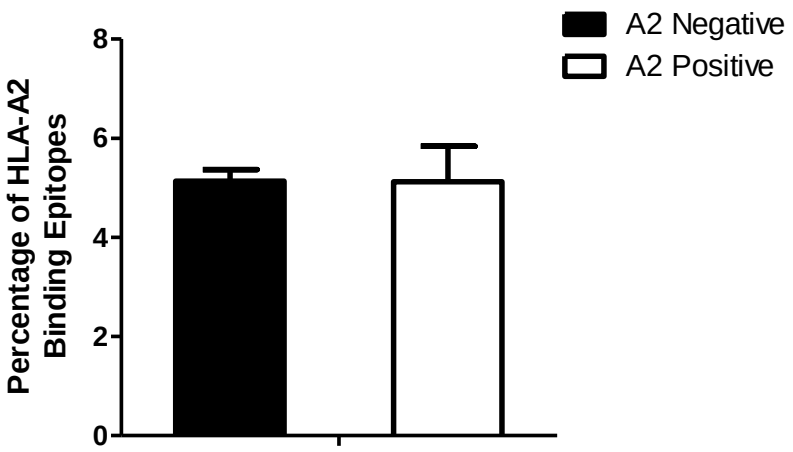


Figure 3.10 Frequency of HLA-A2 peptides present in the CDR3 of BCRs in HLA-A2$^+$ and A2$^-$ Healthy Donors

Amplified BCRs from healthy donors (separated into A2+ and A2- cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A2-binding peptides using the SYFPEITHI algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 18 or above. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance.

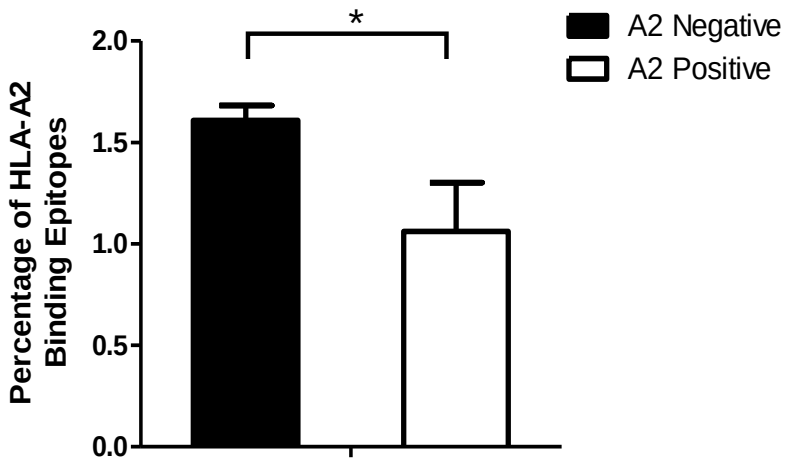


Figure 3.11 Frequency of high and low affinity HLA-A2 peptides present in the CDR3 of BCRs in HLA-A2$^+$ and A2$^-$ Healthy Donors

Amplified BCRs from healthy donors (separated into A2+ and A2- cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A2-binding peptides using the BIMAS algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 5 or above. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance, * $P<0.05$.

The results demonstrate a similar percentage decrease between the two cohorts when compared with the results using the higher threshold although the total percentage of peptides predicted to bind is very high and has increased to ~5%. These results suggest that using the lower threshold may provide similar trends to the higher threshold, but the results may be somewhat hidden by the large percentage of false positive results. Therefore, further analysis using SYFPEITHI will be limited to using the higher threshold of 22.

Using the BIMAS algorithm, the percentage of peptides predicted to bind to HLA-A2 with a low affinity (using a threshold of half-time for dissociation >5), combined with the high affinity peptides, demonstrated a statistically significant increase from the HLA-$A2^-$ cohort (1.61%) compared with the HLA-$A2^+$ cohort (0.97%) (χ^2=6.48, p=0.01) (Figure 3.11). Therefore, the results produced with BIMAS using the lower threshold suggest that there could be immunoediting of the B cell repertoire to remove HLA-A2 binding peptides from within the B cell CDR3 of HLA-$A2^+$ donors.

Analysis of the peptide sequences predicted to bind to HLA-A2 by both algorithms shows that the single peptide predicted to bind to HLA-A2 by SYFPEITHI in the $A2^+$ donors was also predicted to bind to HLA-A2 by BIMAS as well (Appendix A2). However, BIMAS also predicted a further ten peptides to bind to HLA-A2 that SYFPEITHI did not. In the $A2^-$ donors, 8/28 peptides were predicted to bind to HLA-A2 by both algorithms compared to 3/28 predicted by SYFPEITHI alone and 17/28 by BIMAS alone (Appendix Table A2). These results show that although there is some cross-over between the two prediction algorithms, the results obtained from both are not identical reflecting the differences in their programming.

3.6 Frequency of BCRs Containing HLA-A2 Binding Peptides

Although not significant, the above results suggest that there may be an immunoediting process in the B cell repertoire based on the frequency of predicted HLA-A2 binding peptides. However, the increased frequency seen in HLA-$A2^-$ donors could be the result of skewed data where one or two B cells contain a BCR with a large number of peptides within its CDR3 sequence, accounting for the difference in peptide frequency. In order to determine if the frequency of HLA-binding peptides is evenly distributed in the data set, the frequency of BCRs containing more than one HLA-A2 binding peptide was investigated. A similar decrease in the frequency of BCRs containing an HLA-A2 binding peptide would strengthen the argument that there is possibly editing of B cells. Using SYFPEITHI, there is an increased frequency of BCRs containing an HLA-A2 binding peptide in HLA-$A2^-$ donors (1.6%) compared with HLA-$A2^+$ donors (0.0%) (Figure 3.12). A similar trend is seen in the BIMAS analysis using both the high and low thresholds with an increased frequency seen in HLA-$A2^-$ donors 1.6% (20) and 16.0% (5) compared with HLA-$A2^+$ donors 0.0% (20) and 3.7% (5).

Although many of the results do not demonstrate statistical significance, the trends seen suggest that B cells from HLA-$A2^+$ donors, containing CDR3-derived peptides predicted to bind to HLA-A2, are edited from the B cell repertoire. However, in HLA-$A2^-$ donors, those B cells expressing a receptor which contains a peptide predicted to bind to HLA-A2 are not deleted simply because the peptides cannot be presented at the cell surface. Taken together, these results suggest that the original hypothesis may be true and therefore there is a possibility that there is no

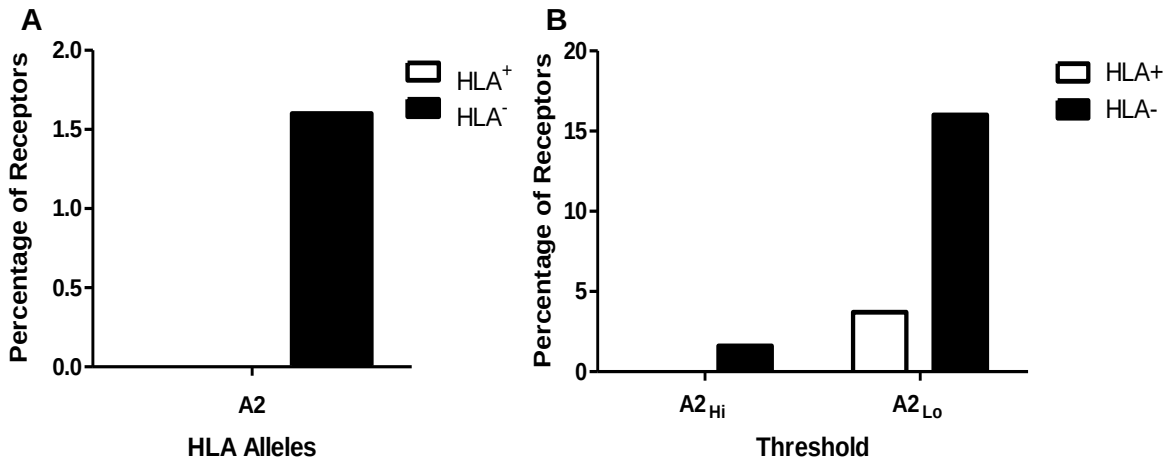


Figure 3.12 Frequency of BCRs containing more than one HLA-A2 binding peptide

BCRs from healthy donors (separated into A2+ and A2- cohorts) were analysed to determine the frequency of those containing more than one HLA-A2 binding peptide using both peptide prediction algorithms. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 22 or above using SYFPEITHI (A), a score of 20 or above using $BIMAS_{hi}$ (B) and a score of 5 or above using $BIMAS_{lo}$ (B). This was done in an attempt to determine whether there was skewed data in the previous analysis of the peptide prediction data due to a single BCR containing a large number of HLA binding peptides within the CDR3.

peripheral tolerance towards B cells expressing a BCR containing CDR3-derived peptides predicted to bind to HLA-A2.

3.7 Performance Analysis of HLA-A1, -B7 and -B8 predictive algorithms

The donors were also chosen to provide an equal number of donors who were positive or negative for HLA-A1, -B7 and -B8 (five donors for the HLA^+ cohort and five donors for the HLA^- cohort). However, in order to analyse the data to determine the presence or absence of CDR3-derived peptides, thresholds for these HLA alleles had to be set. As discussed earlier, in the context of setting the threshold for HLA-A2 (section 3.4, page 109), receiver operator curve analysis was used with a set of peptides taken from IEDB (Figure 3.13). AUCs for each HLA allele and computer algorithm were calculated using GraphPad Prism 5 (version 5.01).

3.7.1 SYFPEITHI

Analysing the peptides using SYFPEITHI, the thresholds were set where no randomly generated peptide sequences, used as those which do not bind to HLA, were predicted to bind to the HLA allele. Above this threshold, it is possible that almost every peptide would bind to HLA and this would provide data with 100% specificity for each of the alleles. However, in setting the threshold this high, it is conceivable HLA-binding peptides with a lower score could be overlooked. Unfortunately, it is not possible to include the lower scoring peptides without including some of the higher scoring non-binding peptides and for this analysis, it was more important to analyse peptides that would almost definitely bind to HLA. Therefore, the thresholds were set

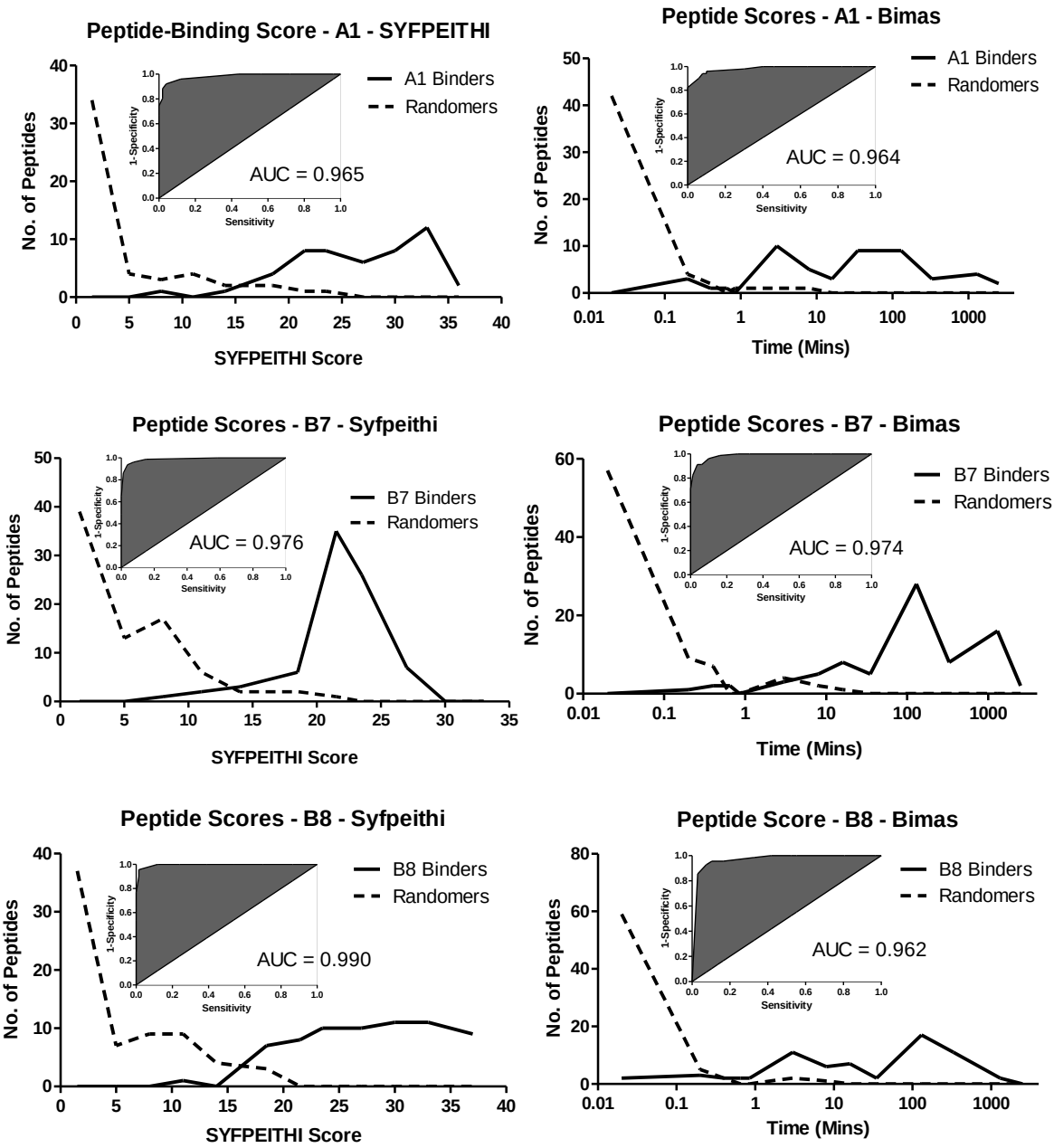


Figure 3.13 ROC analysis to determine thresholds for HLA-A1, -B7 and -B8

Data sets for HLA-binders and HLA-non binders for the chosen alleles, were analysed using both computer algorithms BIMAS and SYFPEITHI. The scores generated from the two data sets were plotted to determine the thresholds to be set for analysis of the TCR and BCR data sets and the computer algorithm for each allele and each peptide prediction algorithm determined by ROC analysis (inset).

at 25 for HLA-A1, 22 for HLA-B7 and 21 for HLA-B8. There was no lower threshold set as previous data using HLA-A2 had shown that lowering the threshold produced a large number of peptides that were unlikely to bind to HLA-A2 and therefore the real A2-binding peptides were hidden. It would be highly likely that the same would occur with the other HLA alleles analysed and therefore only the higher thresholds were used for analysis.

3.7.2 BIMAS

Analysing the same data using the BIMAS computer algorithm, the upper threshold was again set to the time where no randomly generated peptides bound to the HLA alleles. This resulted in the thresholds set as 12 for HLA-A1, 16 for HLA-B7 and 12 for HLA-B8. The lower thresholds were set to 5 for all HLA types as most of the HLA-binding peptides produced a score of greater than 5 and there were very few non-binding peptides with a score greater than 5. This score would provide the maximum sensitivity and maximum specificity for each of the HLA alleles.

3.8 Broader Analysis of HLA-Binding Peptides

Once the results for HLA-A2 had been completed, the sequences were analysed using non-HLA-A2 alleles, HLA-A1, -B7 & -B8 to determine if the results seen with HLA-A2 were consistent across the other HLA types tested.

3.8.1 SYFPEITHI – High Scoring Peptides

Using SYFPEITHI, with the peptide binding thresholds previously described (section 3.7.1), the number of HLA-binding peptides in donors within the HLA positive

cohorts was slightly decreased compared with the HLA negative donors in all of the other HLA types (Figure 3.14). Similarly to HLA-A2, there is a small increase in the peptides predicted to bind to HLA-B7 in the HLA-$B7^-$ cohort (0.05%) compared with the HLA-$B7^+$ cohort (0.00%) (χ^2=2.06, p=0.15). Analysis of the peptides predicted to bind to HLA-B8 showed a decreased percentage in HLA-$B8^+$ donors (0.19%) compared with the HLA-$B8^-$ donors (0.24%, χ^2=0.24, p=0.61). Again, there is a decrease in the peptides predicted to bind to HLA-A1 in the HLA-$A1^+$ cohort (0.37%) compared with the HLA-$A1^-$ cohort (0.47%) (χ^2=0.44, p=0.51). None of the above results were statistically significant but the trend suggests that the hypothesis could be true for all of the HLA types tested. Although further work may be required to generate statistically significant data, the results presented suggest there may be selective depletion of B cells containing CDR3-derived peptides in the HLA alleles tested.

3.8.2 BIMAS – High Scoring Peptides

Using the second peptide prediction algorithm BIMAS, with the higher binding thresholds to incorporate peptides with a higher binding affinity for HLA, there is a general trend seen with all three HLA types with a small increase in the percentage of predicted peptides in the HLA negative donors compared with the HLA positive donors (Figure 3.15). Analysis of all three HLA alleles demonstrated similar results with an increase in $A1^-$ donors (1.75%) compared with the $A1^+$ donors (1.64%) (χ^2=0.16, p=0.69), an increase in $B7^-$ donors (0.59%) compared with $B7^+$ donors (0.238%) (χ^2=0.23, p=0.63) and also an increase in $B8^-$ donors (1.84%) compared with $B8^+$ donors (1.77%) (χ^2=0.05, p=0.82). Although none of the results are statistically significant, the trend shown in all HLA types is similar to that seen when

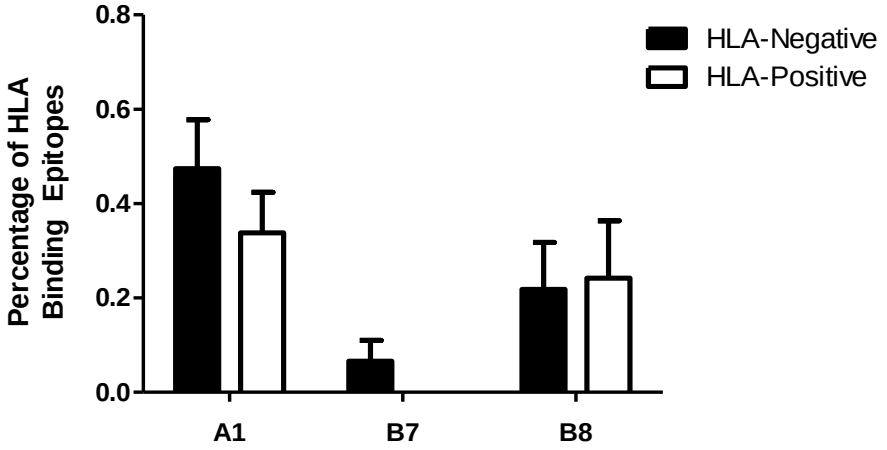


Figure 3.14 Frequency of peptides predicted to bind to HLA-A1, -B7 or -B8 present in the CDR3 of BCRs

BCRs from healthy donors (separated into HLA-positive and HLA-negative cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A1, -B7 and -B8-binding peptides using the SYFPEITHI algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by thresholds 25 for HLA-A1, 22 for HLA-B7 and 21 for HLA-B8. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance.

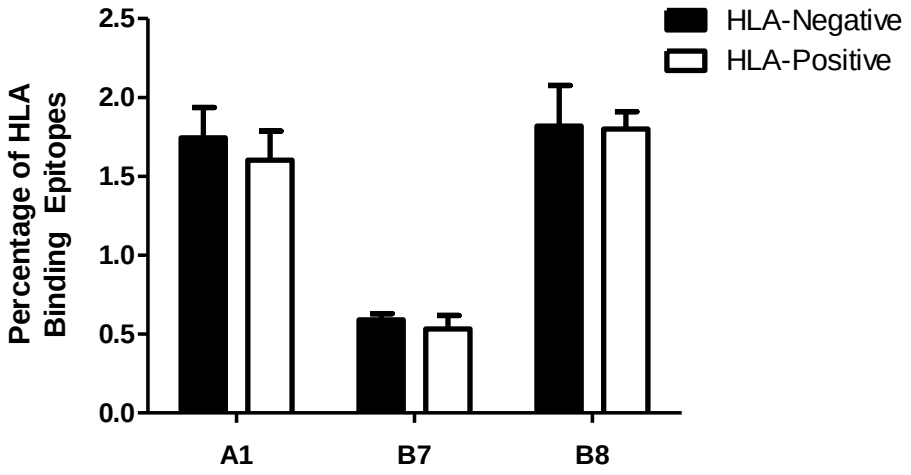


Figure 3.15 Frequency of high affinity peptides predicted to bind to HLA-A1, -B7 or -B8 present in the CDR3 of BCRs.

BCRs from healthy donors (separated into HLA-positive and HLA-negative cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for high affinity HLA-A1, -B7 and -B8-binding peptides using the BIMAS algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by threshold of 12 for HLA-A1, 16 or HLA-B7 and 12 for HLA-B8. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance.

analysing HLA-A2 with a decreased percentage of peptides in the HLA positive cohorts. Therefore, these results suggest that there may be an absence of peripheral tolerance towards these peptides and if this is the case that there could be editing of the B cell repertoire to remove some of these potentially dangerous B cells in all of the alleles tested.

3.8.3 BIMAS – Lower Scoring Peptides

When the BIMAS threshold was reduced to >5, to include the peptides with lower affinity for HLA, there was no difference between the percentage of peptides predicted to bind to HLA in the HLA positive and HLA negative cohorts for HLA-A1 and HLA-B7 (Figure 3.16). The percentages from each cohort were almost identical with 1.75% and 1.84% for $A1^+$ and $A1^-$ (χ^2=0.01, p=0.92), 1.21% and 1.20% for $B7^+$ and $B7^-$ (χ^2=0.00, p=0.96) and 2.73% and 2.82% for $B8^+$ and $B8^-$ donors (χ^2=0.00, p=0.95).

Analysing the sequences of the peptides predicted to bind to these HLA alleles demonstrates that the majority of peptides predicted to bind to HLA alleles by SYFPEITHI are also predicted to bind by BIMAS with a few exceptions. However, the majority of peptides predicted to bind to HLA alleles by BIMAS are not predicted to bind by SYFPEITHI (Appendix A2). This suggests that the SYFPEITHI algorithm is more stringent in what it determines as a peptide that can bind to a given HLA allele. Despite the differences in the peptides predicted to bind to HLA, the trends produced by both algorithms are similar demonstrating a small decrease in frequency of HLA-binding peptides from the CDR3 of HLA^+ donors.

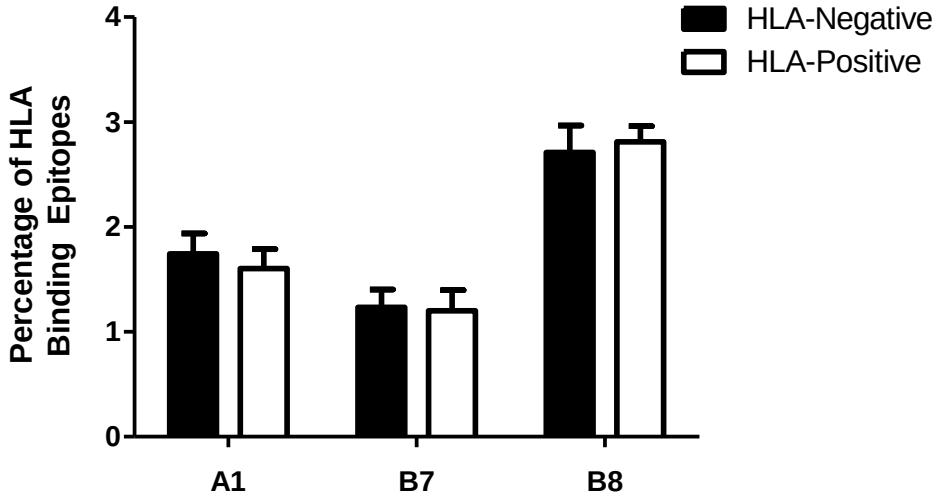


Figure 3.16 Frequency of high and low affinity peptides predicted to bind to HLA-A1, -B7 or -B8 present in the CDR3 of BCRs.

BCRs from healthy donors (separated into HLA-positive and HLA-negative cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for high affinity HLA-A1, -B7 and -B8-binding peptides using the BIMAS algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by threshold of 5 for all HLA alleles. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance.

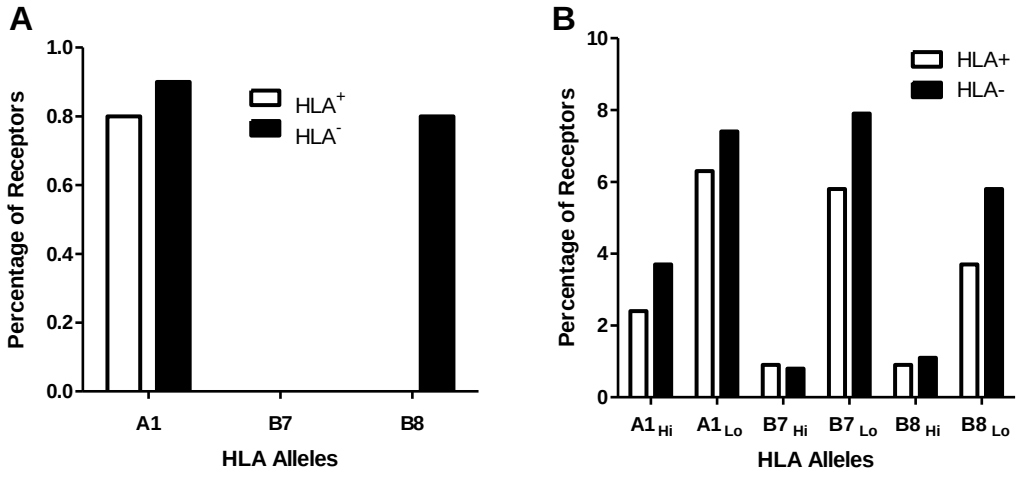


Figure 3.17 Percentage of BCRs containing more than one HLA-binding peptide in the other HLA types investigated

BCRs from healthy donors (separated into HLA-positive and HLA-negative cohorts) were analysed to determine the frequency of those containing more than one HLA-binding peptide in the other HLA alleles investigated using both peptide prediction algorithms SYFPEITHI (A) and BIMAS (B). This was done in an attempt to determine whether there was skewed data in the previous analysis of the peptide prediction data due to a single BCR containing a large number of HLA binding peptides within the CDR3. Data is represented as a percentage of all peptides analysed where peptide binding was defined for both algorithms as previously discussed.

3.8.4 Frequency of BCRs Containing HLA-Binding Peptides

After showing that there was a decreased frequency of BCRs containing an HLA-A2 binding peptide in HLA-A2$^+$ donors, I wanted to determine whether the same was true in the other HLA alleles investigated. Using SYFPEITHI, the frequency of BCRs containing more than one peptide for all HLA alleles was increased in the HLA-negative donors compared with the HLA-positive donors (Figure 3.17). Using BIMAS to analyse the same data, the HLA-A1 and -B8 donors demonstrated similar results to SYFPEITHI at both the lower and upper threshold with the HLA-negative donors containing an increased frequency of BCRs than the HLA-positive donors. However, the frequency of BCRs in HLA-B7 was very similar between the HLA-positive and HLA-negative donors. These results are very similar to the previous results from BIMAS investigating the frequency of peptides where there was little difference in all HLA types between the HLA-positive and HLA-negative donors.

The above results suggest that there may depletion of B cells, possibly due to the absence of peripheral tolerance and presence of potential HLA-binding peptides within the sequence of the BCR. The possibility of editing of the B cell repertoire may be HLA-specific as the results are strongest in HLA-A2$^+$ donors but aren't as pronounced in HLA-A1, -B7 and B8 positive donors. Although the results for the other HLA types demonstrated a small decrease in the frequency of peptides predicted to bind to the HLA molecules in the HLA positive and HLA negative cohorts, the trends seen are similar to the results from HLA-A2. There is a suggestion that this phenomenon may occur in these HLA types, although this is only the case when studying peptides with high affinity and excluding the peptides with a lower affinity. Therefore, the results shown suggest that donors with HLA-A1, -B7 and -B8 alleles

may selectively delete B cells which contain a peptide predicted to bind with a high affinity for HLA-A1, -B7 and -B8 although further studies would be needed to demonstrate statistical signficance.

Overall, the results from both computer algorithms might suggest that there is no peripheral tolerance towards B cells and that this is not unique to HLA-A2 although much of the data is not statistically significant. However, results from BIMAS investigating peptides with a lower affinity for HLA in these alleles suggest that there may be peripheral tolerance towards these peptides as there is a similar frequency of peptides in HLA-positive donors compared with the HLA-negative donors. This is different to that seen in HLA-A2 where there was a statistically significant decrease in peptides predicted to bind to HLA-A2 in HLA-$A2^+$ donors.

3.9 Frequency of CDR3-Derived HLA-A2-Binding Decamer Peptides

HLA-A2 molecules can incorporate longer peptides comprised of 10 amino acids, as well as the more conventional 9 amino acids in the peptide binding groove (Collins et al, 1995 and Takahashi et al, 1999). As a result of this, the frequency of CDR3-derived decamer peptides predicted to bind to HLA-A2 in $A2^+$ and $A2^-$ donors was investigated. Using the SYFPEITHI prediction algorithm and a peptide-binding threshold of 22 to incorporate peptides that are most likely to bind to HLA-A2 only, the frequency of predicted HLA-A2 binding peptides was decreased in $A2^+$ donors (0.35%) compared with the frequency in $A2^-$ donors (0.57%) (χ^2=2.05, p=0.15) (Figure 3.18).

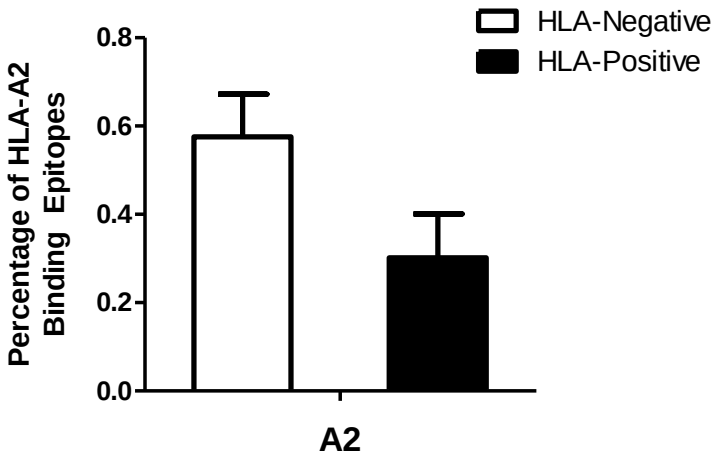


Figure 3.18 Frequency of HLA-A2 decamer peptides present in the CDR3 of BCRs in HLA-A2$^+$ and A2$^-$ Healthy Donors

BCRs from healthy donors (separated into HLA-positive and HLA-negative cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A2-binding peptides of 10 amino acids using the SYFPEITHI algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 22 or above. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance.

Although these results were not statistically significant, the results for the 10 amino acid peptides, with a higher affinity for HLA-A2, showed the same trend for HLA-A2 as that seen with the nonamer peptides. The trend seen with both sets of results produced by both lengths of peptides suggests that there may be a true decrease in the frequency of potentially immunogenic peptides predicted to bind to HLA-A2 from the BCR CDR3s of HLA-$A2^+$ donors.

3.10 Immunoediting of the B Cell Repertoire

Data described so far suggest that there could be a depletion of B cells containing HLA-binding peptides within the CDR3 of BCRs. The next step in studying this depletion was to try and determine at what stage of the B cell maturation this depletion occurs. If this is true, the depletion could occur at almost any stage during the life and maturation of the B cell. One possible scenario is that this potential editing occurs during the maturation of naïve B cells into memory B cells because at this stage B cells undergo somatic hypermutation and affinity maturation which could remove B cells with potential HLA-A2 binding peptides from the B cell repertoire. Naïve and memory B cells differ in their expression of the surface bound molecule CD27, a member of the tumour necrosis factor superfamily of receptors which binds to CD70 (Maurer et al., 1992 and Agematsu et al, 2000). Naïve B cells lack expression of CD27 ($CD27^-$) whereas memory B cells express CD27 ($CD27^+$) (Klein et al, 1998) and therefore can be separated into naïve and memory subsets by CD27 selection. During this stage of maturation, B cells undergo somatic hypermutation and affinity maturation which could remove B cells with potential HLA-A2 binding peptides from the B cell repertoire. The hypothesis tested was that the editing of the B cell repertoire, seen in HLA-A2 donors, occurs at the stage of B cell activation and

generation of memory B cells. There are two possible outcomes testing this hypothesis. Firstly, the frequency of peptides found in memory B cells is similar to that in naïve B cells and in this scenario, the editing will have occurred prior to activation of B cells. Secondly, the frequency of HLA-A2 binding peptides in naïve B cells will be increased compared with memory B cells demonstrating removal of these peptides during the activation of naïve B cells and the formation of memory B cells.

3.11 Separation of PBMCs into Naive and Memory B Cells

In order to test this new hypothesis, B cells were magnetically selected from PBMCs, from an HLA-$A2^+$ donor, into $CD27^+$ and $CD27^-$ fractions using an anti-CD27 PE antibody and anti-PE bead magnetic selection. The cells were separated using two columns to improve the purity of the selected cells with >97% of cells selected staining positive for CD27 by flow cytometry (Figure 3.19 A). The cells in both positive and negative fractions of the separation will contain B cells, T cells and other leukocytes and therefore, in order to determine the sequences of only the B cells, DNA from the cells was amplified using the same oligonucleotide primers as previously described (Table 2.2). After running the PCR amplification of the $CD27^-$ fraction, strong bands could be seen ~500bp for the IgM primers (Figure 3.19 B). However, bands could be seen for both IgA and IgG (which should not be there as B cells in this fraction should be naïve and therefore they wouldn't have undergone class-switching) suggesting that the separation was not 100% effective and even a small number of memory B cells ($CD27^+$) contaminating the fraction would be responsible for this as PCR is extremely sensitive. Further evidence for this is the fact that the bands for these “rogue” amplifications are fainter than the bands seen for IgM. Similarly, after running the PCR amplifications from the $CD27^+$ cells on the

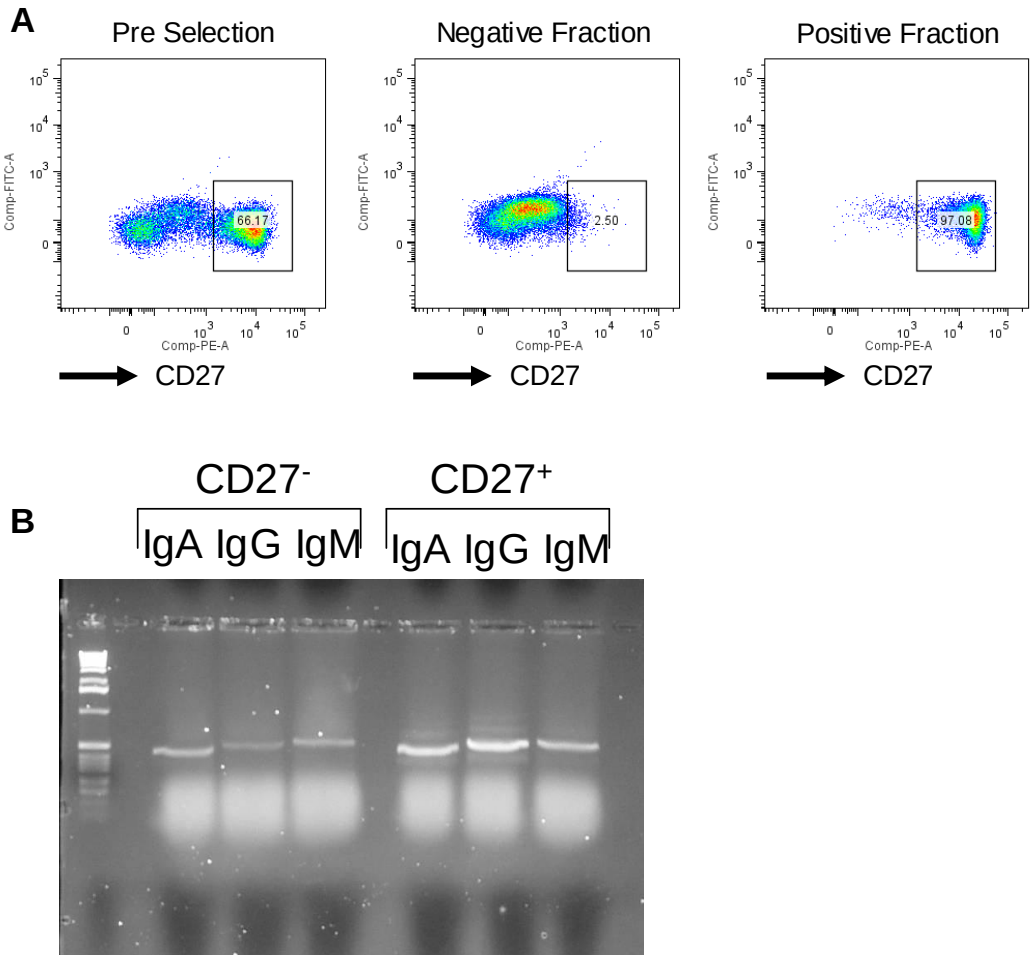


Figure 3.19. CD27 Selection of PBMCs from an HLA-A2$^+$ donor using MACS magnetic separation and PCR amplification of B cell receptors

Unfractionated PBMCs from a single HLA-A2$^+$ donor were stained with CD27-PE antibody to separate naïve B cells (CD27$^-$) from memory B cells (CD27$^+$), washed and incubated with anti-PE microbeads. After extensive washing, the cells were run through two magnetic sorting columns and the fractions analysed on the flow cytometer (A). RNA was extracted from the CD27$^-$ (naïve) and CD27$^+$ (memory) B cell fractions and cDNA synthesised as previously described. This was used as template DNA for PCR amplification using a V-region primer and all three of the BCR constant region primers (IgA, IgG and IgM) (B).

same gel, bands could be seen for all 3 Ig heavy chain constant regions. This may again be due to the fact that the separation of cells was not 100% effective and again, the expected IgA and IgG bands appeared stronger than the "rogue" IgM bands. As $CD27^-$ naive B cells should only contain IgM expressing B cells, the PCR product using IgM primers was collected. Similarly, the $CD27^+$ memory B cells should have undergone class switching and therefore the majority will express either IgA or IgG. I collected the PCR products amplified with the IgA or IgG specific primers for the constant regions. The amplified DNA from both subsets ($A2^+/CD27^+$ & $A2^+/CD27^-$) was purified and the BCRs separated using the TA cloning kit as described previously. A few different plasmids containing a single BCR for each of the subsets were sequenced locally to confirm the presence of IgA, IgG or IgM in the correct subsets prior to sequencing a large number of these. The initial sequencing results returned the correct sequences from the correct subsets (Table 3.1) with only the CDR3 of IgM^+ B cells being sequenced from the $CD27^-$ subsets and both the CDR3s of IgA^+ and IgG^+ B cells being sequenced from the $CD27^+$ subsets. This meant that the molecular biology work had been successful and the remaining BCRs could be sequenced.

There were 96 purified BCR CDR3s from each of the subsets sent for sequencing. Analysis of these results will focus on the comparison of HLA-A2 binding peptides between $CD27^+$ and $CD27^-$ B cells in the $A2^+$ donor. In testing the hypothesis that editing of the B cell repertoire occurs at the stage of activation and class-switching, there are two possible outcomes. Firstly and bearing in mind that the B cell repertoire from $A2^+$ individuals has already been edited, if there is a decreased frequency of the remaining HLA-A2 binding peptides in memory B cells compared with the frequency

	CDR3 Sequence
$A2^+$ $CD27^-$ IgM^+	SEDTAVYYCALNTYYYGSGSYYGYWGQGTLVTVSSGSASAPTL
$A2^+$ $CD27^+$ IgA^+ IgG^+	DTAFYYCARIHCGVGICYWDWGQGTLVTVSSASTKGPS
	DTAVYYCARRSDIWHAFDPWGPGTMVTVSSASTKGPS
	DTGTYYCARGGGQTWGQGVLVTVSSASTKGPS

Table 3.1 Examples from CDR3 sequencing from the B Cell subsets

BCRs were sequenced from each of the four purified B cell subsets. The sequences shown begin with the 3' region of the variable region, leading into the CDR3 region (underlined) and then moving into the 5' region of the constant region.

of HLA-A2 binding peptides in naïve B cells, this would suggest that the selective depletion of B cells containing a potentially immunogenic peptide within the CDR3 occurs after activation of B cells and before a pool of memory B cells is formed. Secondly, if there is a similar frequency of HLA-A2 binding peptides from the CDR3s of both the memory and naïve B cells in an $A2^+$ individual, this would suggest that the editing of the B cell repertoire occurs at earlier developmental stages, possibly still within the bone marrow or earlier in the lymph node. Sequences returned provided 83 sequences that were $CD27^-$ IgM CDR3s, providing 2903 potential peptides (9 amino acids in length), and 90 sequences from the $CD27^+$ IgA/G, 25 of which were IgA and 65 were IgG, providing 3019 peptides. The sequences of the memory B cells demonstrated that all sequences had been somatically hypermutated whereas the naïve B cell sequencing data demonstrated two sequences that had been somatically hypermutated and these sequences were removed from the data set.

3.12 Analysis of Predicted HLA-A2-Binding Peptides in Naïve and Memory B Cells

3.12.1 SYFPEITHI - High Scoring Peptides

Using the SYFPEITHI prediction algorithm and a predicted HLA-A2 binding cut-off of 22 as used previously, the frequency of potential HLA-A2 binding peptides in $CD27^-$ IgM^+ naïve B cells (0.10%) from an $A2^+$ donor was slightly decreased compared with the memory B cells (0.20%) (χ^2=0.89, p=0.35) (Figure 3.20). This result suggests that there has been no editing of the repertoire at this stage and that there is possibly positive selection of BCRs containing potential HLA-A2 binding peptides in the memory B cells. This may also suggest that the process of somatic

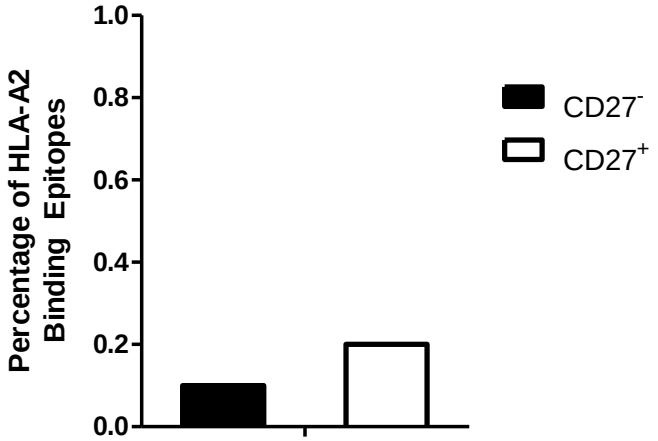


Figure 3.20 Frequency of HLA-A2 peptides present in the CDR3 of BCRs in an HLA-A2$^+$ Healthy Donor

BCRs from naïve and memory B cells from a single HLA-A2$^+$ donor were sequenced and the CDR3 analysed for HLA-A2-binding peptides using the SYFPEITHI algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 22 or above. Data was analysed using the chi-squared test to determine statistical significance.

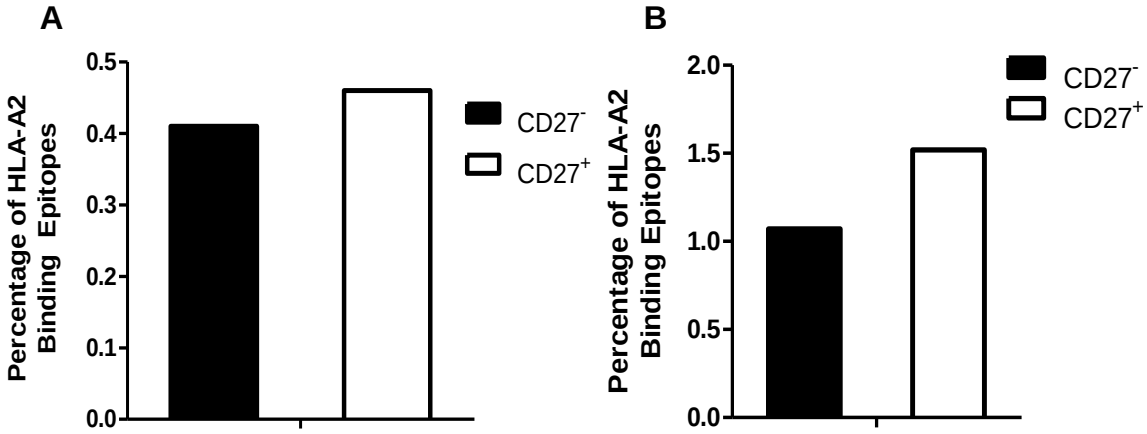


Figure 3.21 Frequency of high and low affinity HLA-A2 peptides present in the CDR3 of BCRs

BCRs from naïve and memory B cells from a single HLA-A2$^+$ donor were sequenced and the CDR3 analysed for high affinity (A) and high and low affinity (B) HLA-A2-binding peptides using the BIMAS algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 20 or above for high affinity peptides and 5 or above for low affinity peptides. Data was analysed using the chi-squared test to determine statistical significance.

hypermutation and/or affinity maturation may increase the frequency of peptides contained within the CDR3 of memory B cells. This would therefore mean that if there is immune editing in $A2^+$ individuals it occurs at an earlier stage of B cell maturation as described before and that the potential editing of the B cell repertoire does not occur as B cells become activated and mature into memory B cells.

3.12.2 BIMAS - High Scoring Peptides

In order to validate the data from the SYFPEITHI prediction algorithm, the same data was analysed using the BIMAS prediction algorithm as well. Initially, I used a peptide dissociation half-life of 20 minutes to incorporate peptides with a high affinity for HLA-A2. There is little difference between the frequency of HLA-A2 binding peptides found within the CDR3 of naïve and memory B cells in the HLA-$A2^+$ donor (0.41% to 0.46%) (χ^2=0.09, p=0.77), although the results from the memory B cells are slightly increased similar to that seen in the results returned from SYFPEITHI (Figure 3.21 A). Similar to the results generated from SYFPEITHI, this result suggests that somatic hypermutation and/or affinity maturation increase the frequency of HLA-A2 binding peptides within the CDR3 of memory B cells compared with naïve B cells. This would further suggest that the potential process of immune editing of the B cell repertoire would occur before the generation of mature naïve B cells.

3.12.3 BIMAS - Lower Scoring Peptides

The data set was re-analysed using a lower peptide-binding threshold of 5 to determine the frequency of predicted peptides with a low affinity as well as those with a high affinity for HLA-A2. Results from the HLA-$A2^+$ donor demonstrate an increased frequency of peptides from memory, $CD27^+$ B cells predicted to bind to

HLA-A2 (1.52%) compared with peptides predicted to bind to HLA-A2 from $CD27^-$ naïve B cells (1.02%) (χ^2=2.40, p=0.12) (Figure 3.21 B). The result for peptides with high and low affinity for HLA-A2 suggest that, similarly to the results from SYFPEITHI, there is an increased frequency of peptides within the CDR3 of B cells after they have undergone somatic hypermutation and affinity maturation.

The results suggest that if editing of the B cell repertoire does occur, it would already have occurred before the B cells have reached the peripheryand that rather than the decrease in frequency of HLA-A2 binding peptides in the memory B cell subset as outlined in the hypothesis, there is actually an increase in the frequency of HLA-A2 binding peptides in the memory B cell subset. The results from the BIMAS algorithm would appear to validate the previous results which were produced from the SYFPEITHI algorithm. In relation to the suggested hypothesis, the potential process of immune editing of the B cell repertoire does not appear to occur at this stage of B cell maturation and would probably occur at an earlier stage of B cell development.

3.13 Immunoediting of the B Cell Lymphoma Repertoire

The previous results suggested that there may be editing of the B cell repertoire to remove BCRs containing CDR3-derived peptides in healthy donors. If this is true, there would have to be a process in B cells whereby the majority of B cells containing these peptides are removed in healthy volunteers. Since our group has a particular interest in haematological malignancies, it was very interesting to me to find out whether there could be a selection process occurring in patients with various B cell lymphomas. This led me to test the hypothesis that there may not be peripheral tolerance towards CDR3-derived peptides expressed by B cells from patients with B

cell lymphomas and therefore, patients will undergo immunoediting of the B cell repertoire. In order to test this hypothesis, 38 genomic DNA samples (15 HLA-$A2^+$ and 23 HLA-$A2^-$) from patients with multiple myeloma (MM), follicular lymphoma (FL) and primary central nervous system lymphoma (PCNSL) were analysed, kindly donated by Prof. C. Ottensmeier (University of Southampton).

3.13.1 Analysis of Predicted HLA-A2 Binding Peptides in B Cell Lymphoma

3.13.1.1 SYFPEITHI

The peptide data set from the patient samples was initially analysed using the SYFPEITHI prediction algorithm with a threshold of 22 to investigate the peptides with a high binding affinity for HLA-A2. There was an increased frequency of potential HLA-A2 binding peptides contained within the CDR3s of B cells sequenced from the HLA-$A2^-$ patient cohort (0.4%) (Figure 3.22) compared with those from the $A2^+$ patients (0.0%) (χ^2=1.98, p=0.16). This result suggests that there could be editing of the B cell repertoire in patients with B cell lymphomas although there would need to be an increased data set to determine statistically if such a process does occur.

3.13.1.2 BIMAS

Subsequently, the same data set was analysed using the BIMAS peptide prediction algorithm using a threshold of 20, to incorporate peptides with a high affinity for HLA-A2. Similarly to the results produced by SYFPEITHI, there was an increased frequency of potential HLA-A2 binding peptides within the CDR3s of B cells from HLA-$A2^-$ patients (1.06%) compared with those from the HLA-$A2^+$ patients (0.20%) (χ^2=3.10, p=0.08) (Figure 3.23). This result again suggests that there may be a mechanism, present in B cells, to delete cells which contain a peptide predicted to

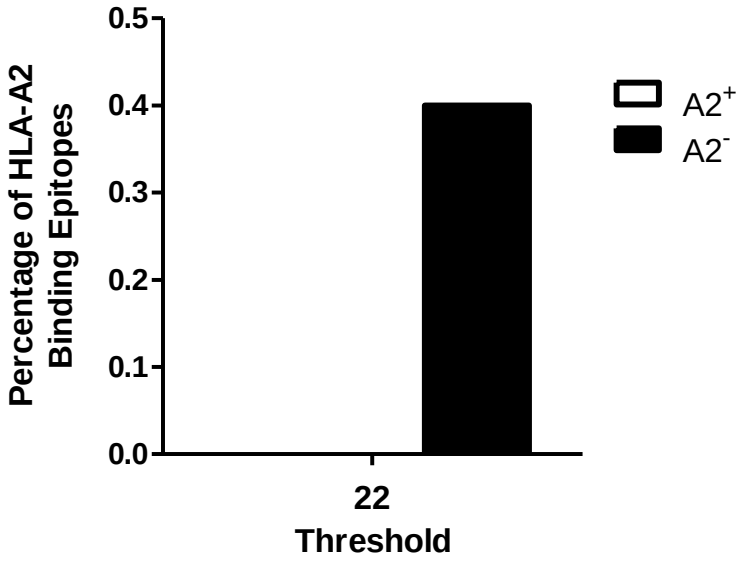


Figure 3.22 Frequency of HLA-A2 peptides present in the CDR3 of BCRs in HLA-A2$^+$ and A2$^-$ patients

CDR3 from BCRs, from patients with various B cell malignancies, which had been previously sequenced were analysed for HLA-A2-binding peptides using the SYFPEITHI algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 22 or above. Data was analysed using the chi-squared test to determine statistical significance.

bind to HLA-A2 within the CDR3 in HLA-A2$^+$ patients. However, inclusion of potential peptides predicted to bind to HLA-A2 with a low affinity as well produces similar results as those generated from SYFPEITHI. There is a decreased frequency of potential HLA-A2 binding peptides in the CDR3s of B cells from HLA-A2$^-$ patients (1.85%) compared with the HLA-A2$^+$ patients (2.20%) (χ^2=0.19, p=0.66) (Figure 3.23).

If the results incorporating the peptides with lower affinity for HLA-A2 are included, these results suggest that there is no editing of the B cell repertoire to remove B cells which contain a peptide predicted to bind to HLA-A2. Therefore, there could be peripheral tolerance towards CDR3-derived peptides by T cells, which could recognise the peptide if it was presented by HLA-A2 molecules at the surface of the B cell. However, if the results from the peptides with a higher affinity for HLA-A2 are true, there would appear to be no peripheral tolerance towards the CDR3-derived peptides and therefore there is the possibility that there could be a process of editing the B cell repertoire to remove these potentially dangerous B cells before they have the potential to cause an auto-immune reaction. Peptide prediction analysis from healthy donors and patients suggest there is a decrease in the frequency of HLA-A2 binding peptides, albeit not statistically significant, contained within the CDR3 of the BCR in HLA-A2$^+$ donors. Initially, CDR3-derived peptides were going to be targeted to generate a naïve T cell response. However, this work was not carried out because there is a low frequency of peptides predicted to bind to HLA-A2 contained within the CDR3 of BCRs and although it would be possible to locate a potential peptide to generate a naïve T cell response against, it would prove too difficult and time consuming. Therefore, even though this work can be undertaken in the future, it was

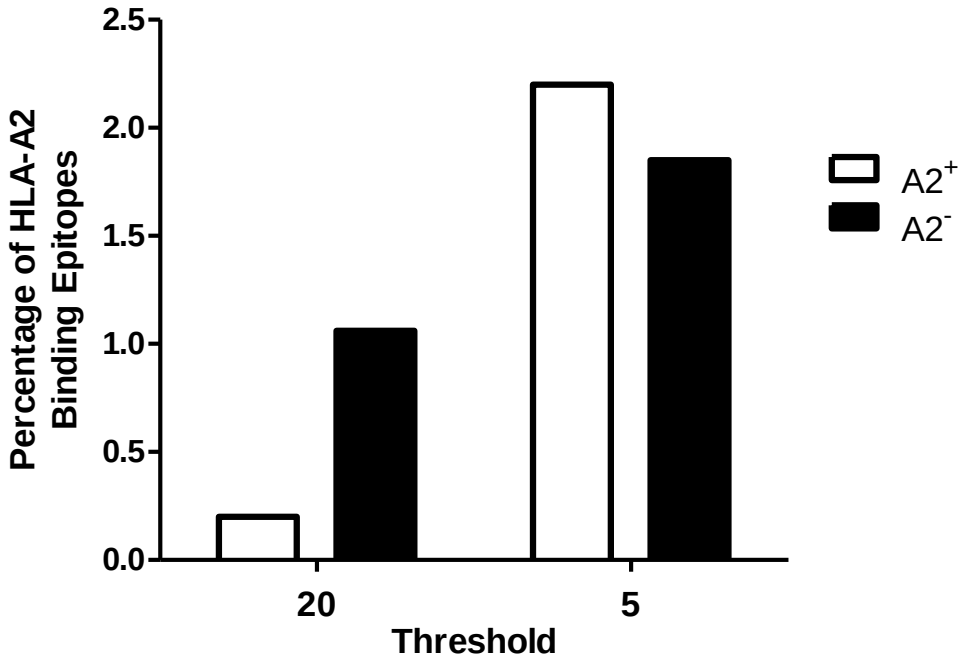


Figure 3.23 Frequency of high and low affinity HLA-A2 peptides present in the CDR3 of BCRs in HLA-A2$^+$ and A2$^-$ patients

CDR3 from BCRs, from patients with various B cell malignancies, which had been previously sequenced were analysed for high and low affinity HLA-A2-binding peptides using the. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 20 or above for high affinity peptides and 5 or above for low affinity peptides. Data was analysed using the chi-squared test to determine statistical significance.

not carried out at the time. Instead, an attempt was made to generate a model to investigate the antigen processing of CDR3-derived peptides in order to understand further whether these peptides are presented or not at the cell surface in the context of HLA molecules (discussed later).

3.14 Discussion

In this chapter, the hypothesis tested was that there is no peripheral tolerance directed towards CDR3-derived peptides from the B cell receptor in an HLA-A2 setting. If this hypothesis is true, there would be editing of the B cell repertoire to remove BCRs containing HLA-A2 binding peptides in an HLA-$A2^+$ donor as the presentation of these non-self peptides could result in the initiation of an auto-immune reaction. If editing of the B cell repertoire does occur as a result of the presence of CDR3-derived peptides, there would be a decreased frequency of HLA-A2 binding peptides in an HLA-$A2^+$ donor compared with an HLA-$A2^-$ donor. An HLA-$A2^-$ donor would not need to remove the HLA-A2 binding peptides from the CDR3 as they do not express HLA-A2 and therefore the peptide would not be presented at the cell surface.

The results for HLA-A2 demonstrated a decreased frequency of CDR3-derived nonamer peptides in HLA-$A2^+$ donors compared with HLA-$A2^-$ donors although the differences seen were not statistically significant. This suggests that there could be editing of the B cell repertoire to remove B cells which express a BCR containing HLA-A2 binding peptides and that there could be a lack of peripheral tolerance towards these peptides, proving the original hypothesis is true. The results from analysis using two different HLA-peptide binding prediction algorithms demonstrated a decreased frequency (although not statistically significant) demonstrating that the

results seen are not due to any artefacts in the programming of the prediction algorithms. Similarly, when CDR3-derived decamer peptides were analysed using SYFPEITHI, there was a decreased frequency of peptides found within the CDR3 from HLA-$A2^+$ donors compared with HLA-$A2^-$ donors. The results from this second peptide analysis would appear to support the initial results that there could be a process of editing of the B cell repertoire based on CDR3 sequence and therefore that there is possibly decreased peripheral tolerance towards CDR3-derived peptides in an HLA-A2 setting.

Similar results are seen in 3 other HLA alleles tested (HLA-A1, -B7 and -B8) although the decreased frequency of HLA-binding peptides in HLA-positive donors compared with HLA-negative donors is less striking than in HLA-A2. The results for these alleles may become stronger if a larger data set was used by sequencing more BCRs from HLA positive and negative donors. These results suggest that there may be a process of immunoediting seen in B cells which removes CDR3-derived peptides which could bind to any of the HLA alleles found in any donor. The removal of these peptides could be very important in B cells due to the secretion of antibody which would also contain the CDR3-derived peptide. Antibodies can move throughout the body and could be taken up by many different cell types including dendritic cells, macrophages and other B cells via cell-surface expressed receptors e.g. Fc receptors. If antibodies contained CDR3-derived peptides that could bind to an HLA allele expressed by the cells, there is the possibility that they could process the antibody and present these peptides on their surface. Consequently, CD8 T cells which recognise the CDR3-derived peptide could generate an autoimmune response against the

parental B cell. If the results seen are true, removal of these peptides from the CDR3 by a process of immunoediting would prevent the chances of this happening.

These may be very significant results suggesting that there may be a third selection process in the maturation process of B cells, alongside positive and negative selection, whereby B cells must express self peptides from the CDR3 of the BCR and those with a potentially immunogenic peptide are deleted before they can populate the periphery. The potential deletion of these B cells, possibly by CDR3-peptide-specific T cells resident in the bone marrow (Figure 3.2), may prevent potentially harmful autoimmune reactions once these B cells have matured into plasma cells and have begun producing antibodies.

The CDR3-derived peptide data shown above is completely dependent on the two computer algorithms used and, as such, any errors in the computer algorithm would result in errors in the data set. The two algorithms used were picked predominantly because they are the most well known, are thought to be the best characterised and have been used in many previous publications (Hansson et al, 2003; Zirlik et al, 2006 & Strothmeyer et al, 2010). Other algorithms have been used (Harig et al, 2001) but the majority of prediction studies have used BIMAS and SYFPEITHI. Although the prediction algorithms are used extensively they are not 100% accurate and therefore the data should be viewed accordingly. There is some reassurance in the fact that many of the peptides predicted to bind to HLA in SYFPEITHI are also predicted to bind to HLA by BIMAS. However, the reverse is not true with the majority of peptides predicted to bind to HLA by BIMAS not predicted to bind to HLA by SYFPEITHI. This may be due to the thresholds used to analyse the data and as the

two computer algorithms are not linked in any way and do not use a similar scoring system, this could account for some of the differences seen.

Although attempts were made to use appropriate thresholds by using receiver operator curve analysis for each HLA allele and algorithm, the thresholds used are open to debate. Previous publications have relied on thresholds (Hansson et al, 2003), similar to the results shown here, whereas other publications have avoided the use of thresholds by analysing a percentage of the highest scoring peptides regardless of score (Strothmeyer et al, 2010). It could be reasonably assumed that changing the threshold would alter the results but there is a difference in changing the frequency of peptides predicted to bind and changing the results so there is no longer a difference in the frequency. Attempts were made to analyse the data set using different thresholds to try and solve this problem. In every case, changing the thresholds for any given HLA allele resulted in a change to the frequency of peptides predicted to bind to HLA. However, in many cases, especially using SYFPEITHI, the overall results remained the same with a decrease in the frequency of HLA-binding peptides in HLA^+ donors compared with HLA^- donors although changing the threshold often resulted in a change to the significance of the result. This was not the case for all HLA alleles tested but it possibly goes some way to demonstrate that changing the threshold does not alter the outcome dramatically but does alter the significance of the result. From the list of CDR3-derived peptides predicted to bind to the HLA alleles, tested, none of them were found to be the same as pathogenic epitopes. This may not be surprising if there truely is editing of the B cell repertoire as it would be suggested that pathogenic epitopes in the CDR3 would be edited from the repertoire, preventing an immune response being generated against the CDR3-derived peptides.

Statistical support was received from supervisors and experienced staff but it may be of interest to seek further statistical support in an effort to determine what could be done to show statistical significance regarding the CDR3-derived peptide editing.

One question that arose as a consequence of these results was: at what stage of B cell maturation could the process of immunoediting of B cells occur? Theoretically, the editing could occur at any stage of maturation of B cells and one potential stage is during the activation of naïve B cells in the periphery. During activation B cells undergo somatic hypermutation and affinity maturation, losing expression of the cell surface glycoprotein CD27 (Agematsu et al, 2000). Naïve B cells were separated from activated B cells by magnetic selection of CD27 to test the hypothesis: does immunoediting of the B cell repertoire occur during activation of B cells.

Testing this hypothesis using HLA-A2 as the model, there was a slightly increased frequency of peptides found within the CDR3 of memory B cells compared with naïve B cells. The results were similar when using both peptide prediction algorithms and suggest that they are not due to an artefact from one of the algorithms. The result suggests that the hypothesis being tested is false and that if immunoediting of B cells does occur, it is before the cells are mature naïve B cells, possibly occurring during selection of B cells in the bone marrow. This could be another possibility of where the potential immunoediting could occur because B cells undergo positive and negative selection at this stage and therefore it would be possible for the B cells to undergo a further round of selection at this stage dependant on the sequence of the CDR3. This theory is given further credence by the presence of a large number of memory $CD8^+$ T

cells in the bone marrow (Slifka et al, 1997 and Cavanagh et al., 2005) which are activated but not through recent stimulation with antigen (Di Rosa & Santoni, 2002). In this theoretical model, B cells which had already passed positive and negative selection would present peptides from their CDR3 on the surface to bone marrow resident $CD8^+$ T cells. Those B cells containing a CDR3-derived peptide recognised by the resident T cells would be killed and those without a CDR3-derived peptide would pass this round of selection and could populate the periphery. Further work could be undertaken into the presence of MHC class II peptides present in the CDR3 of BCRs. MHC class II work is made more difficult due to the variation in length of peptide and therefore the prediction of peptides which bind. However, previous studies suggest the presence of $CD4^+$ T cells in the bone marrow as well (Di Rosa & Santoni, 2003) and these may have a role in shaping the B cell repertoire as well.

The increased frequency of peptides found in the CDR3 of memory B cells compared with naïve B cells is very surprising. It would be expected that once the HLA-A2 binding peptides had been edited from the CDR3 region, they would not be found later in the life and maturation of B cells. This increased frequency of HLA-A2 binding peptides in memory B cells may be due to the effects of somatic hypermutation and/or affinity maturation as these processes, which occur at this stage of B cell maturation, are thought to be random events (Wilson et al., 1998). This means that they may introduce amino acids into the CDR3 resulting in a new HLA-binding peptide. If the immunoediting scenario is true and the immune system has gone to great lengths to remove potentially hazardous B cells from the repertoire, why would mature memory B cells be allowed to mutate their BCR to contain HLA-A2 binding peptides? Expression of CDR3-derived peptides may result in killing of the B

cell due to recognition by circulating T cells. However, expression of these peptides at the surface of the B cell may lead to interaction with T cells which aids in the activation of the B cell or possibly to prolong the life of a memory B cell. Further investigation of this phenomenon may lead to some interesting and surprising results.

As our group is interested in lymphoma, the next hypothesis tested suggested that a process of immunoediting could occur in the malignant B cell repertoire. DNA from patients with B cell lymphomas including multiple myeloma, follicular lymphoma and primary central nervous system lymphoma were HLA-A2 typed and the CDR3 sequence analysed for HLA-A2 binding peptides. There was a decreased frequency of HLA-A2 binding peptides (although not statistically significant) found within the CDR3 of HLA-$A2^+$ donors compared with HLA-$A2^-$ donors. The results were similar when using the two different peptide prediction algorithms again suggesting that the results are not an artefact of either algorithm. These results suggest that there could be a process of immunoediting of the BCR in B cell lymphomas, proving the hypothesis true. If there is a process of immunoediting, it would also mean that there would probably be no peripheral tolerance towards CDR3-derived peptides in patients, meaning that the B cells could be targeted if immune responses could be mounted to CDR3-derived peptides (Hsu et al., 1997 and Bendandi et al., 1999). However, the apparent lack of CDR3-derived peptides in BCRs from HLA-$A2^+$ patients with B cell lymphoma suggests that CDR3-derived peptides, known to be targeted after idiotype vaccination (Bertinetti et al., 2006 & Navarrete et al., 2008) in a percentage of patients, may not be a useful $CD8^+$ T cell target for the majority of patients. If the BCRs from B cell lymphomas do not contain HLA-binding peptides within the CDR3, there would be no peptide for CD8 T cells to target.

These results could have a significant impact on potential anti-idiotype therapies available for patients and may explain why many of the idiotype based therapies and vaccine trials have such poor efficacy (Timmerman et al, 2002; Levy et al, 2008). If this is the case, it would mean that the idiotype, one of the best defined tumour antigens, would be very difficult to target. However, the small number of patients makes analysis of the data set very difficult, but both computer algorithms produce similar results suggesting that there is authenticity regarding these results. In order to prove that the patient samples do or do not edit B cells, dependent on their CDR3 sequence, the number of patients in both the $A2^+$ and $A2^-$ cohort would need to be increased in an attempt to show statistical significance.

3.15 Conclusion

In this chapter, the hypothesis that there was no peripheral tolerance towards CDR3-derived peptides from the BCR and therefore there was the requirement of immunoediting of the B cell repertoire to prevent an autoimmune reaction was tested. There was a decreased frequency of HLA-binding CDR3-derived peptides in HLA-positive donors compared with HLA-negative donors (although not statistically significant). This suggests that there may be a process of immunoediting of the B cell repertoire to remove potentially dangerous B cells. Furthermore, there could also be a similar process of immunoediting of malignant B cells in patients with various B cell lymphomas. Despite attempts to identify the timing of this apparent immunoediting, it was discovered that if immunoediting does occur, it would probably have taken place before B cells mature into naïve B cells and exit the bone marrow.

4. Immunoediting of the T Cell Receptor Repertoire.

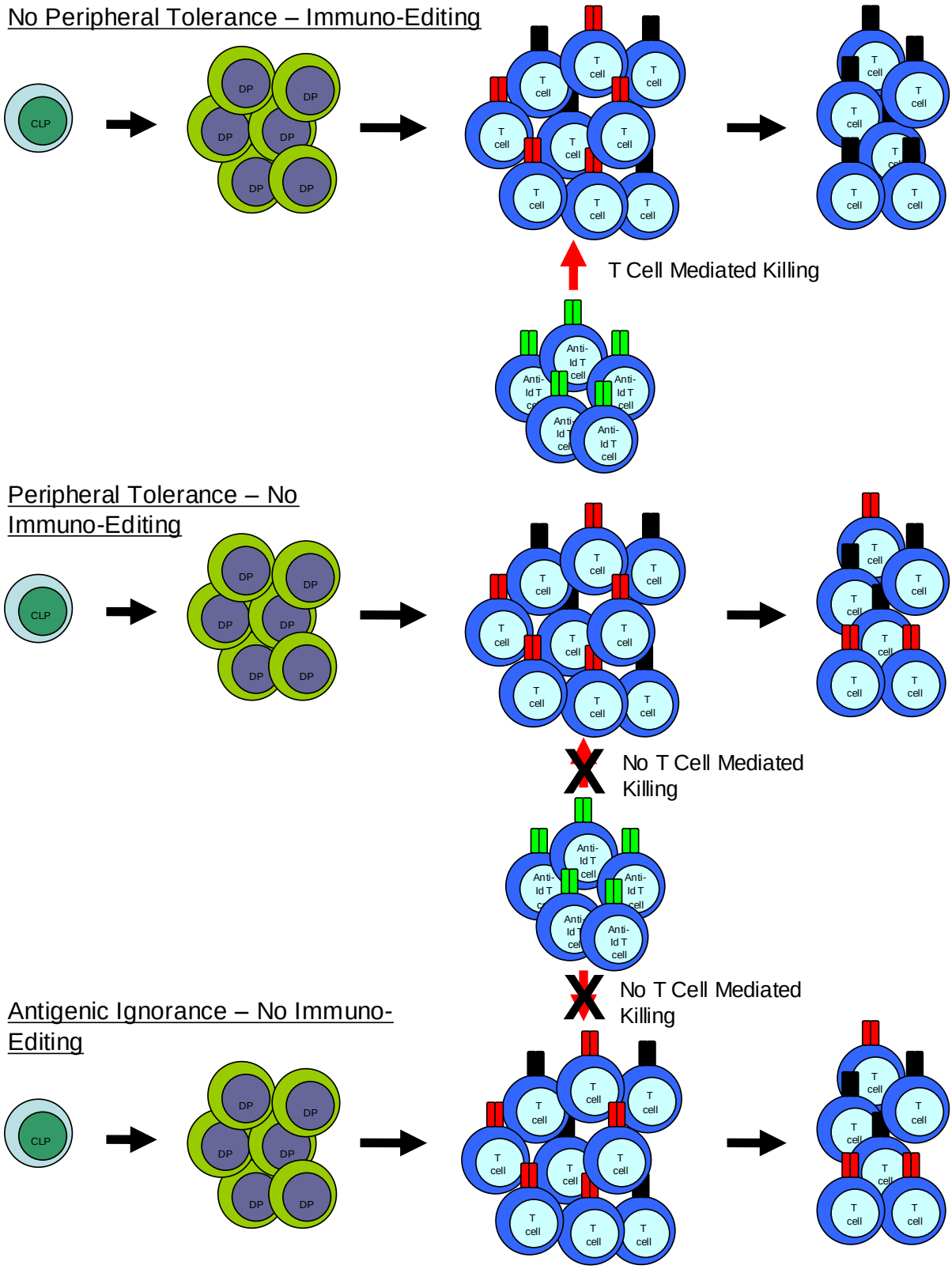
4.1 Introduction

T cell receptors (TCR) could contain peptides within the "non-self" CDR3, which could potentially initiate an immune response if they were to be presented at the surface of the T cell in complex with MHC molecules. There has been no previously published study to determine the presence or absence of CDR3-derived peptides in patients or healthy donors.

The first hypothesis tested was that T cells bearing TCRs containing HLA-binding peptides within their CDR3s are not subject to peripheral immunological tolerance and are edited out of the T cell repertoire by anti-CDR3 peptide derived specific T cells after positive and negative selection has occurred in the thymus, describing a pathway in terms of how the final T cell repertoire is defined. Deletion of these T cells would only allow T cells expressing receptors which do not contain HLA-binding peptides within the CDR3 to populate the periphery (Figure 4.1). In this model, there would be a decreased frequency of CDR3-derived HLA-binding peptides in HLA-positive donors compared to that of HLA-negative donors (Figure 4.2). There are two competing hypotheses which would explain the lack of editing in the T cell repertoire. Firstly, peripheral tolerance towards the peptides presented from the T cell receptor, similar to peripheral tolerance against any self-peptide, could protect the T cell, meaning that there would be no functional CDR3-specific T cells populating the periphery, having been deleted in the thymus or tolerised in the periphery. Secondly, the TCR could be prevented from

Figure 4.1 Cellular hypothesis for deletion of T cells containing predicted HLA-A*0201 peptides within their CDR3

If there is no peripheral immunological tolerance, a T cell from an HLA-A*0201^{+} individual containing potential A2-binding peptides derived from the CDR3 region (Red) would undergo deletion, by anti-idiotype-specific T cells (Green), as the peptide could be presented on the surface of the T cell in complex with HLA-A2. TCRs which do not contain a potential HLA-A2 binding peptide within the CDR3 region (Black) would populate the periphery. Therefore, peripheral tolerance towards CDR3-derived peptides would result in a decreased frequency of HLA-A2 binding peptides within the CDR3 region of HLA-A2^{+} donors. However, if there is immunological tolerance, or antigenic ignorance, towards CDR3-derived peptides, the T cells containing a TCR with a CDR3-derived peptide would be allowed to populate the periphery and the anti-idiotype-specific T cells would not recognise the T cells. Therefore, the frequency of HLA-A2 binding peptides, within the CDR3 region, in HLA-A2^{+} donors would be similar to that from HLA-A2^{-} donors.


No Peripheral Tolerance – Immuno-Editing
CLP
DP
T cell
T Cell Mediated Killing
Anti-Id T cell
Peripheral Tolerance – No Immuno-Editing
No T Cell Mediated Killing
Antigenic Ignorance – No Immuno-Editing
No T Cell Mediated Killing

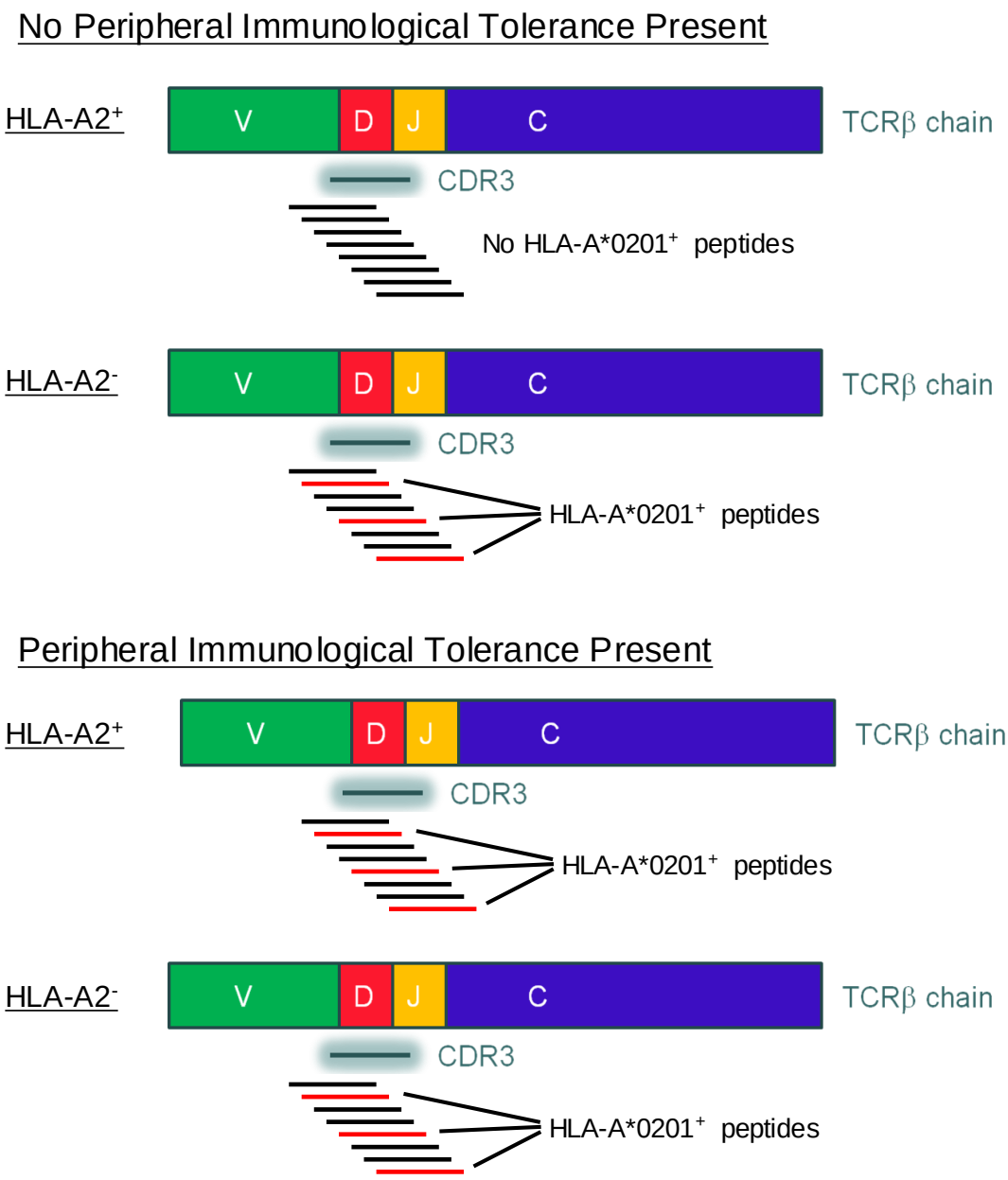


Figure 4.2 Molecular hypothesis for deletion of T cells containing predicted HLA-A*0201 peptides within their CDR3

If there is no immunological peripheral tolerance, a T cell from an HLA-A*0201$^+$ individual containing potential A2-binding peptides derived from the CDR3 region would undergo deletion as the peptide could be presented on the surface of the T cell in complex with HLA-A2. This would result in a decreased frequency of HLA-A2 binding peptides within the CDR3 region of HLA-A2$^+$ donors compared with HLA-A2$^-$ donors. However, if there is immunological tolerance towards CDR3-derived peptides, the frequency of HLA-A2 binding peptides in HLA-A2$^+$ donors would be similar to that from HLA-A2$^-$ donors.

entering the antigen processing pathway, in a form of antigenic ignorance, to prevent the expression of CDR3-derived peptides at the cell surface. In these models, shown in Figure 4.1, where there is a form of peripheral tolerance towards these peptides, the frequency of potentially immunogenic peptides within the CDR3 in HLA-positive donors would be similar to the frequency in HLA-negative donors (Figure 4.2).

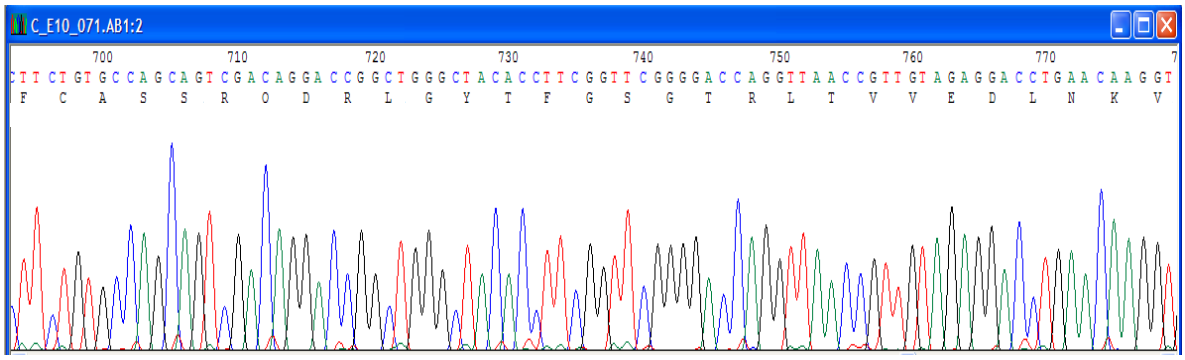
4.2 Amplification of T Cell Receptors

The TCR β-chain contains an extra, diversity region within the CDR3 and as a result is thought to have more variation in its CDR3 compared to the α-chain. As a result of this, it is possible that there is an increased incidence of HLA-binding peptides within the CDR regions of the TCR β-chain compared with the TCR α-chain. Therefore, this work initially focussed on the TCR β-chain and could potentially investigate the CDR3 of the TCR α-chain in the future.

4.3 Frequency of Predicted HLA-A2 Binding Peptides from TCR CDR3

TCRs were sequenced from the beginning of the variable region to the beginning of the constant region, incorporating the CDR3 (Figure 4.3). The sequence shows the end of the variable region moving into the diversity region followed by the joining region, comprising the CDR3. The sequence finishes with the beginning of the constant region.

A



B

NAGVTQTPKFQVLKTGQSMTLQCAQDMNHEYMSWYRQDPGMGLRLIHYSVGAGITDQG
EVPNGYNVSRSTTEDFPLRLLSAAPSQTSVYFCASSRQGRLGYTFGSGTRLTVVEDLNKVF
PPEVAVFEPSEAEISHTQKATLVCLATGFFPDHVELSWWVNGKEV

V Beta 6.5

CDR3 Region (Including D Beta 1.1 & J Beta 1.2)

C Beta 1

Figure 4.3. Sequencing the CDR3 of a TCRβ chain

The data returned for the sequencing is processed using the ChromasPro sequencing program which turns the coloured peaks (A: Green, C: Blue, G: Black and T: Red) into the nucleotide base sequence. The base sequence is then translated into the amino acid sequence encompassing the variable region (orange) leading into the CDR3 regions (blue) which incorporates the diversity and joining regions. The 3' oligonucleotide primer is situated within the constant region of the TCR β-chain and this results in the truncated sequence of the β-chain (green).

4.3.1 SYFPEITHI – High Scoring Peptides

Initially, the HLA-A2 positive and negative cohorts were analysed for the presence or absence of CDR3-derived peptides that could bind to HLA-A2. 92 TCRs from five HLA-$A2^+$ donors and 82 TCRs from five HLA-$A2^-$ donors, resulting in a total of over 5000 potential 9 amino acid peptides (2971 for HLA-$A2^+$ donors and 2668 for HLA-$A2^-$ donors), were analysed. Using the SYFPEITHI algorithm, there was no decreased incidence of predicted HLA-A2 binding peptides in HLA-$A2^+$ donors when compared with HLA-$A2^-$ donors as was first hypothesised. Surprisingly, there was actually a statistically significant increase in HLA-A2 binding peptides in HLA-$A2^+$ donors with the CDR3s derived from $A2^+$ donors containing almost 2% HLA-A2 binding peptides compared with only 1.24% from the $A2^-$ donors (χ^2=4.53, p=0.01) (Figure 4.4 A). The results are very surprising and would appear to suggest that TCRs containing HLA-A2 binding peptides are in some way being positively selected during T cell maturation. The results are the opposite to the original hypothesis proposed and would appear contradictory to what would have been expected as the presence of HLA-binding peptides in the TCR could lead to the presentation of those peptides on the surface of T cells.

4.3.2 SYFPEITHI – Lower Scoring Peptides

Previously, a lower threshold of 18 was also used when analysing the BCR to determine if the results were similar to that of the higher threshold. The results were similar but there was a high degree of background with non-binding peptides found within the A2-binding results. The lower threshold was used again in the analysis of

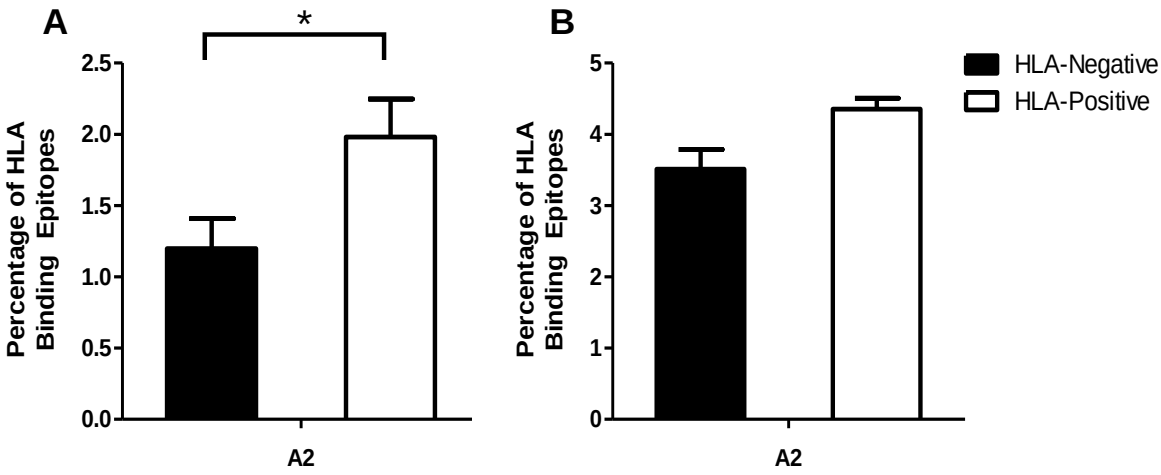


Figure 4.4 Frequency of predicted HLA-A2 binding peptides from in HLA-A2$^+$ and HLA-A2$^-$ donors

TCRs from healthy donors (separated into HLA-A2$^+$ and HLA-A2$^-$ cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A2-binding peptides using the SYFPEITHI algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 22 or above (A) or 18 and above (B). Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance where * P<0.05.

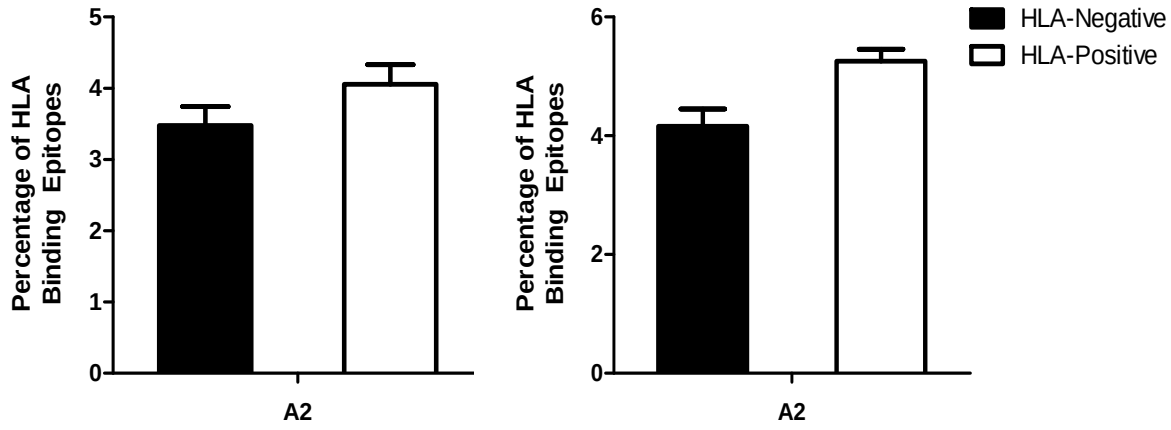


Figure 4.5. Frequency of high affinity HLA-A2 binding peptides from the CDR3 of the TCR β-chain

TCRs from healthy donors (separated into HLA-A2+ and HLA-A2- cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A2-binding peptides using the BIMAS algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 20 or above (A) or 5 and above (B). Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance.

TCR sequences to determine if similar results were seen. There is a similar trend where HLA-A2$^+$ donors contain an increased percentage of HLA-A2-binding peptides (4.21%) compared with the HLA-A2$^-$ donors (3.45%) although this result is not statistically significant (χ^2=2.19, p=0.14) (Figure 4.4 B). However, the frequency of peptides returned using the lower threshold is very high compared with the higher threshold and although the results appear similar, there seems to be a lot of false positive peptides in the A2-binding peptides and as a results of this, the lower threshold was discarded.

The result generated using the higher threshold was very surprising, demonstrating an increased frequency of HLA-A2 binding peptides in HLA-A2$^+$ donors. In order to try to authenticate this result, the CDR3 sequences were analysed with the second HLA-binding algorithm, BIMAS.

4.3.3 BIMAS - High Scoring Peptides

Using the BIMAS algorithm, similar results are seen with an increase in the frequency of high affinity HLA-A2 binding peptides (BIMAS threshold of 20) in the HLA-A2$^+$ cohort (4.04%) compared with the HLA-A2$^-$ donors (3.60%) (Figure 4.5 A). Although these results were not statistically significant (χ^2=0.74, p=0.39) they suggest that there is peripheral tolerance towards CDR3-derived peptides and consequently, no editing of the T cell repertoire.

4.3.4 BIMAS – Lower Scoring Peptides

Incorporating the peptides with a lower affinity for HLA-A2 (shown in BIMAS by a half-time for dissociation greater than 5), the percentage of HLA-A2 binding peptides in HLA-$A2^+$ donors is again increased (5.12%) compared with the HLA-$A2^-$ donors (4.24%) (Figure 4.5 B), although again the results are very high and not statistically significant (χ^2=2.43, p=0.12). The results using the BIMAS HLA-binding algorithm also suggest that TCRs, containing HLA-A2 binding peptides, may be positively selected because there is an increased incidence of HLA-A2 binding peptides seen in the $A2^+$ donors compared with the $A2^-$ donors. It would appear that the affinity of the peptides for HLA-A2 does not alter the incidence in HLA-A2 binding peptides because there is a similar increased incidence of HLA-A2 binding peptides when incorporating those with a lower affinity.

Analysis of the peptide sequences predicted to bind to HLA-A2 by both algorithms shows that the majority of peptides predicted to bind to HLA-A2 by SYFPEITHI in the $A2^+$ donors are also predicted to bind to HLA-A2 by BIMAS as well, 14/26 (Appendix Table A4). However, BIMAS also predicted a further eight peptides to bind to HLA-A2 that SYFPEITHI did not and SYFPEITHI predicted a further five peptides that BIMAS did not predict. Similar results were seen after analysing the $A2^-$ donors, 12/17 peptides were predicted to bind to HLA-A2 by both algorithms compared to 3/17 predicted by BIMAS alone and 2/17 by SYFPEITHI alone (Appendix Table A4). Similar to the results seen when analysing the BCR peptides, the results show that although there is some cross-over between the two prediction algorithms, the results obtained from both are not identical reflecting the differences in their programming.

4.4 Frequency of TCRs Containing HLA-A2 Binding Peptides

The above results appear to show no editing of the T cell repertoire based on the sequence from the CDR3. However, the increased frequency of HLA-A2 binding peptides in HLA-$A2^+$ donors could be a result of many peptides contained within a few TCRs. Therefore, the frequency of TCRs containing an HLA-A2 binding peptide was determined in order to demonstrate that there was no editing of T cells.

Using SYFPEITHI, there is an increased frequency of TCRs containing more than 1 HLA-A2 binding peptide in HLA-$A2^+$ donors (3.3%) compared with HLA-$A2^-$ donors (2.4%) (Figure 4.6 A). These results correspond to the similar increase in CDR3-derived peptide frequency from HLA-$A2^+$ donors compared with HLA-$A2^-$ donors. Using the BIMAS algorithm, there is a higher frequency of peptides predicted to bind to HLA-A2, at the upper and lower thresholds, compared to SYFPEITHI but there is still an increased frequency of TCRs containing more than 1 HLA-A2 binding peptide in HLA-$A2^+$ donors using both thresholds (29.3% and 54.3%) compared with HLA-$A2^-$ donors (19.5% and 36.%) (Figure 4.6 B).

These two algorithms are the most heavily used in many previous publications and because the results from SYFPEITHI are confirmed using BIMAS, it would suggest that the results are a true reflection of HLA-binding peptides within the CDR3 of TCRs.

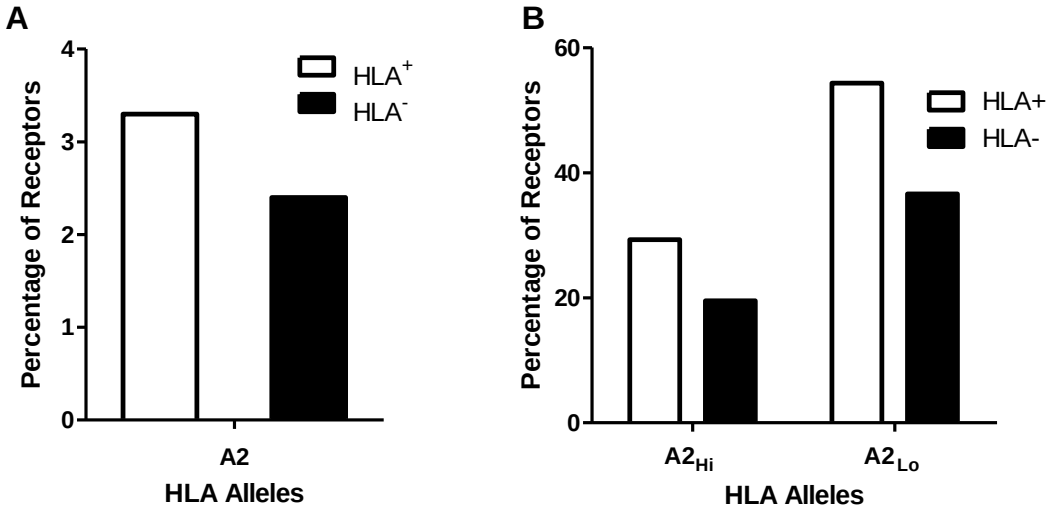


Figure 4.6. Frequency of TCRs containing more than one HLA-A2 binding peptide.

TCRs from healthy donors (separated into HLA-A2+ and HLA-A2- cohorts) were analysed to determine the frequency of those containing more than one HLA-A2 binding peptide using both peptide prediction algorithms. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 22 or above using SYFPEITHI (A), a score of 20 or above using $BIMAS_{hi}$ (B) and a score of 5 or above using $BIMAS_{lo}$ (B). This was done in an attempt to determine whether there was skewed data in the previous analysis of the peptide prediction data due to a single TCR containing a large number of HLA binding peptides within the CDR3.

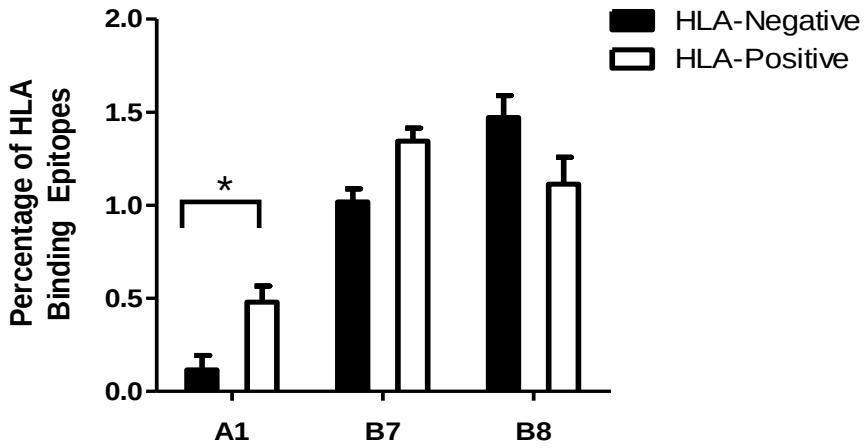


Figure 4.7 Frequency of predicted HLA-binding peptides from other HLA-types in healthy donors

TCRs from healthy donors (separated into HLA-positive and HLA-negative cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A1, -B7 and -B8-binding peptides using the SYFPEITHI algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by thresholds 25 for HLA-A1, 21 for HLA-B7 and 22 for HLA-B8. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance where * $P<0.05$.

4.5 Broader Analysis of HLA Binding Peptides

After the surprising results from the HLA-A2 donors, the sequenced CDR3s were re-analysed for other HLA alleles, HLA-A1, -B7 and –B8 to see if the results were consistent over different HLA alleles. The thresholds used for this analysis are the same as previously discussed.

4.5.1 SYFPEITHI – High Scoring Peptides

Analysis of the peptides produced using the higher thresholds for the other HLA types using SYFPEITHI produced interesting results as well. For HLA-A1, similar results were found where the number of potential HLA-binding peptides was significantly increased in the HLA-$A1^+$ donors (0.39%) compared with those from the $A1^-$ donor (0.07%) (Figure 4.7) (χ^2=6.44, p=0.01). Analysis of the HLA-B7 donors also demonstrated an increased incidence of CDR3-derived HLA-B7 peptides in $B7^+$ donors (1.36%) compared with $B7^-$ donors (0.99%) although this result was not statistically significant (χ^2=1.68, p=0.20). These results would appear to corroborate the previous results seen with potential HLA-A2 binding peptides and again suggest that the TCRs with a potential HLA-binding peptide within the CDR3 are being positively selected. However, analysis of HLA-B8 binding peptides in $B8^+$ and $B8^-$ donors showed the opposite to the other HLA types studied with a small increase in the percentage of HLA-B8 binding peptides in the $B8^-$ donors (1.50%) compared with the $B8^+$ donors (1.04%) (Figure 4.7), although this result was not statistically significant (χ^2=2.34, p=0.13).

4.5.2 BIMAS – High Scoring Peptides

The same data set was used with the BIMAS HLA-binding prediction algorithm to again try to authenticate the results seen with the SYFPEITHI algorithm. The same thresholds were used as previously set, to detect the presence of high affinity peptides within the CDR3. There is an increased incidence of HLA-binding peptides from HLA-$A1^+$ and -$B7^+$ donors (1.43% & 1.88) when compared with those from the HLA-$A1^-$ and $B7^-$ (1.23% & 1.44%) (Figure 4.8 A) although the increase seen here is not statistically significant (χ^2=0.45, p=0.50 & χ^2=1.70, p=0.19). However, using the algorithm for HLA-B8, there was an increased frequency of CDR3-derived peptides in HLA-$B8^-$ donors (1.61%) compared with the HLA-$B8^+$ donors (1.21%) (χ^2=1.62, p=0.20) (Figure 4.8 A). The differences seen here are similar to those seen when analysis of the higher affinity peptides was completed with SYFPEITHI suggesting that those results may be verified by this BIMAS data.

4.5.3 BIMAS – Lower Scoring Peptides

Incorporating lower affinity peptides (Threshold of 5) in the study as well demonstrates similar results to those from the study of low affinity peptides only when analysed with SYFPEITHI. There is an increased frequency of predicted HLA-binding peptides from HLA-$A1^+$ and -$B7^+$ donors (3.69% and 2.36%) compared with those of HLA-$A1^-$ and $B7^-$ (3.55% and 2.09%) (χ^2=0.09, p=0.77 & χ^2=0.48, p=0.49) (Figure 4.8 B). Although the difference between the two cohorts was smaller than previously seen, the trend remains the same when investigating the lower affinity peptides as well. Similarly to the previous

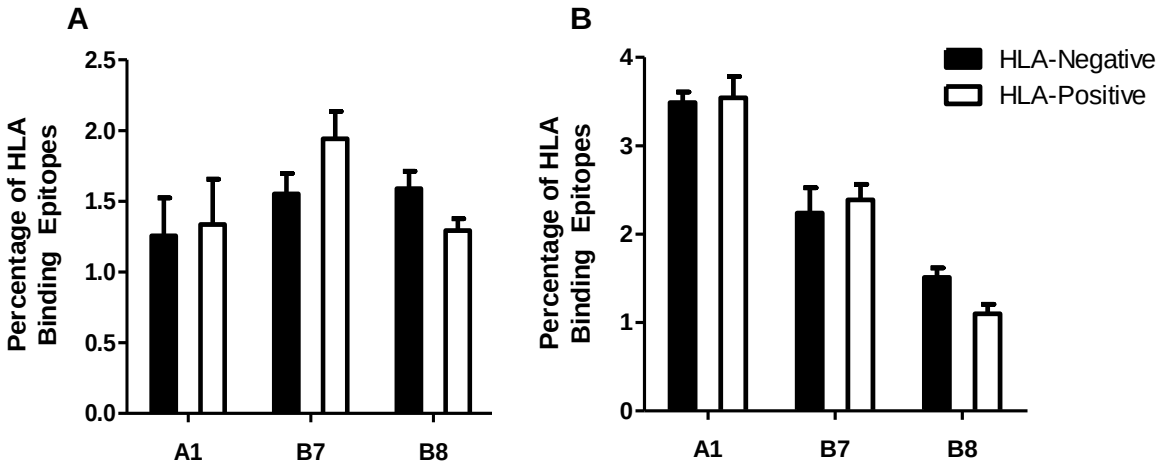


Figure 4.8. Frequency of high and low affinity HLA-binding peptides from the CDR3 of the TCR β-Chain

TCRs from healthy donors (separated into HLA-positive and HLA-negative cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for high affinity (A) and low affinity (B) HLA-A1, -B7 and -B8-binding peptides using the BIMAS algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by thresholds 12 for HLA-A1, 16 for HLA-B7 and 12 for HLA-B8 (A) and 5 for all alleles (B). Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance.

HLA-B8 results, there was a decreased frequency of CDR3-derived peptides in the HLA-B8$^+$ donors (1.08%) compared with the HLA-B8$^-$ donors (1.54%) (χ^2=2.31, p=0.13) (Figure 4.8 B).

4.6 Frequency of TCRs Containing CDR3-Derived Peptides

After showing that there was an increased frequency of TCRs containing more than 1 HLA-A2 binding peptide in HLA-A2$^+$ donors, the other HLA alleles were investigated to determine whether there was a similar difference in frequency. Using SYFPEITHI the results in Figure 4.9 demonstrate that there are no TCRs which contain more than one peptide using the algorithms for HLA-A1 and –B7. Similar to the previous results, HLA-B8$^+$ donors demonstrate a decreased frequency of TCRs containing at least one CDR3-derived HLA-binding peptide (0.0%) compared with the HLA-B8$^-$ donors (1.2%).

Using BIMAS, similar trends can be seen, using either the higher or lower thresholds, with the HLA-positive donors in –A1 and -B7 demonstrating an increased frequency of TCRs compared with the HLA-negative donors (Figure 4.9). The trend seen previously with –B8 can also be seen here with an increased frequency of TCRs in HLA-B8$^-$ donors (1.2% (high) and 13.6% (low)) compared with HLA-B8$^+$ donors (1.1% (high) and 7.5% (low)) using both the higher and lower BIMAS thresholds.

These results suggest that there is peripheral tolerance towards CDR3-derived peptides from TCRs in all HLA alleles tested except HLA-B8. Therefore, there is selection pressure on TCRs based on CDR3 sequence but instead of selecting TCRs without any

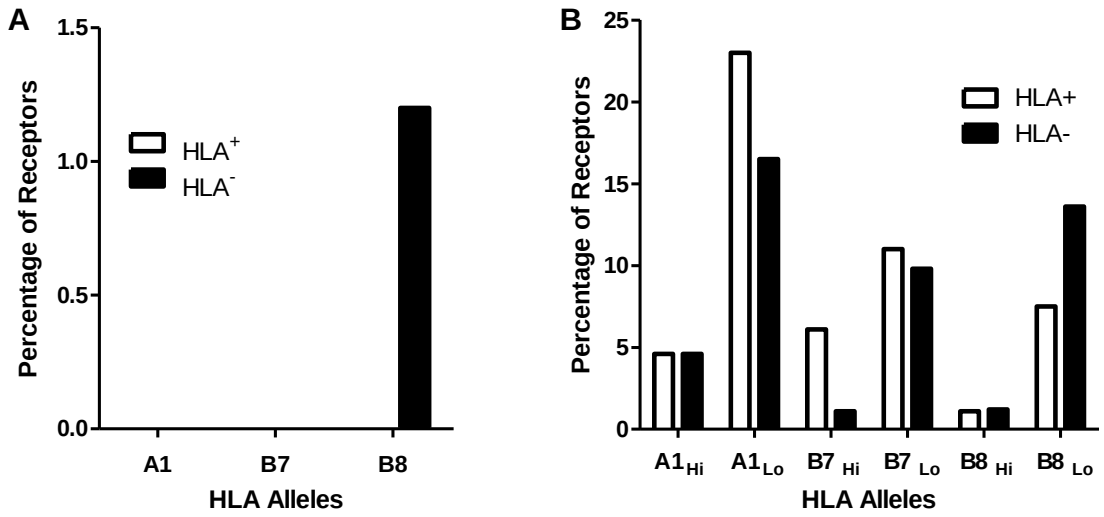


Figure 4.9 Frequency of TCRs containing more than one HLA-binding peptide in the other HLA types investigated

TCRs from healthy donors (separated into HLA-positive and HLA-negative cohorts) were analysed to determine the frequency of those containing more than one HLA-binding peptide in the other HLA alleles investigated using both peptide prediction algorithms SYFPEITHI (A) and BIMAS (B). Data is represented as a percentage of all peptides analysed where peptide binding was defined for both algorithms as previously discussed. This was done in an attempt to determine whether there was skewed data in the previous analysis of the peptide prediction data due to a single BCR containing a large number of HLA binding peptides within the CDR3.

HLA-binding peptides within the CDR3 as was first hypothesised, TCRs are actually selected to contain an HLA-binding peptide within their CDR3, except from those in the HLA-$B8^+$ cohort. This is due to the fact that for both algorithms tested, there was an increased incidence of CDR3-derived peptides from HLA positive donors compared with HLA negative donors.

4.7 Frequency of CDR3-Derived HLA-A2 Binding Decamer Peptides

The above results are for CDR3-derived peptides which are 9 amino acids long, however, it is well known that a significant percentage of HLA class I peptides can be increased in length to 10 amino acids (Collins et al, 1995 and Takahashi et al, 1999). Therefore, a second analysis of the sequences of TCR CDR3s was performed to determine whether the results seen in HLA-A2 donors for peptides which were 9 amino acids in length was repeated in peptides which are 10 amino acids in length.

Using SYPEITHI, there was a statistically significant increase in the higher affinity peptides, 10 amino acids in length, predicted to bind to HLA-A2 in TCRs from HLA-$A2^+$ donors (1.24%) compared with HLA-$A2^-$ donors (0.56%) using a threshold of 22 (χ^2=7.17, p=0.01) (Figure 4.10), similar to that seen in the above results for peptides 9 amino acids in length. Although this result does not validate all of the previous results for all of the different HLA types, the similarities in the different analyses gives a degree of confidence that the results are authentic and not a mathematical artefact due to the HLA peptide prediction algorithms.

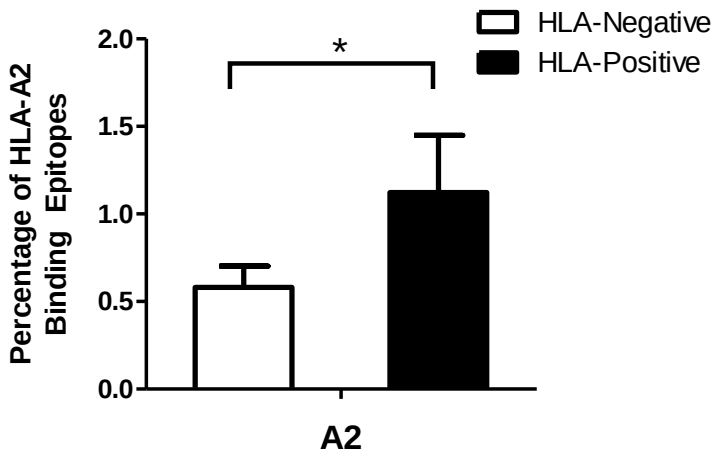


Figure 4.10 Frequency of predicted HLA-A2 binding decamer peptides from HLA-A2$^+$ and HLA-A2$^-$ donors

TCRs from healthy donors (separated into HLA-positive and HLA-negative cohorts) were sequenced from unfractionated PBMCs and the CDR3 analysed for HLA-A2-binding peptides of 10 amino acids using the SYFPEITHI algorithm. Data is represented as a percentage of all peptides analysed where peptide binding was defined by a score of 22 or above. Error bars denote mean + standard error mean. Data was analysed using chi-squared test to determine statistical significance where * $P<0.05$.

4.8 T Cell Response to CDR3-Derived Peptides

The above results show the presence of potentially immunogenic HLA-binding peptides within the CDR3s of TCR β-chains from healthy donors in several HLA alleles. Even though these data disproved the original hypothesis where a decreased percentage of HLA-binding peptides contained within the TCRs of HLA-positive donors compared with HLA-negative donors was expected, this led to the development of a second hypothesis, that the CDR3-derived peptides could be processed and presented on MHC class I molecules on the surface of the T cells and therefore I could generate an immune response towards them. Firstly, a peptide contained within the CDR3 of a TCR was required so it could be targeted. $CD8^+$ T cells were cloned using CMV peptides as antigens to allow growth of T cell clones and the TCR α & β-chains sequenced to find a potentially immunogenic peptide within the CDR3. Previously, Winter et al., 2003 had determined that T cell responses could be generated towards peptides from the V region as well as the CDR3 of T cells from CTCL patients. Further work demonstrated the ability of these T cells to recognise and lyse autologous CTCL cells in a peptide-MHC I dependent manner. Once a TCR expressing an HLA-binding peptide had been discovered an immune response against the idiotype-derived peptide was mounted. HLA-A2 was used for this work as the algorithm for peptide prediction is the most robust of the HLA alleles that have been studied.

4.8.1 TCR CDR3-Derived HLA-A2 Binding Peptides

Five T cell clones were grown from two different HLA-$A2^+$ donors, with T cells specific for a number of CMV-derived peptides. Sequencing analysis of the TCR α &

β chains demonstrate the presence of an HLA-A2 binding peptide, ALCDTGRRAL, within the TCRα chain of the ELR-specific T cell clone from donor DB (Table 4.1). This peptide, which scored 24 in the SYFPEITHI-derived HLA A2 algorithm but only scored 17.5 using the BIMAS-derived algorithm, was considered the best candidate. However, whilst sequencing the TCR alpha and beta chains for this clone, I realised that there was a possibility that the T cells were not clonal. The TCR from the ELR "clone" contained two different TCR beta chain sequences (Table 4.1) which suggests that this particular T cell could have at least two different T cell clones growing together in culture. In order to confirm this finding I used the flow cytometer to stain the T cells for the different V-beta chain usage to determine whether there was more than one TCR beta chain variable (TCRVβ) chain being used by the T cells. If there was staining for more than one TCRVβ chain in the whole T cell culture, this would confirm that there was more than one T cell clone growing. The sequencing results suggest that the ELR1 "clone" contains one T cell using the TCRVβ17.2 variable region and another T cell using the Vβ14 variable region and therefore the antibody staining should follow these results. However, the sequencing results may not have picked up all of the different T cell CDR3s and there could be more than two different T cells in the culture. The T cells were stained using all 24 TCRVβ antibodies and the results showed that the ELR1 T cell clone was 7.1% positive for Vβ17.2 and 90% positive for Vβ14 (Figure 4.11 A) with no staining for the other Vβ antibodies. These results suggest that there are only two T cell clones growing together in the culture which correlates with the results from the sequencing. In order to have a

T Cell Clone	Sequence
VTE 2 β-Chain	SAMYLCASSLEQVRPYEQYFGPGTRLTVTEDLKNV
NLV 1 α-Chain	SATYLCAVKATNYGQNFVFGPGTRLSVLPYIQNPDPA
NLV 1 β-Chain	TSVYFCASPDYGDNEQFFGPGTRLTVLEDLKNV
ELR 1 α-Chain ($V\beta14^+$)	SAMYYCALCDTGRRALTFGSGTRLQVQPNIQNP
ELR1 α-Chain ($V\beta17.2^+$)	SAMYFCASSDRGQPQHFGDGTRLSILNIQNPDPA
ELR 1 β-Chain (A)	SAVYLCASRGVGSPLHFGNGTRLTVTEDLNKVFPP
ELR 1 β-Chain (B)	TAFYLCASSIDPLRVGPNGDSPLHFGNGTRLTVTEDLNKNV
TPR 2 α-Chain	SAVYICAVNTGNQFYFGTGTSLTVIPNIQNPDPA
TPR 2 β-Chain	SAVYFCASSQEFPPRANTGELFFGEGSRLTVLEDLKNV
RPH 1 α-Chain	SAMYLCASSIDSFNEQFFGPGTRLTVLEDLKN

Table 4.1. Sequencing TCR α and β chains from CMV-specific $CD8^+$ T cell clones

DNA encoding the TCR α or β-Chain from CMV-specific CD8 T cell clones were sequenced. The sequence above shows the variable region, through the CDR3 region (underlined) and into the constant region. The sequences could then be used to determine the presence of HLA-A2 binding peptides within the CDR3 region using SYFPEITHI and BIMAS. The ELR1 Clone was found to be made of two different clones and therefore contains two sequences for the alpha and beta chains. The two clones could be selected using magnetic beads (Figure 4.11).

clonal T cell culture, I attempted to separate the two T cell clones using the TCR Vβ17.2 antibody, which was conjugated with FITC, and anti-FITC microbeads. The two T cell clones were isolated using magnetic selection over two columns to try to ensure the purity was as high as possible so that the $V\beta14^+$ T cells are only in the negative fraction and the $V\beta17.2^+$ T cell are only in the positive fraction. The results of the T cell selection/separation showed that, after running the cells over two columns, the two T cell clones were separated and very pure (Figure 4.11 B-D) with 99.6% of the CD8 T cells in the positive fraction also staining positive for TCRVβ17.2 and less than 0.1% in the negative fraction. This result allowed the determination of which T cell clone the CDR3-derived HLA-A2 binding peptide (ALCDTGRRAL) belonged to. After growing both T cell clones, the alpha chains of both the $V\beta14^+$ and the $V\beta17.2^+$ cells were sequenced and it was determined that the $V\beta14^+$ cells contained the TCR alpha chain with the HLA-A2 binding peptide, ALCDTGRRAL (Table 4.1).

4.8.2 CDR3-Derived Peptide-Specific T Cells

Previously, studies had generated T cell responses against peptides from the Ig framework regions (Trojan et al., 2000). The specificity of the TCR from the T cell clone where the peptide was found did not concern me as this T cell will eventually become the target for any T cells generated against the CDR3-derived peptide. The peptide was predicted to bind to HLA-A2, with a binding score produced by SYFPEITHI of 24, so a CD8 T cell response could be generated against it using previously discussed methods (Ho et al, 2006). Dendritic cells were generated from PBMCs, from an HLA-$A2^+$ donor, and stained after 3 days with antibodies to markers expressed on mature dendritic cells (CD80, CD83 and CD86). The

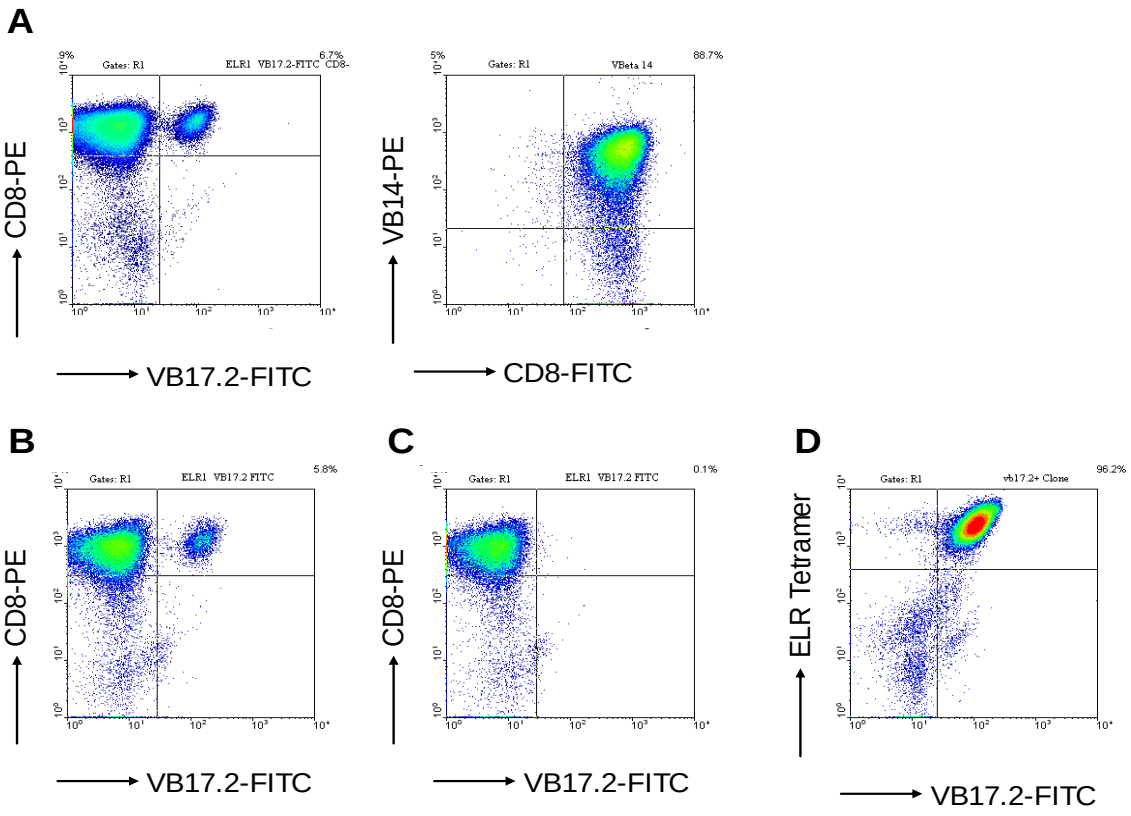


Figure 4.11 Antibody staining and magnetic selection of TCRVβ at the surface of ELR1 cells

ELR1 T cells were stained with a panel of antibodies to determine their β-chain variable region usage. Cells were stained with antibodies specific for individual TCRVβ regions as well as with anti-CD8 (FITC or PE) for 30 minutes before being washed and analysed on the flow cytometer (A). ELR1 T cells were stained with the Vβ17.2-FITC antibody for 30 minutes and washed before addition of anti-FITC microbeads. Cells were washed extensively and were then separated using two magnetic columns to separate the antibody stained cells from the cells not stained with antibody. Cells prior to separation (B) and the negative (C) and positive (D) fractions were then analysed on the flow cytometer.

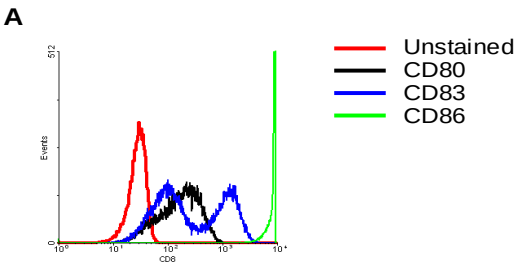


Figure 4.12 Antibody staining of dendritic cells matured for 3 days

PBMCs were plated out and adherent cells were matured into dendritic cells using cytokines and growth factors over 3 days (Ho et al, 2006). Dendritic cells were stained on day 3 with antibodies to determine their maturation state. The cells were incubated with antibodies against CD80 (Black), CD83 (Blue) and CD86 (Green) for 30 minutes. After washing, the cells were analysed on the flow cytometer.

cells stained with antibodies were positive (Figure 4.12) with all three of them staining more than 85% of the dendritic cells. These results strongly suggest that the cells are dendritic cells and have matured using the cytokine cocktail described. Dendritic cells were used to generate a naïve T cell response against the CDR3-derived peptide. After 3 rounds of stimulation, T cells were cloned (as described in the materials and methods) and 10 T cell clones recognised peptide-pulsed PBMCs shown by release of IFN-γ (Figure 4.13 A) whilst not recognising unpulsed PBMCs suggesting that these clones are specific for the CDR3-derived peptide. The peptide was predicted to bind to HLA-A2 but peptide binding studies have not confirmed this possibly in part due to the cysteine residue at position 3. This amino acid contains the reactive sulphydryl group that can either be in a reduced or oxidised state. Cysteine residues could form a dimer (cystine), joined by a disulphide bridge, and this may alter the presentation of the peptide to the $CD8^+$ T cells. The peptide will act differently in each of its two states which makes determining HLA restriction difficult. A B cell line, JY (A2, B7 and Cw7 homozygous), was used to further determine the HLA-restriction of the CDR3-derived peptide. JY cells, pulsed with ALCD peptide, were strongly recognised by the T cell clones using an IFN-γ ELISA whereas unpulsed JY cells were not recognised (Figure 4.13 B). This data demonstrates the specificity of the T cell clones for the ALCD peptide and helps to narrow the HLA restriction as well. Recognition of the peptide-pulsed JY cells means the peptide can only bind to A2, B7 or Cw7. The peptide is unlikely to bind to HLA-B7 due to the presence of leucine at position 2, generally regarded as an anchor for HLA-A2 whereas HLA-B7 binding peptides generally prefer proline at position 2. This is seen in the HLA-B7 peptide prediction

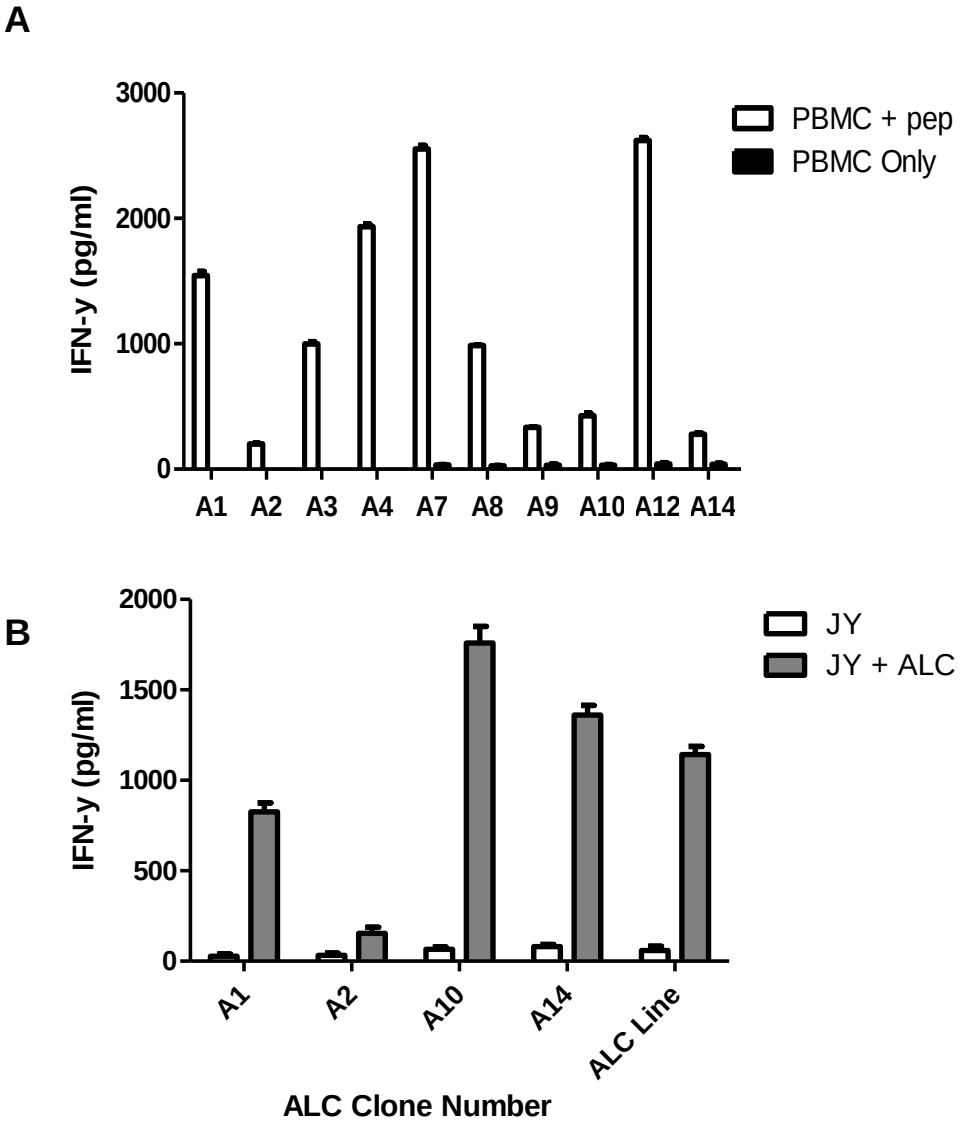


Figure 4.13. Recognition of peptide pulsed target cells by ALC clones

Initially 10 clones thought to be specific for the CDR3-derived peptide ALC were tested for reactivity using IFN-γ ELISA. Autologous PBMCs were pulsed with ALC peptide (PBMC + Pep) or left unpulsed (PBMC) and after washing, target cells were incubated with the ALC-specific T cells overnight with unpulsed PBMCs acting as a negative control. The media was taken to assay for IFN-γ by ELISA (A). Four surviving T cell clones were used to test for reactivity against ALC to help ascertain the HLA-restriction of the peptide. Cells from the B cell line JY were pulsed with ALC peptide (JY + ALC) or left unpulsed (JY) and after washing, the target cells were incubated with the ALC-specific T cells overnight with unpulsed JY cells acting as a negative control. The media was taken to assay for IFN-γ by ELISA (B). Error bars denote mean + standard error mean.

score of 14 from SYFPEITHI which tends to suggest that this peptide won't bind to HLA-B7. However, HLA-B7 prediction from BIMAS suggests that the peptide may indeed bind with a score of 12.00 compared with only 17.52 for HLA-A2. SYFPEITHI doesn't have a prediction algorithm for HLA-Cw7 so it is not possible to determine potential binding of the ALCD peptide. However, BIMAS contains a prediction algorithm for HLA-Cw7, scoring ALCD 1.01, suggesting that the peptide does not bind to HLA-Cw7. These results suggest that the peptide used binds to HLA-A2 and not to HLA-B7 or HLA-Cw7 but this may need verification.

4.8.3 Recognition of Parental T Cell Clone by Peptide-Specific T Cells

One of the hypotheses tested, that runs throughout all of this work, was whether or not a peptide originally found within the CDR3 of a TCR could be processed by a T cell and expressed on the surface of the cell in complex with MHC class I molecules. Originally, a proteomic approach using mass spectrometry was going to be employed in an attempt to determine the presence of the CDR3-derived peptide on the cell surface. However, the number of T cells required to do this was very high (~10^9) and despite lengthy attempts to generate this number of T cells, it wasn't possible. Therefore, to test this hypothesis ALC-specific T cells were cultured with the original T cell clone to determine whether there was recognition of the ELR T cell clone by the ALC-specific T cell clone. The ALC peptide used to generate CD8 T cell clones was originally discovered in one of the TCR α-chains from the $V\beta14^+$ fraction ($V\beta17.2^-$ fraction) of the T cell "clone" ELR1. Previously, I determined that the ALC-specific T cell clones and line recognise peptide strongly, by generating a large IFN-γ response, and don't recognise JY cells that have not been pulsed with peptide. In order to determine the presence of the ALC peptide on the cell surface in complex

with an HLA molecule, a second, more sensitive method, was used. ALC specific T cells and the ELR1 Vβ14^{+} T cell clone were co-cultured to test whether the ELR1 target cells express the ALCD peptide on the surface of the cell which could be detected by the ALC-specific T cells. The ELR T cell clone that was Vβ17.2^{+}, and therefore did not contain the ALC peptide within the α-chain, was also used as a control target cell that is almost identical to the ELR1 target cell. After 24 hours co-culture, the culture supernatant was taken and tested for IFN-γ with the presence of IFN-γ demonstrating recognition of ALC peptide at the cell surface. An initial experiment to demonstrate recognition of the ELR-specific T cell clone by the ALC-specific T cells showed a low IFN-γ response when the two cells were cultured together, (Figure 4.14) demonstrating T cell recognition. There is an increased amount of IFN-γ released in this experiment by the clone recognising the parental T cell expressing the TCR containing the ALC peptide compared with all of the controls although the amount of IFN-γ produced was lower than previous experiments where the ALC-specific T cells recognised peptide-pulsed target cells. This result suggests that the ALCD peptide is expressed on the surface of the target T cells, although at a lower number of peptide-MHC complexes than when the PBMCs or JY cells were peptide-pulsed because there was a decrease in IFN-γ production when the ALCD-specific T cells were cultured with the ELR1 T cells.

Both target and responder T cells could potentially produce IFN-γ and it was important to test both cell lines for spontaneous IFN-γ release. There is a very low background release of IFN-γ by both cell lines, however, the amount of IFN-γ

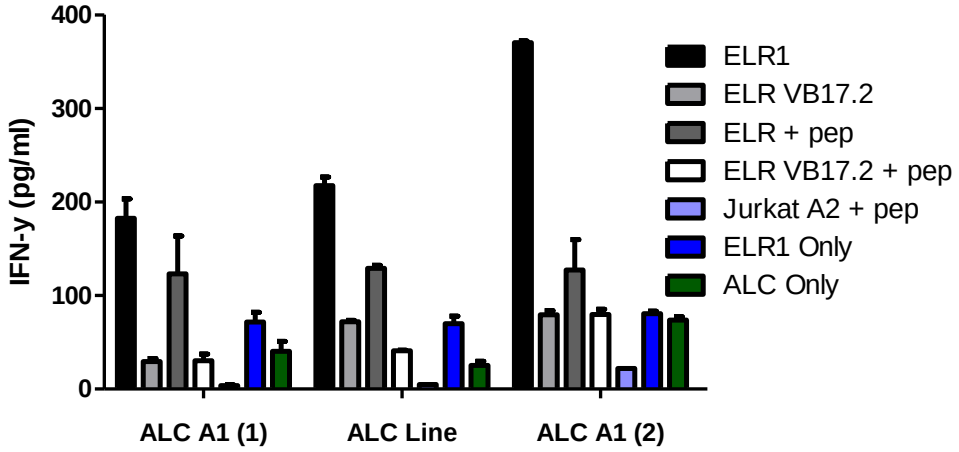


Figure 4.14. Recognition of ELR1 T cell clone by the ALC-specific T cell clone

The ELR1 T cell clone contains the ALC peptide within the TCR α-chain whereas the ELR Vβ17.2 T cell clone does not contain the ALC peptide within its sequence. Two T cell clones, either ALC + ELR 1 or ALC + ELR Vβ17.2 were incubated together overnight to determine whether the ALC-specific T cells could recognise the ELR1 or ELRVβ17.2 T cells and the media taken for IFN-γ ELISA. Peptide-pulsed T cells (ELR + pep & ELR Vβ17.2 + pep) or cell lines (Jurkat A2 + pep) were used as control. In order to demonstrate there wasn't spontaneous release of IFN-γ, the T cell clones were cultured on their own (ELR1 only and ALC only) overnight and the media assayed. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance.

produced by the co-culture of the two cell lines produced a significant increase in IFN-γ. This would suggest that the IFN-γ release could be specific for ALC peptides, in complex with HLA, present on the surface of the ELR T cell clone. The ELR T cell clone was derived from an HLA-$A2^+$ donor who was also HLA-B8 but not HLA-B7. This would suggest that the ALC peptide was probably presented on the cell surface by HLA-A2 as we already know the peptide is restricted through either A2 or B7. Since the cells used don't contain HLA-B7 or HLA-Cw7, this would suggest that the peptide is indeed presented by HLA-A2.

Although the peptide pulsed LCLs (acting as positive control) were not recognised by the peptide specific T cells, the results suggest that there is recognition of endogenously processed CDR3-derived peptides. This would suggest that potentially immunogenic CDR3-derived peptides gain access to the MHC class I processing pathway. These experiments were repeated to prove that there was an IFN-γ response towards the ELR1 T cell clone but, whilst trying to produce further data, the ALCD-specific T cell clones and lines died so no further work was possible with this part of the project.

4.9 HLA and TCR Expression on Jurkat-A2 Cells

Initially, experiments were focused on the immortalised Jurkat T cell line, which was HLA-$B7^+$ but HLA-$A2^-$. I had to use a T cell to introduce the TCR β-chain into as there would need to be an endogenous TCR α-chain to pair with the newly introduced β-chain. This would allow the mutated TCR β-chain to be expressed at the surface of the cell as a fully intact TCR and would mean that the TCR would be degraded in the same manner as a normal TCR. One of the most important considerations in

establishing this model was to reach as close to physiological conditions as possible so the results would be indicative of what happens in functional T cells. The Jurkat T cell line that we were given (Dr V. Engelhard, University of Virginia) had already been transduced with HLA-A2 so I had an immortal T cell line that was HLA-$A2^{+}$. The cells were stained with BB7.2-PE (anti-HLA-A2 antibody) and BB7.1-PE (anti-HLA-B7 antibody) in order to confirm that the cells growing were the same HLA type as expected (Figure 4.15 A). The Jurkat-A2 cells were strongly positive for both HLA-A2 and -B7, demonstrating that the cells could be confidently used for future experiments. As mentioned before, in order for the TCR β-chain, that is going to be introduced into the cells, to be expressed at the cell surface, it must pair with an endogenous α-chain. The staining (Figure 4.15 B) demonstrates the expression, albeit slightly low, of TCRαβ protein at the surface of the cell. This confirms that there is an endogenous α-chain that the introduced TCR β-chain could pair with and therefore be expressed at the surface. The Jurkat T cell line is a CD4 T cell line which already contains a T cell receptor. The T cell receptor used for mutagenensis studies contained the TCR variable region TCRVβ7. It was important to first ensure that the Jurkat T cell receptor was TCRVβ7 negative. Using the TCRVβ7 antibody (Abcam, Cambridge, UK) conjugated to phycoerythrin demonstrated that the Jurkat T cell line is TCRVβ7 negative (Figure 4.15 C). PBMCs, which will contain a small percentage of T cells expressing TCRVβ7, were used as a positive control to test that the antibody does bind with 4.2% staining positive as expected. These results confirm that if the mutated T cell receptor β-chain is introduced into the cell, antibody staining could be used to test for the expression at the surface of the T cells.

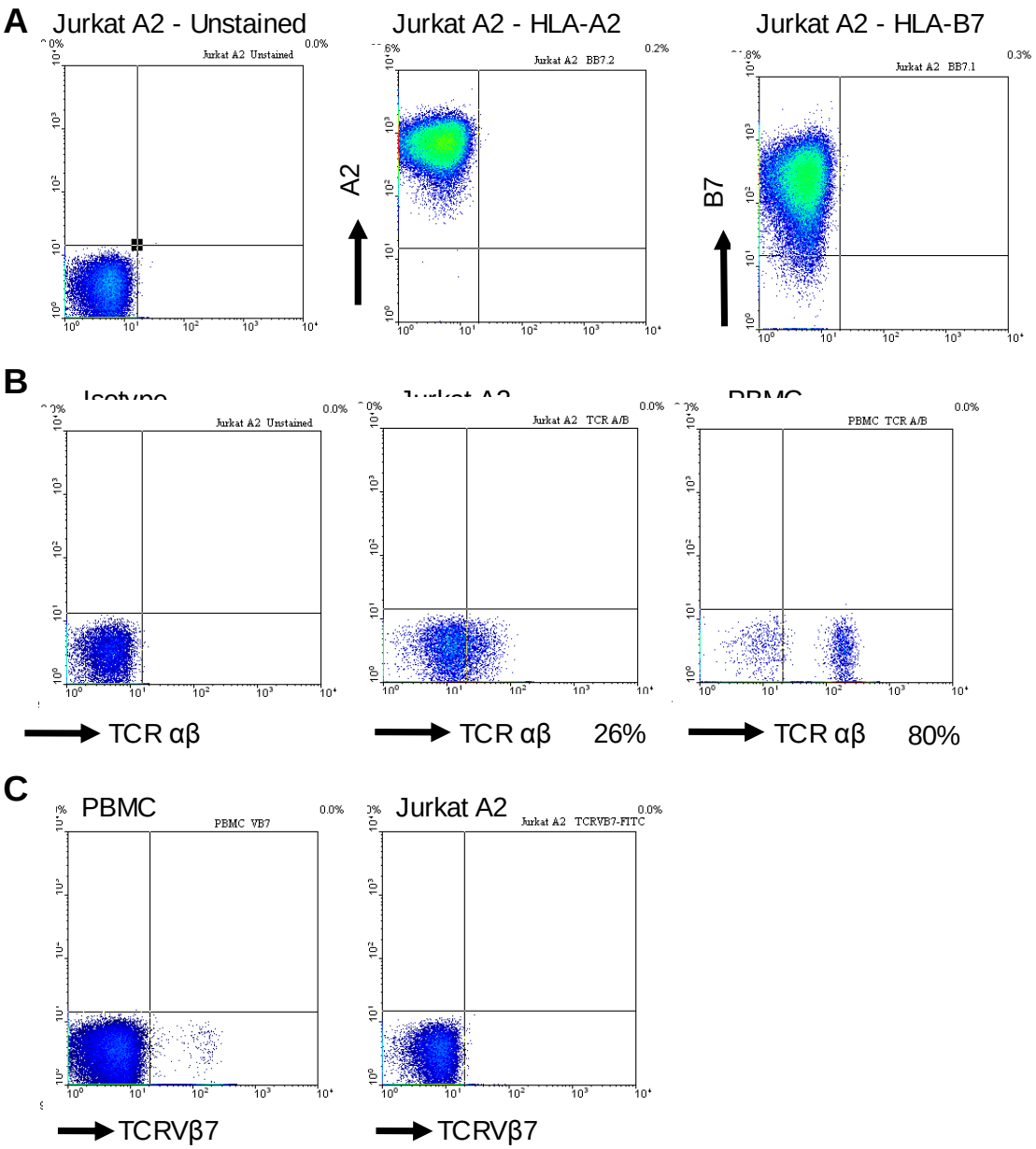


Figure 4.15. Flow cytometric staining Jurkat A2 cells

Jurkat A2 cells were stained with antibodies against surface HLA-A2 and HLA-B7 (A) for 30 minutes, washed and analysed on the flow cytometer. Jurkat A2 cells and PBMCs were stained with either an isotype control antibody or an antibody against surface TCRαβ expression for 30 minutes, washed and analysed on the flow cytometer (B). PBMCs and Jurkat A2 cells were stained with the TCRVβ7-PE antibody for 30 minutes, washed and analysed on the flow cytometer (C).

4.10 Introduction of Foreign DNA into Cells

Initially, I had to decide which method was the easiest and most reproducible method for inserting DNA encoding the T cell receptors into the cell of interest. The methods tested were standard transfection, with an array of different transfection reagents, transfection leading to stable integration, using a specially prepared kit from Invitrogen, nucleofection, using the Amaxa system, and transduction, using lentiviral vectors. After determining which of the protocols generated the best results, further experimental work would proceed with this method.

4.10.1 Green Fluorescent Protein

Initially, green fluorescent protein (GFP) was used as an easy way to detect protein for my first attempts using Jurkat T cells. Once GFP expression was detected in Jurkat T cells, the same protocol could be used to introduce the TCR DNA. Although standard transfection of Jurkat A2 cells using Lipofectamine LTX (and the PLUS reagent) or Lipofectamine 2000 didn't work, a different transfection reagent, TransIT, demonstrated encouraging results (Figure 4.16). TransIT is specifically tailored for Jurkat T cells as they have been described as a cell line that is difficult to transfect. Flow cytometric analysis of the transfected cells showed 1-2% of cells were GFP-positive after 24hrs which increased to 6-10% after 96 hours in Jurkat A2 T cells. After 7 days less than 1% of the transfected cells were GFP positive demonstrating the loss of expression of GFP after 7 days and suggesting that the expression seen after 96 hours was transient and not stable. Although the cells lost expression of GFP, this method was considered for introduction of mutated TCR DNA into the Jurkat A2 cells.

4.10.2 Nucleofection

Jurkat cells are very difficult to transfect and nucleofection is considered to be a useful method in transferring DNA into cells which are difficult or impossible to transfect. Based on electroporation, nucleofection uses a combination of factors including voltage and time to make cells porous and allow transfer of DNA into the cell. Using this method, the conditions are completely dependant on the cell type, not the type of nucleic acid being used. Unlike other transfection methods, the cells do not need to be actively dividing when DNA is being transferred into the cells.

Nucleofection of Jurkat A2 cells was performed using the pcDNA3.1/Hygromycin vector. Using the Amaxa nucleofector the conditions used for nucleofection of Jurkat A2 cells could not be obtained. However, 48 hours post-nucleofection, the cells were analysed for GFP expression using the flow cytometer. Less than 0.5% expressed GFP demonstrating that nucelofection did not transfer DNA efficiently into the Jurkat A2 cells. Therefore, transfection of DNA using nucleofection as a method for introducing the mutated TCR DNA into Jurkat A2 cells ceased.

4.10.3 Flp-In Transfection

GFP contained within the pcDNA5 vector and transfected into Jurkat A2 T cells

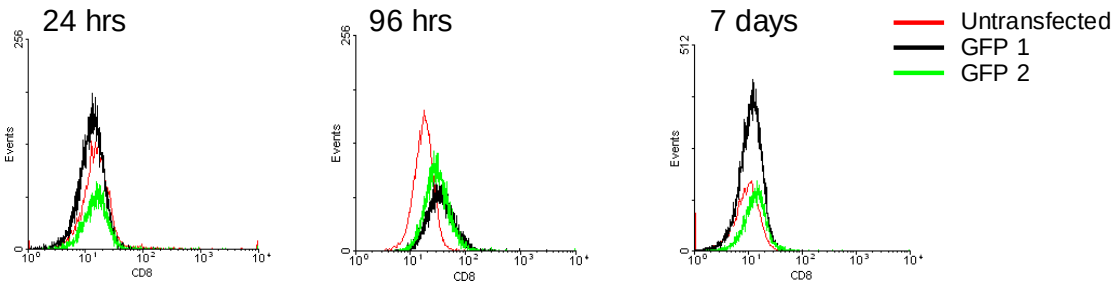


Figure 4.16. Expression of green fluorescent protein at the surface of Jurkat A2 cells

Green fluorescent protein (from two different plasmids) was transfected into Jurkat A2 cells using the TransIT transfection reagent (MirusBio). Cells were analysed on the flow cytometer after 24 hours when there was little GFP expression. After 96 hours, both of the transfected cell lines demonstrated an increased percentage of GFP staining (black and green) compared with the untransfected control cells (red). However, staining after 7 days demonstrated the loss of GFP from both transfected cell lines.

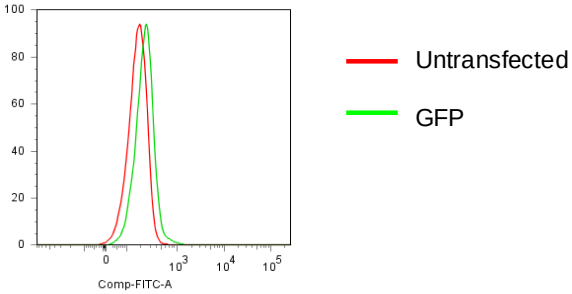


Figure 4.17 GFP expression in Jurkat-Flp In after transfection using Flp-In system

The Jurkat-Flp In cells were transfected with the pcDNA5/Hygro plasmid containing GFP. 48 hours after transfection, the cells were analysed on the FACS machine and ~4% of transfected cells were GFP positive (Green) compared with the untransfected control (Red). Expression of GFP was lost soon after.

demonstrated 4% GFP-positive cells (Figure 4.17) when tested 48 hours post-transfection. The cells were grown in media containing 300ug/ml hygromycin to try to select the transfected cells. However, when analysed again on the flow cytometer after 7 days, no cells survived suggesting that the hygromycin selection had killed all of the cells and that the protein production seen after 3 days was transient. Therefore, due to the inability of this system to generate and select stable transfectants, it was not used in any further work.

4.11 Expression of GFP by Lentivirus

Lentiviral trasnduction (Bai et al., 2003) began by attempting to generate pTRIP-GFP lentiviral particles, using different transfection reagents (Lipofectamine 2000, Lipofectamine LTX, GeneJuice and NanoJuice) to transfect 293T cells in order to determine which transfection reagent produces the most lentiviral particles. After generating the virus particles, Jurkat A2 cells were transduced with each of the GFP-viruses and after 48 hours, the cells were analysed on the flow cytometer (Figure 4.18 A-B). The greatest percentage of GFP-transduced cells was seen in the Lipofectamine LTX transfection reagent (99.5%) when compared with the untransduced control cells. Lipofectamine 2000 (42%) and NanoJuice (18%) also produced GFP-positive cells although at a lower percentage and GeneJuice didn't produce any GFP-positive cells. This suggested that the Lipofectamine LTX reagent, along with the PLUS reagent, was the best protocol to produce lentivirus particles in 293T cells. Previously, the problem encountered was the transient nature of the protein production following introduction of the gene of interest. Therefore, the transduced cells were cultured to allow quantitation of GFP expression over the following months to determine

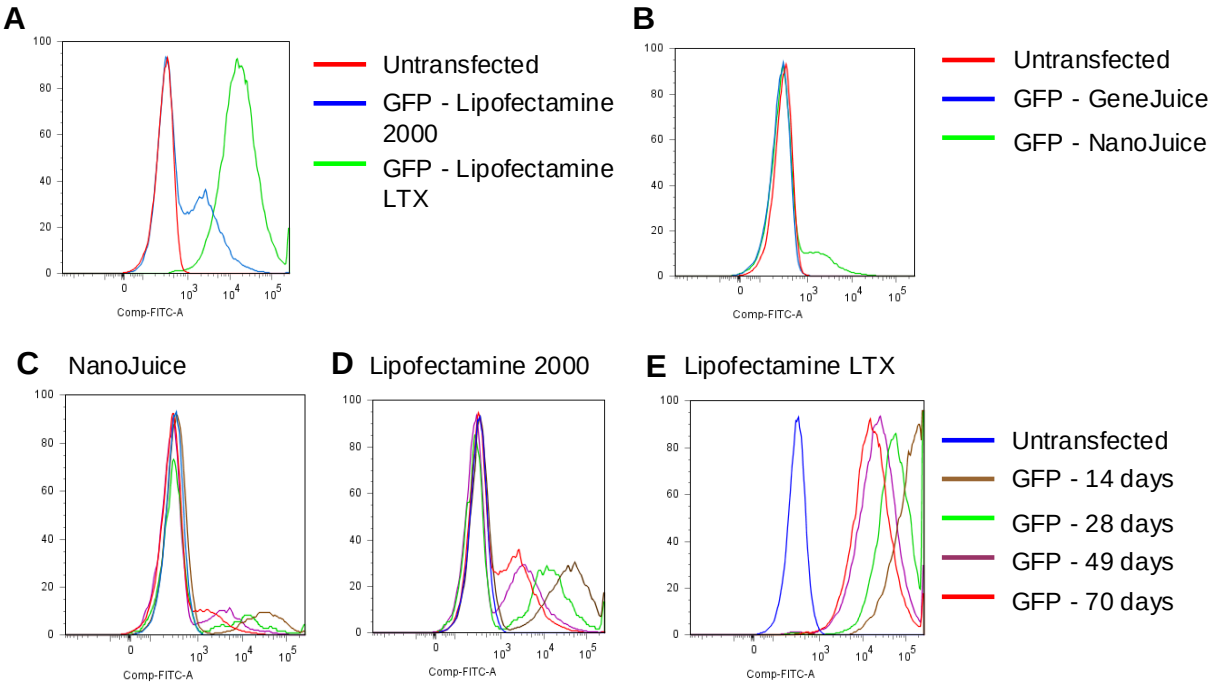


Figure 4.18. Expression of GFP after lentiviral transduction of Jurkat A2 cells

GFP lentivirus was produced in HEK-293T cells transfected with different transfection reagents and the lentivirus particles used to transduce Jurkat A2 cells (A-B). Cells were transduced with virus from flasks of 293T cells transfected with Lipofectamine LTX (A: Green), Lipofectamine 2000 (A: Blue), NanoJuice (B: Green) or GeneJuice (B:Blue). Untransduced Jurkat-A2 cells (Red) were used as control cells. Cells transduced with GFP lentiviral particles produced from either NanoJuice (C), Lipofectamine 2000 (D) or Lipofectamine LTX (E) were analysed on the flow cytometer 4 times over 10 weeks. Cells were removed from culture, washed and analysed using the flow cytometer.

whether the introduction of the DNA was transient or stable. The Jurkat T cell line, transduced with lentiviruses produced with the various reagents, were cultured and the percentage of GFP-producing cells determined. The cells were tested every few weeks and the percentages of GFP-positive cells were noted to see whether the cells continued to produce the protein. Up to 10 weeks after transduction, similar percentages of cells were still expressing GFP (Figure 4.18 C-E) suggesting that the transduction produces stable integration of the gene of interest. Following the results with pTRIP-GFP viruses, pLVX-IRES-Puromycin lentivirus particles containing GFP were generated using standard 293T cells. The transfection was done using the Lipofectamine LTX and PLUS reagent, which were used for all subsequent transductions.

4.12 Expression of GFP in Selectable Lentivirus

Previously, the selection of transfected cells was unsuccessful and in order to test the pLVX lentiviral system, I transduced Jurkat A2 cell line and HEK-293T cells with pLVX-IRES-Puromycin virus containing GFP. After 48 hours, the cells were analysed on the flow cytometer to determine the transduction efficiency using the new lentivirus with the pTRIP-GFP virus, used previously, as a control (Figure 4.19 A-B). The Jurkat A2 cell line transduced with the pLVX-GFP virus contained 6% GFP positive cells compared with the mock transduced cells whereas the HEK-293T cells transduced with the pLVX-GFP virus contained 21% GFP positive cells compared with the untransduced control cells. The pTRIP-GFP virus produced an increased percentage of GFP positive Jurkat-A2 and HEK-293T cells (74% and 92%) demonstrating that the transduction efficiency of the

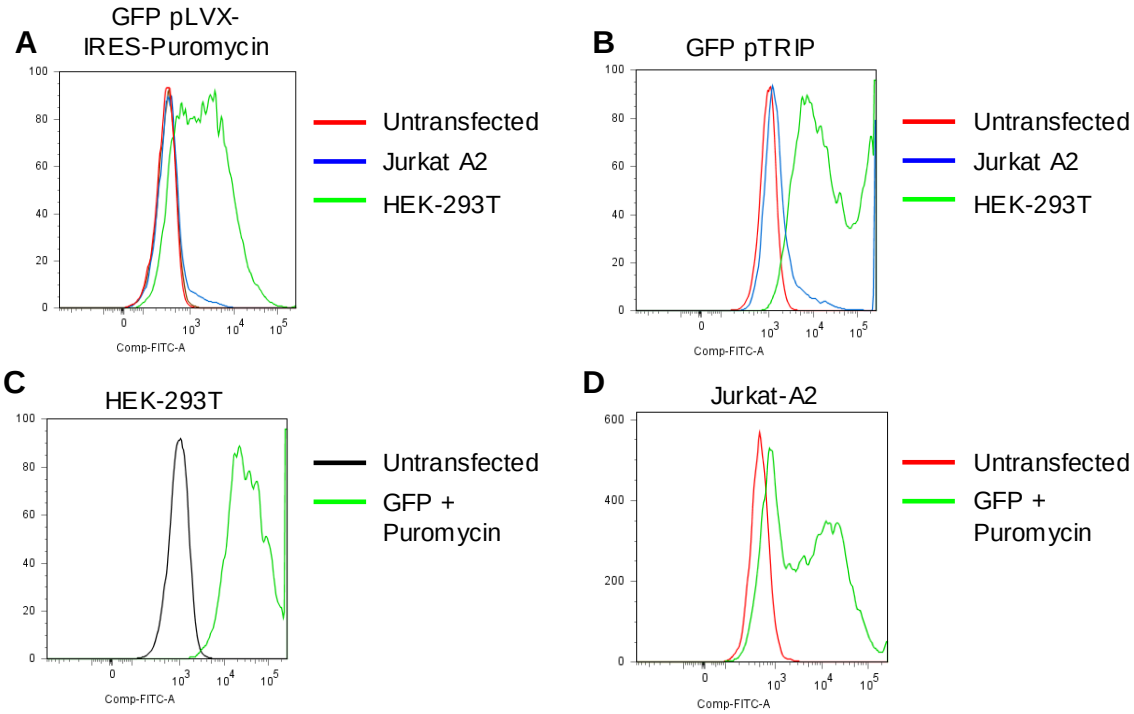


Figure 4.19 Transduction and selection of Jurkat-A2 and HEK-293T cells with pLVX-IRES-puromycin lentivirus containing GFP

Jurkat-A2 cells (Blue) and HEK-293T cells (Green) were transduced with GFP lentiviral particles (A-B). The lentivirus was produced from Lipofectamine LTX transfected HEK-293T cells using either pLVX-IRES-Puromycin (A) or pTRIP (B). The cells were washed and analysed on the flow cytometer 28 hours after transduction. The cells transduced with the pLVX-IRES-Puromycin virus (A) were grown in media containing 1µg/ml puromycin. After 5 days culture the HEK-293T cells (C) and Jurkat-A2 cells (D) transduced with the pLVX-IRES-Puromycin virus were washed and assayed for GFP expression using the flow cytometer. Untransduced cells were used as negative control (C: black and D: red).

pTRIP-GFP virus is higher than that of the pLVX-GFP virus but the pLVX virus can be selected to increase the GFP-positive percentage. Puromycin (1μg/ml) was added to the media 48 hours after transduction to try and select the transduced GFP-positive cells from the untransduced, GFP negative cells. The HEK-293T cells were then analysed on the flow cytometer 3 days after selection began with the percentage of GFP-expressing cells increasing from 21% to 62% after 3 days (data not shown). This demonstrated the ability of puromycin to select the transduced HEK-293T cells from the untransduced cells as expected. After 5 more days of culture, both cell lines were analysed on the flow cytometer and the percentage of GFP-expressing HEK-293T cells had increased again to 99.2% (Figure 4.19 C-D) whereas the percentage of GFP-expressing Jurkat A2 cells had increased to 57% compared with the mock transduced control cells. Therefore demonstrating that the population of transduced cells can be selected using puromycin so there are very few untransduced cells surviving in the puromycin-supplemented media. After demonstration that the pLVX virus system worked well with GFP in HEK-293T cells and that the transduced cells could be selected very easily with puromycin, this method of introducing DNA into Jurkat A2 cells was used as well.

Following attempts to introduce GFP into cells using 4 different methods, the two methods that demonstrated the largest increase in GFP expression in Jurjat A2 cells were standard transfection, using the TransIT reagent, and transduction using lentivirus. Experiments moved on in an attempt to introduce the mutated TCR DNA into the same cell line using each of these methods.

4.13 Expression of T Cell Receptor β–Chain by Transfection

Using the MirusBio TransIT reagent to transfect Jurkat A2 cells, they stained positive for TCRVβ7 after 2 days (range 5.7-12.2%) (Figure 4.20 A). The results suggested that the T cell receptor had reached the surface in a small percentage of cells which was expected as transfection is known to have a very low efficiency. Importantly, this result demonstrated that the mutations made to the T cell receptor β-chain did not alter the ability of the T cell receptor to fold properly and pair with the endogenous TCR α-chain. Upon culture, the expression of the TCR β-chain was lost after 5 days (Figure 4.20 B). This would appear to suggest that the transfection of DNA was transient and this is probably the reason it was lost after such a short period of time. As standard transfection wasn't producing stable protein expression, a new method of introducing the TCR DNA was used.

4.14 Expression of TCR in Jurkat-A2 Cells

4.14.1 Standard Transduction of Lentivirus

The Jurkat-A2 cell line was transduced with unconcentrated virus containing each of the mutated TCRs (Table 2.6) as well as the unmutated TCR as a positive control. After 48 hours culture, cells were stained with an antibody specific for TCRVβ7 to determine the presence of the protein at the cell surface. The initial results (Figure 4.21) were very disappointing demonstrating that there was little or no expression of the mutated TCR protein at the surface of any of the transduced cells (Range 0.03% - 0.41%). After these poor results viruses containing the TCRs with NLVPMVATV and DYSNTHSTRYV in the CDR3 were produced and concentrated up to 50x greater than the previous lentivirus concentration used. The concentrated virus particles

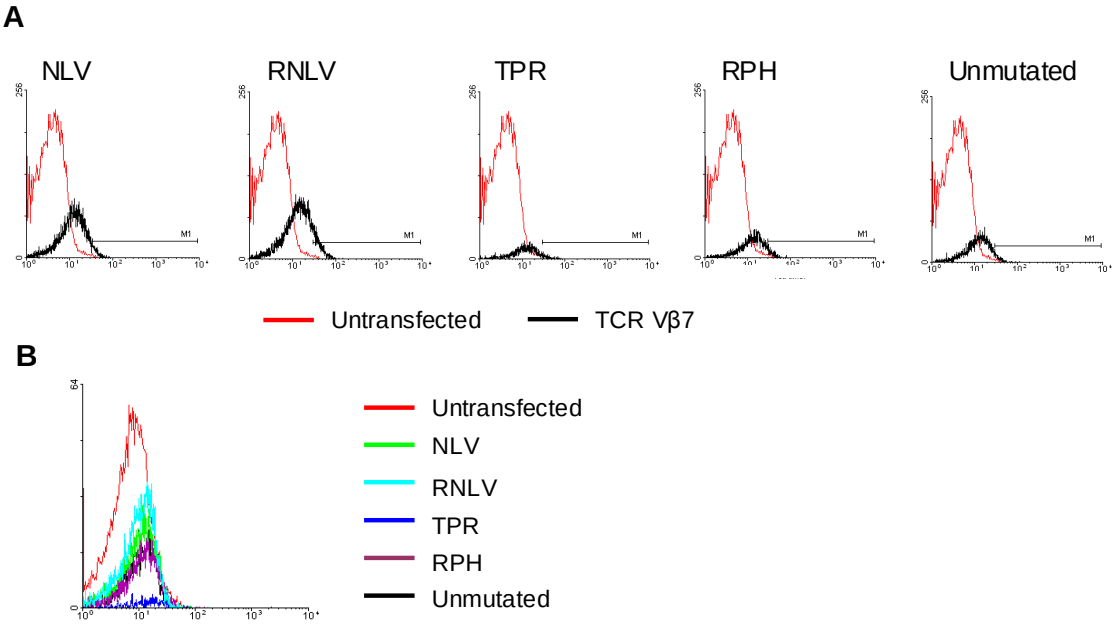


Figure 4.20. Expression and selection of mutated TCR β-chains at the surface of Jurkat A2 cells

Mutated TCR β-chains were transfected into Jurkat A2 cells using the TransIT transfection reagent (MirusBio). Cells were stained, 48 hours post transfection, using anti-TCRVβ7-PE antibody for 30 minutes. After staining with the antibody, cells were washed and analysed on the flow cytometer (A: black). Untransfected cells stained with the same antibody (A: red) were used as control cells. Cells were grown in media containing hygromycin (300µg/ml). After 5 days, cells were again stained with anti-TCR Vβ7-PE antibody for 30 minutes, washed and analysed using the flow cytometer (B).

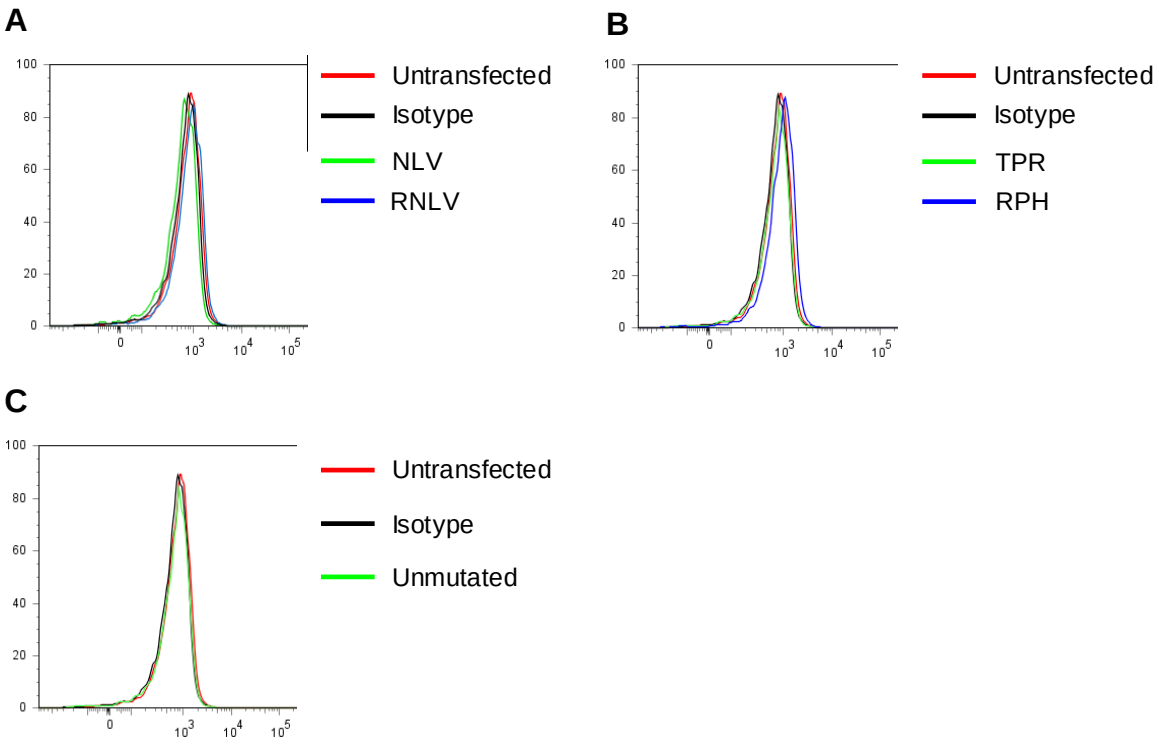


Figure 4.21 Transduction of Jurkat A2 cells using lentivirus containing mutated TCRs

Lentiviral particles containing mutated TCRs were produced by Lipofectamine LTX transfection of HEK-293 lenti X cells. Jurkat-A2 cells were transduced with the mutated TCR viruses NLV (A, Green), RNLV (A, Blue), TPR (B, Green), RPH (B, Blue) and unmutated (C. Green) with mock transduced cells (A-C Red) and isotype stained cells (A-C black) acting as negative controls. Cells were stained, 48 hours post-transfection, with the anti-TCRVβ7-PE antibody for 30 minutes, washed and analysed using the flow cytometer.

were stored for each of the mutated TCRs and Jurkat-A2 cells were transduced with each of the viruses and assayed for protein production after 48 hours on the flow cytometer. The results using the concentrated virus were similar to the previous attempts with less than 1% of cells staining positive for the TCR β-chain (data not shown). After this result, a new method to transduce Jurkat-A2 T cells was used where the T cells were co-cultured with the transfected 293 cells (see materials and methods 2.6.3.3).

4.14.2 Co-Culture Transduction

After culturing the T cells with the transfected 293 cells for 48 hours, the cells were separated and stained for analysis on the flow cytometer. The results using the co-culture transduction (Figure 4.22) were much better than previous results with 4% of Jurkat-A2 cells transduced with the NLV-mutated TCR staining positive for TCRVβ7 (A) and those cells transduced with the DYSN-mutated TCR also stained positive for 7% (B). After showing that there were positively transduced cells, they were grown in puromycin in an attempt to select TCRVβ7 expressing cells.

The Jurkat-A2 cells transduced using the co-culture method were put on antibiotic selection using 1μg/ml puromycin. However, 5-7 days after beginning selection there were no cells alive suggesting that the cells had still been sensitive to puromycin and that the transduction had not been successful.

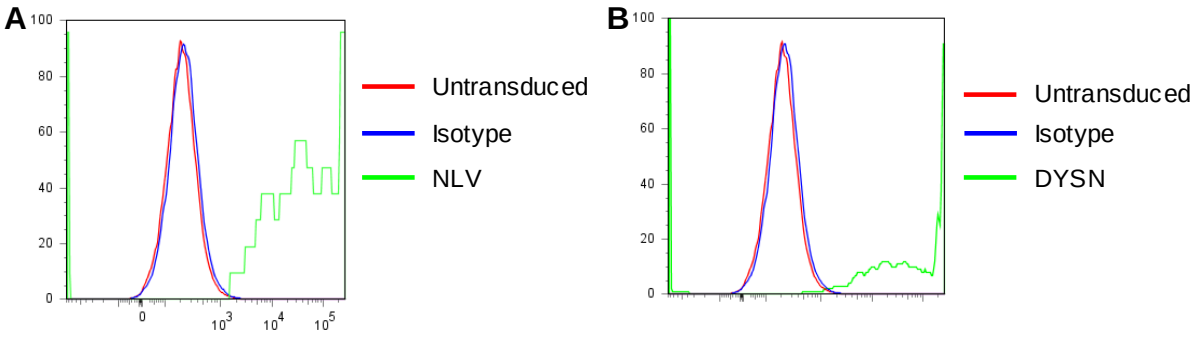


Figure 4.22 Transduction of Jurkat A2 cells using co-culture method

Lenti-X 293T cells were transfected with the mutated TCR DNA containing NLV (A) and DYSN (B) and after 72 hours $1x10^6$ Jurkat-A2 cells were added to the flask. The co-culture was incubated for 48 hours and Jurkat-A2 cells were removed after 48 hours and stained with either an isotype control (A-B blue) or an anti-TCRVβ7 antibody conjugated with phycoerythrin (A-B green). Untransduced cells (A-B red) acted as negative controls throughout. The cells were washed and analysed by flow cytometry with standard Jurkat-A2 cells stained with the antibody as control.

4.15 Discussion

In this chapter, the hypothesis tested was that there is no peripheral tolerance towards CDR3-derived peptides. Therefore in an HLA-$A2^+$ donor, peptides binding to HLA-A2 would need to be removed to prevent the possibility of T cells expressing the CDR3-derived peptides at the cell surface and generating an autoimmune reaction. In this scenario, an HLA-$A2^-$ donor would not need to remove any HLA-A2 binding peptides as they would not contain the HLA-A2 molecule required to present the peptide at the cell surface. If immunoediting does occur in the T cell repertoire, HLA-$A2^+$ donors would exhibit a decreased frequency of HLA-A2 binding peptides within the CDR3 compared with those from HLA-$A2^-$ donors.

The results for HLA-A2 demonstrated an increased frequency of CDR3-derived nonamer peptides in HLA-$A2^+$ donors compared with HLA-$A2^-$ donors, suggesting the original hypothesis is false. This suggests that there is no immunoediting process in the T cell repertoire and therefore suggests that there is peripheral tolerance towards these peptides that would prevent induction of an immune reaction if the peptides were expressed at the surface of the T cell and recognised by other $CD8^+$ T cells. Thus, peptides from the TCR CDR3 are similar to other self-peptides which cannot be recognised by self T cells. Similar results were produced when CDR3-derived decamer peptides predicted to bind to HLA-A2 were investigated. The results from this second peptide analysis would appear to validate the initial results. All of the peptide prediction analysis was consistent using two different peptide prediction algorithms suggesting it is not an artefact of either algorithm. Furthermore, the results appear to suggest that there is positive selection of T cells expressing a TCR containing an HLA-A2 binding peptide within the CDR3. Therefore, why would

TCRs from an HLA-A2^{+} donor containing HLA-A2 binding peptides be selected to populate the periphery? One reason may be that memory T cells, those which have previously encountered antigen and circulate in the periphery looking for the antigen, require periodical stimulation for long-term survival and the presentation of CDR3-derived peptides by memory T cells are recognised by CDR3-peptide specific T cells. The "gentle" stimulation of the memory T cell after being recognised by a CDR3-specific T cell may provide signals or soluble factors that prevent T cell death. This theory has foundations in the idiotype network theory first postulated by Niels Jerne in 1974 where the idiotype from each lymphocyte can be recognised by and can recognise the idiotype from other lymphocytes. In presenting peptides from their CDR3, memory T cells may receive periodical stimulation thereby surviving to combat infection.

This is a very significant finding suggesting that there may be a selection process during the lifecycle of T cells whereby T cells express self peptides from the CDR3 and those with a potentially immunogenic peptide are positively selected to survive. This selection process in T cells could occur very early on in the maturation process of T cells in the thymus during positive and negative selection. At this stage of T cell development, there is a lot of T cell death in the thymus, normally attributed to positive and negative selection, and the 3rd selection process suggested here could take place at the same time.

Similar results were also seen in two further HLA alleles tested (HLA-A1 and -B7) with HLA-B8 being the only allele tested to produce different results. The results for HLA-A1 and -B7 suggest that the selection of TCRs containing HLA-binding

peptides is not restricted to HLA-A2 only and therefore gives some credibility to the results. However the results for HLA-B8 suggest that the original hypothesis is true, that there is immunoediting of the T cell repertoire, as there is a decreased frequency of peptides found with the CDR3 sequence from HLA-B8^{+} donors compared with HLA-B8^{-} donors. It is unlikely that there is positive selection of TCRs in certain HLA alleles and editing of TCRs in other HLA alleles dependent on the sequence of the CDR3. Therefore, these results suggest that either the results from HLA-B8 are wrong in some way or the results from all of the other alleles tested are wrong. If this were the case, the computer algorithms would likely be the problem but the ROC analysis for all of the alleles gave similar results and suggests that there are no problems with either algorithm for any of the HLA alleles. If the results from the HLA-B8 allele are wrong, this may be due to the lack of lysine and/or methionine amino acid residues within the CDR3 which are often found in peptides that are predicted to bind to HLA-B8. If this is the case, the selection process dependent on the CDR3 may be HLA allele specific due to the lack of certain amino acids within the CDR3.

After discovering that TCRs from HLA-A2^{+} donors contain an increased frequency of HLA-A2 binding peptides, work began to generate an immune response against a CDR3-derived peptide from a T cell clone. Previous studies have shown T cell responses towards cutaneous T cell lymphoma (CTCL) cells (Bagot et al., 1998). However, there has been very little studying the presentation of CDR3-derived peptides on the surface of the T cell with Berger et al., 1998 demonstrating the potential presentation and CTL response towards a CDR3-derived peptide. After generating a naïve T cell response, the hypothesis tested was: peptides contained within the CDR3 of TCRs are processed by the MHC class I processing pathway and

presented at the cell surface. There was a possibility that the non-self nature of the TCR CDR3 would mean that the TCR would be specifically excluded from the MHC processing pathways by the cell to prevent the presentation of potentially immunogenic peptides. However, results showed that the parental T cell clone could be recognised by the peptide-specific T cells generated suggesting that the CDR3-derived peptide can be processed and presented by HLA-A2 at the cell surface, meaning that the hypothesis was true.

However, the results from the experiment were not without problems shown by the lack of response towards the positive control peptide-pulsed Jurkat-A2. These cells should give a strong IFN-γ response because the peptide should be presented by the MHC class I molecules on the surface of the cells but there is a decrease in the IFN-γ produced. This is unusual as previous results demonstrated the specificity of the ALC-specific T cells when the targets were peptide pulsed LCLs. The failure of the positive control casts some doubt on the results because essentially the positive control hasn't worked, it may be explained by the fact that the peptide contains a cysteine which could dimerise and alter the recognition of the peptide. There is a possibility that the peptide used to generate the T cell clone, and the one present on the ELR specific T cell clone, is the reduced peptide whereas the peptide used for the IFN-γ co-culture experiment had become oxidised. This would explain why the ALC-specific T cells can recognise the ELR T cell clone with the endogenously processed and presented ALC peptide but they cannot recognise the exogenously peptide pulsed LCLs. The other controls placed in this experiment have shown that there is not a non-specific release of IFN-γ in the culture.

If the results from the T cell co-culture are true, then not only are the TCRs selected so there are more HLA-binding peptides contained within the CDR3, these peptides appear to be presented at the cell surface. The expression of these non-self or foreign protein peptides further suggest that there is some form of peripheral tolerance towards these peptides as theory dictates that they could initiate an autoimmune reaction if they are recognised by functional CD8 T cells. Expression of these peptides at the cell surface may be a natural part of the processing and presentation of self proteins on the cell surface. However, it may be a specific process to present CDR3-derived peptides in order to fulfil the immune network theory as described previously. The results described here agree with Winter et al., 2003 suggesting that CDR3-derived peptides are presented on the surface of T cells.

In order to determine the antigen processing and presentation pathway the TCR enters, a model was designed to investigate this. A T cell receptor β-chain was mutated to contain known viral peptides within the CDR3 and expressed in a T cell line where it could pair with the endogenous α-chain and be expressed at the cell surface. Introduction of the mutated TCR into cells did demonstrate the ability of the mutated TCR to be expressed at the cell surface. This is a very important result as mutation of the TCR could have caused mis-folding and aggregation of the protein, preventing pairing of the introduced TCR β-chain with an endogenous α-chain. A lentiviral system was set up and, after this method showed long-term stable transduction of GFP, was used to transduce Jurkat-A2 cells with the mutated TCRs. Standard transduction of Jurkat A2 cells produced poor results with less than 1% of cells expressing the TCRVβ7 on the cell surface. Co-culture transduction increased the percentage of cells transduced to 6-8%. However, antibiotic selection using

puromycin to select positively transduced cells failed to increase the percentage and there were no cells alive after 7 days selection. Therefore suggesting that the transduction had been transient in nature and had not produced stable integration of the protein into the cell genome.

4.16 Conclusion

In this chapter, the hypothesis that there was no peripheral tolerance towards CDR3-derived peptides from the TCR and therefore there was the requirement of immunoediting of the T cell repertoire to prevent an autoimmune reaction was proven false in healthy donors. There was an increased frequency of HLA-binding peptides in HLA-positive donors compared with HLA-negative donors analysing the CDR3 for nonamer and decamer peptides. A naïve T cell response was generated to a CDR3-derived peptide which, when cultured with the parental T cell clone, produced an IFN-γ response demonstrating the presentation of CDR3-derived peptides at the surface of the T cell. An attempt was made to generate a model to study antigen processing of CDR3-derived peptides using mutated TCRs containing viral peptides. Cells could express the mutated TCRs after lentiviral transduction but the expression was lost after only a few days. Repeated attempts to transduce and select the cells using antibiotics proved fruitless.

5. Antigen Processing and Presentation of Peptides Tagged to Antibodies

5.1 Introduction

Work presented in chapter 3 demonstrated that the CDR3 of surface immunoglobulin are deficient in HLA-A2 binding peptides in HLA-$A2^+$ donors when compared to HLA-$A2^-$ donors suggesting that there may be an underlying immunoediting process. The physiological basis for this potential immunoediting is unclear nor is it clear why any editing should exist or why it is only observed in the antigen receptors within the B cell compartment and not the T cell compartment. Some aspects relating to these questions are explored further in this chapter.

One key difference between the B cell antigen receptor in contrast to their T cell receptor counterparts is that they are secreted in the form of soluble antibodies and thus gain access to all tissue compartments. Moreover, antibodies serve to bind and capture antigens, and through interactions with complement and Fc receptors have direct access to antigen presenting machinery and thus ideally positioned for T cell recognition.

Here it is postulated that there are two theoretical adverse consequences of not editing the CDR3 repertoire of B cell antigen receptors. Both of these are the result of the enormous antigenic diversity of the BCR. Firstly, the secretion of an unedited diverse antibody repertoire may lead to the development of autoimmune disease in the case where a small proportion of antibodies contain, by chance, sequences which are identical or very similar to pathogen immunodominant epitopes. In this case T cells

would have the potential to destroy bystander cells which uptake antibodies bearing epitopes which are close or identical to immunodominant pathogen-derived epitopes. Secondly, a competing hypothesis may suggest the reverse: that maintaining peripheral tolerance to the entire secreted antibody-derived proteome may abrogate or compromise the T cell repertoire by inducing an anergic T cell repertoire or cause large-scale deletion of circulating naive T cells. The diversity of lymphocyte receptors can be potentially dangerous (previously discussed chapter 1.14) and maintaining peripheral tolerance to the antibody-derived proteome may have consequences for the ability of T cells to recognise all foreign antigens.

It is difficult to experimentally test each of these hypotheses, but both require antibodies as antigens to be efficiently processed and recognised by T cells. Therefore, an experimental model was developed whereby antibodies were constructed containing immunodominant T cell epitopes. Due to other work performed in the laboratory, T cells specific for cytomegalovirus were readily accessible and had been well characterised providing an ideal opportunity to assess the immunological consequences of antibodies which contain immunodominant viral epitopes.

5.2 Developing a model of peptide-conjugated antibodies

Initially, a simple model was explored to test whether antibodies bearing immunodominant viral T cell epitopes could stimulate cognate T cell responses *in vitro*. It was reasoned that the position of the viral peptide epitope may not be important, as the proteolytic machinery employed in both Class-I and Class-II antigen processing pathways lacks location-specificity. Moreover, some immunoglobulin

derived HLA-restricted peptides which have been characterised are derived from the multiple regions spanning both heavy and light immunoglobulin chains.

The heterobifunctional molecule sulpho-SMCC was employed to link synthetic peptides to antibodies. Sulpho-SMCC contains both an amine-reactive succinimide group and a sulphydryl-reactive maleimide group (Figure 5.1) providing a mechanism to link cysteine extended peptides to free primary amine groups of the antibody in a random manner. The NHS ester on the sulpho-SMCC reacts with free primary amine groups found on lysine residues and the amino terminus of the antibody polypeptide.

The position of the cysteine within the peptide was chosen to limit the susceptibility of the peptide-antibody complex to carboxypeptidases present in the cell culture media and indeed for future *in vivo* experiments. Therefore, by linking the peptide at the C-terminus to the antibody would render the antibody-peptide complex resistant to carboxypeptidases through steric hindrance. The chemistry used in the peptide conjugation is well known and has been used in many previous publications (Walker et al, 1995 & Patri et al., 2004).

5.3 Rituximab as a model of antibody-antigen processing.

Rituximab was used for the initial peptide-conjugations due to its wide therapeutic application, and therefore potential future therapeutic use, in the treatment of B cell leukaemia and lymphoma. Rituximab was initially conjugated with the HLA class-I CMV peptide NLVPMVATV (Wills et al., 1996) and elongations of this peptide (Table 5.1). In order to determine the presence of peptides conjugated to the antibody,

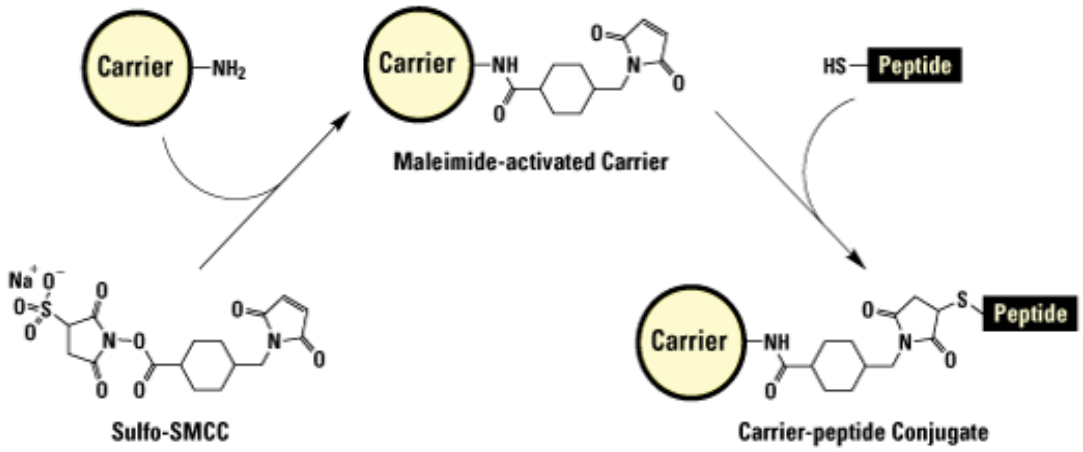


Figure 5.1 Mechanism of action of sulfo-SMCC to conjugate a cysteinylated peptide to a carrier/antibody

The amine-reactive NHS ester group links sulfo-SMCC to free amine groups on the antibody to give the malemide-activated antibody. Addition of a cysteinylated peptide allows linkage of the peptide to the malemide group on the sulfo-SMCC via the sulfhydryl group from the cysteine residue.

HLA Class I	HLA Class II
NLVPMVATVC	DYSNTHSTRYVC
RNLVATVPMVC	DDYSNTHSTRYVC
RNLVATVPMVQC	DDYSNTHSTRYVTC
ARNLVPMVATVC	PDDYSNTHSTRYVC
LARNLVPMVATVC	IPDDYSNTHSTRYVC
Biotin-EPFRPHERNGFTVL	LIPDDYSNTHSTRYVC
	Biotin-LIPDDYSNTHSTRYVC

Table 5.1 HLA class I and class II peptides used for conjugation to antibodies

The antibodies were conjugated with the peptides via the cysteine group at the C terminus of the peptide. Biotinylated peptides were used to assess the conjugation of peptide to antibody using a biotinylation ELISA.

a biotinylated peptide was first used to confirm binding of peptide to antibody and a readout given by ELISA. Indeed, Rituximab conjugates bound to plates were shown to contain biotin groups confirming successful conjugation using this heterobifunctional cross-linking approach (Figure 5.2). This was repeated on multiple occasions to ensure the reliability of the protein chemistry. After conjugation of peptides, cell labelling was undertaken to determine whether the antibody retained its ability to bind to its cellular receptor. Peptide-conjugated Rituximab was shown to bind to target cells with a similar frequency as commercial anti-CD20, therefore demonstrating that conjugation of peptides does not affect antibody binding.

5.4 T cell recognition of processed peptide-antibody

B cell LCLs were used as target cells firstly because they express CD20 and secondly as professional antigen presenting cells, they can present both class-I and class-II peptides on their surface although it has been shown previously that these cells are inefficient at cross-presenting exogenous antigens on MHC class-I molecules (Münz et al., 2000).

The incubated cells were cultured with peptide-specific $CD8^+$ T cells and IFN-γ production assayed by ELISA. The initial results for HLA class-I peptides (Figure 5.3) were very disappointing as there appeared to be no recognition of target cells by the effector T cells. The effector T cells were specific for the peptide seen in the positive control where cells pulsed with exogenous peptide were recognised very strongly, seen by a large increase in the production of IFN-γ. There was no recognition towards any of the peptides tested including those with the elongated carboxy and amino termini. After the poor results seen with the HLA class-I peptides,

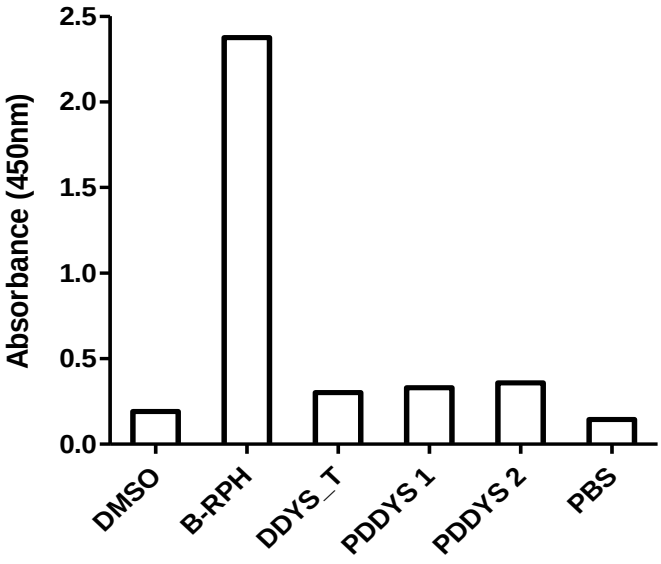


Figure 5.2 Biotinylation ELISA to determine presence of biotinylated peptides conjugated to antibody

Peptide-conjugated antibodies were coated to a plate and tested for the presence of biotin. Extravidin-peroxidase was added to the wells and TMB used to provide a colour change if biotin is present in the well. The plate was read at 450nm using GloMax plate reader.

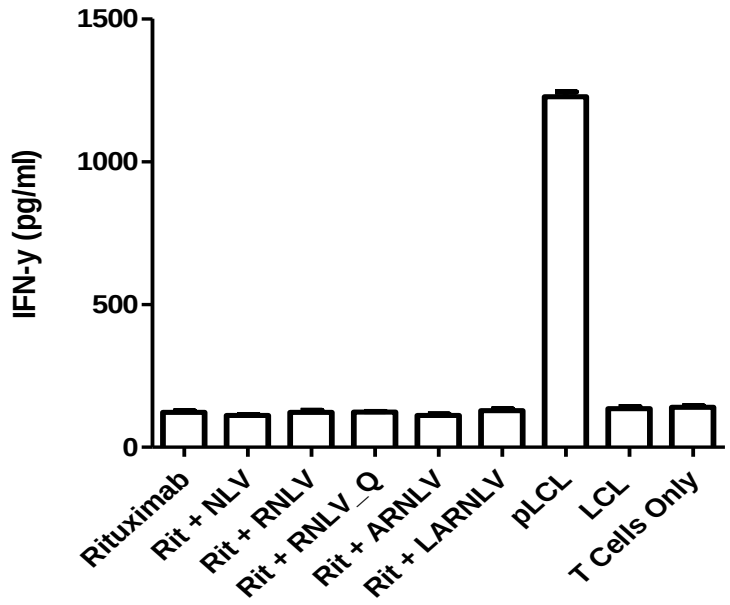


Figure 5.3 IFN-γ Recognition of target cells stained with the HLA class I peptide conjugated CD20 antibody (Rituximab)

Target cells were stained with Rituximab conjugated with class I peptides (NLV - LARNLV) or unconjugated (Rituximab) and used to stain target LCLs. The antibody was washed off and the target cells were incubated overnight at 37°C. Peptide-specific CD8 T cells were added (E:T ratio – 1:5) and incubated overnight with the supernatant taken to assay for IFN-γ production by ELISA. Control wells containing unpulsed target cells (LCL), and T cells cultured alone (T cells only) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide specific T cells (pLCL) were used to demonstrate the ability of the target cells to present the NLV peptide. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance.

Rituximab was then used for conjugation with an HLA class-II peptide (Table 5.1), a DRβ01*07 restricted CMV peptide (Crompton et al, 2008), with similar elongations of the carboxy and amino termini as used in the class-I peptides. After confirming conjugation efficiency, LCLs were incubated with the peptide-antibody conjugates. Similarly to the HLA class-I peptide-conjugated Rituximab, there was no recognition of any of the cells bound by peptide-antibody complexes by the effector T cells after 6 hours (Figure 5.4). There was strong recognition of the exogenously peptide pulsed LCLs as positive control (1500pg/ml), demonstrating that the $CD4^+$ T cells used in the assay are functional. It was thought that this result may be due to the inability of cells to internalise Rituximab as previously described (Einfeld et al, 1988). Therefore, in order to re-test the hypothesis, an antibody that is known to internalise was used.

5.5 BU69 as a model of antibody-antigen processing

BU69 is an anti-CD70 antibody and is internalised rapidly after binding (Israel et al., 2005 & Adam et al., 2006). It was one of the antibodies produced in our laboratory and CD70 is present on the surface of B cells from various B cell lymphomas including ~50% CLL and 33% Follicular lymphoma (Israel et al., 2005). The HLA class-I and class-II peptides were attached to the BU69 antibody using the same method as previously described and the conjugation was confirmed by ELISA (data not shown). Cell lines from our stocks were incubated with CD70 using a commercial antibody to determine surface expression (Figure 5.5 A,B). The LCLs stained ~99% positive for anti-CD70 using the commercial antibody and also >85% positive when using the biotinylated peptide conjugated to BU69 confirming biological reactivity post conjugation.

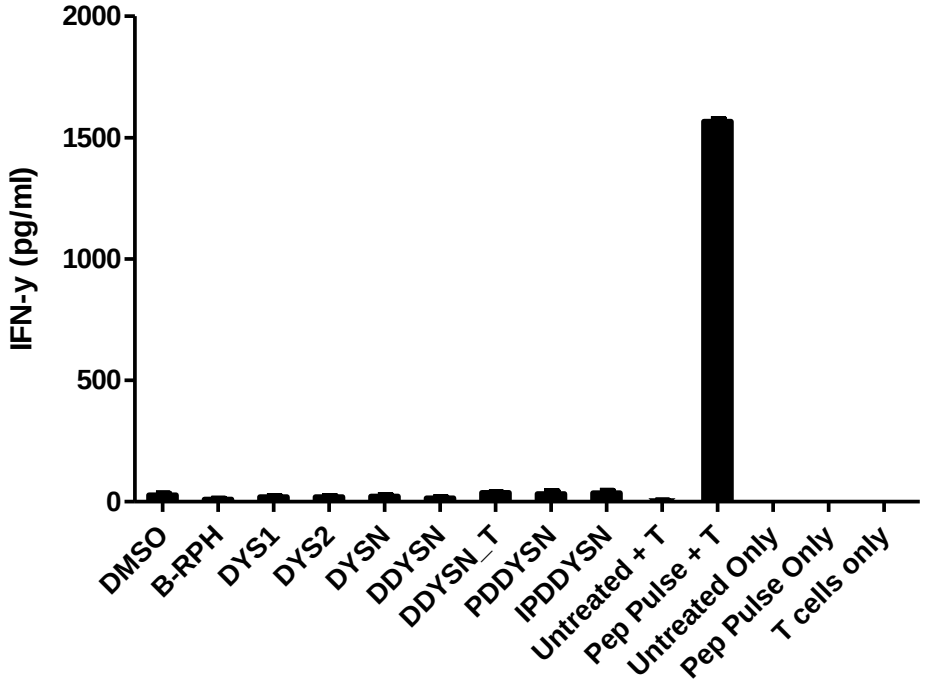


Figure 5.4 IFN-γ recognition of target cells stained with HLA-class II peptide conjugated Rituximab

Target cells were stained with Rituximab conjugated with class II peptides (DYS1 - IPDDYSN), control peptide (B-RPH) or unconjugated (DMSO) and used to stain target LCLs for 30 minutes. After washing off the antibody, the LCLs were incubated overnight before addition of peptide-specific CD4 T cells (E:T ratio 1:5) for 6 hours. The supernatant was assayed for IFN-γ production by ELISA. Control wells containing peptide pulsed target cells without T cells (Pep pulse only), unpulsed target cells (Untreated only), and target cells cultured with peptide-specific T cells (Untreated + T) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide specific T cells (Pep pulse + T) were used to demonstrate the ability of the target cells to present the DYSN peptide. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance.

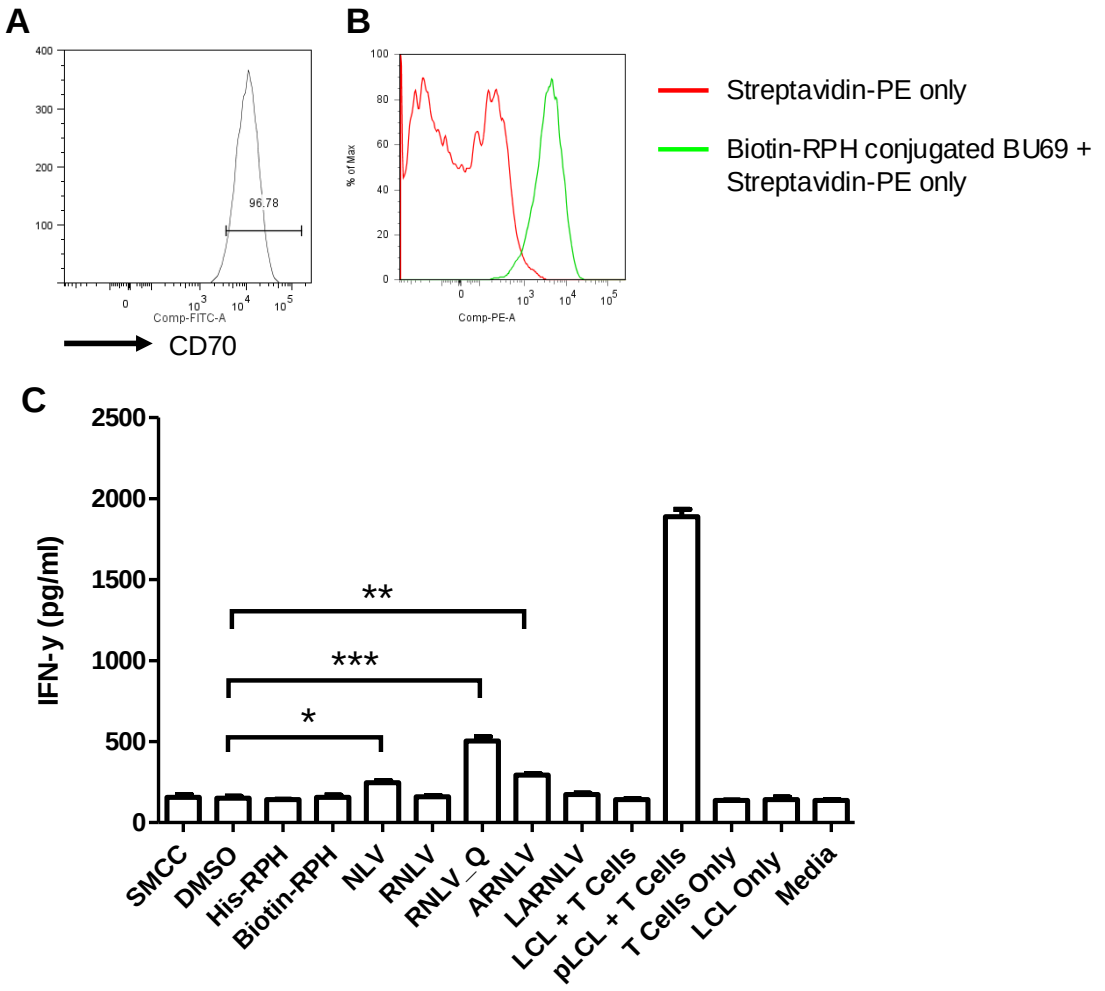


Figure 5.5 IFN-γ recognition of target cells stained with HLA-class I peptide conjugated BU69 (anti-CD70)

LCLs were stained with commercial anti-CD70 FITC (A) and BU69 conjugated with biotinylated peptide (B) for 30 minutes and washed. Streptavidin-PE was added to the BU69 antibody and incubated for 30 minutes, washed and analysed on the flow cytometer. Target cells were stained with CD70 conjugated with class I peptides (NLV - LARNLV), control peptides (His-RPH and Biotin-RPH) or unconjugated (SMCC & DMSO) for 30 minutes and washed before being incubated overnight. T cells were added (E:T ratio – 1:5) and the cell culture incubated overnight before removal of the supernatant to test for production of IFN-γ by ELISA (C). Control wells containing T cells cultured alone (T cells only), unpulsed target cells (LCL only), and target cells cultured with peptide-specific T cells (LCL + T cells) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide specific T cells (pLCL + T cells) were used to demonstrate the ability of the target cells to present the DYSN peptide. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance * P<0.05, ** P<0.01 and *** P<0.001.

5.5.1 MHC class-I peptide conjugation

LCLs were incubated with the BU69 tagged with class-I peptides and cultured with peptide-specific $CD8^+$ T cells. There was an IFN-γ response to 3 of the 7 peptides tested (Figure 5.5 C). The strongest response was seen with extended peptide conjugates, RNLVPMVATVQ and ARNLVPMVATV. There was no response to the BU69 conjugated to irrelevant peptides (RPH) or unconjugated (DMSO) confirming responses seen as peptide specific. Although the IFN-γ responses to the peptide-tagged BU69 were significantly decreased compared to the IFN-γ response towards the peptide-pulsed LCLs (positive control), they are significantly increased against the background IFN-γ response. These data suggest B cells can internalise antibody-bound surface membrane proteins and present the antibody-derived peptide in complex with HLA class-I.

5.5.2 MHC class-II peptide conjugation

After this encouraging result LCLs were assessed for their capacity to present antibody-derived HLA class-II restricted peptides. As expected, there was no increase in IFN-γ production when target cells were incubated with BU69 conjugated with an irrelevant peptide or without a peptide. However, there was an increase in IFN-γ production to 6 of the 7 class-II viral derived peptide antibody conjugates (Figure 5.6), with the only peptide not producing an IFN-γ response being the naturally processed peptide which had been cysteinylated (DYSNTHSTRYVC). Similarly to BU69 conjugated with HLA class-I peptides, the strongest response was seen when the cells were incubated with the BU69 conjugated with the peptide which had been extended by one amino acid at both ends, (DDYSNTHSTRYVT). The target cells pulsed with exogenous peptide (DYSN) produced a large IFN-γ response

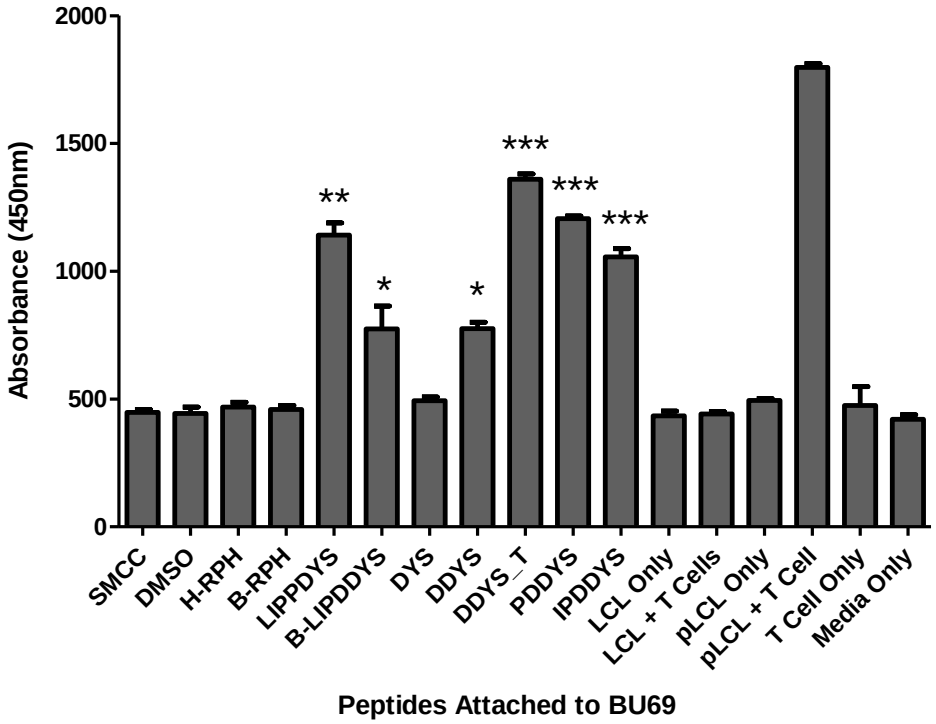


Figure 5.6 IFN-γ recognition of target cells stained with HLA-class II peptide conjugated BU69 (anti-CD70)

LCLs were stained with BU69 conjugated with class II peptides (LIPDDYS - IPDDYS), control peptides (H-RPH and B-RPH) or unconjugated (SMCC and DMSO) for 30 minutes and washed before being incubated overnight. T cells were added (E:T ratio – 1:5) and the cell culture incubated overnight before removal of the supernatant to test for production of IFN-γ by ELISA. Control wells containing peptide pulsed target cells without T cells (pLCL only), unpulsed target cells (LCL only), T cells cultured alone (T cell only) and target cells cultured with peptide-specific T cells (LCL + T cells) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide specific T cells (pLCL + T cell) were used to demonstrate the ability of the target cells to present the DYSN peptide. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance * $P<0.05$, ** $P<0.01$ and *** $P<0.001$.

whereas the target cells which were not pulsed with peptide did not produce any IFN-γ above the background, confirming the peptide-specificity of the $CD4^+$ T cells. Similarly to the class-I peptide results, the amount of IFN-γ produced by recognition of the cells incubated with the peptide-conjugated BU69 was lower than the peptide-pulsed LCLs (positive control). The large increase in IFN-γ suggests that the LCLs can process and present MHC class-II peptides linked to the antibody possibly in the classical HLA class-II antigen processing pathway. Taken together, these results suggest that the earlier hypothesis is true and that peptides conjugated to antibodies, subsequently internalised after binding to B cells, can be processed and presented in complex with both MHC class-I and class-II.

5.5.3 Free, unconjugated peptide binds to cellular MHC

During these first few experiments to attach peptides to an antibody, HiTrap desalting columns were used to remove excess sulpho-SMCC and excess peptide from the peptide-conjugated antibody. However, it was critical to confirm that the excess peptide was completely removed using the desalting column. If the column was not separating the peptide from the antibody-peptide complex, there was the possibility that the results seen previously were due to excess peptide binding to surface MHC on the target cells and not due to the peptide-conjugated antibody. Therefore, the hypothesis tested was that the recognition of target cells by peptide-specific T cells was due to the internalisation of the complex and the processing and presentation of the peptide by the target LCL. In order to test this hypothesis, peptides were conjugated to the antibody in the presence or absence of sulpho-SMCC. In order to conjugate the peptide to the antibody, the antibody must be activated with sulpho-SMCC first. If there is no SMCC attached to the antibody, consequently there would

be no peptide conjugated to the antibody (Figure 5.1). The IFN-γ recognition assay was repeated with target LCLs incubated with antibody conjugated with peptides in the presence or absence of SMCC. The peptide PDDYS was used as this had previously shown a large response. There was a large increase in IFN-γ release when LCLs were incubated with the antibody conjugated to the peptide without SMCC compared with the background release (Figure 5.7). This was presumed to be excess cysteinylated peptide, which hadn't been removed during the purification step, binding to the surface MHC molecules on the LCLs which could then be recognised by the T cells, therefore bypassing the antibody conjugation that was being investigated. This suggests that the HiTrap desalting column was not effective at removing excess peptide and that the responses seen are due to the excess peptide. This may relate to the chromatographic properties of the G25 Superdex® utilised for the separation which has a globular exclusion limit of 5,000Da close to the size of any disulphide linked peptide dimers which may have formed through peptide oxidation.

However, when the target cells were incubated with the antibody conjugated to the peptide in the presence of SMCC, there was further increased IFN-γ release seen when compared with that from the conjugation in the absence of sulpho-SMCC. The results seen with the antibody conjugated in the presence of SMCC demonstrate an increased IFN-γ response compared to the results with the antibody conjugated to peptide in the absence of SMCC, which would appear to suggest that the peptide conjugated to the antibody is processed and presented by the LCLs.

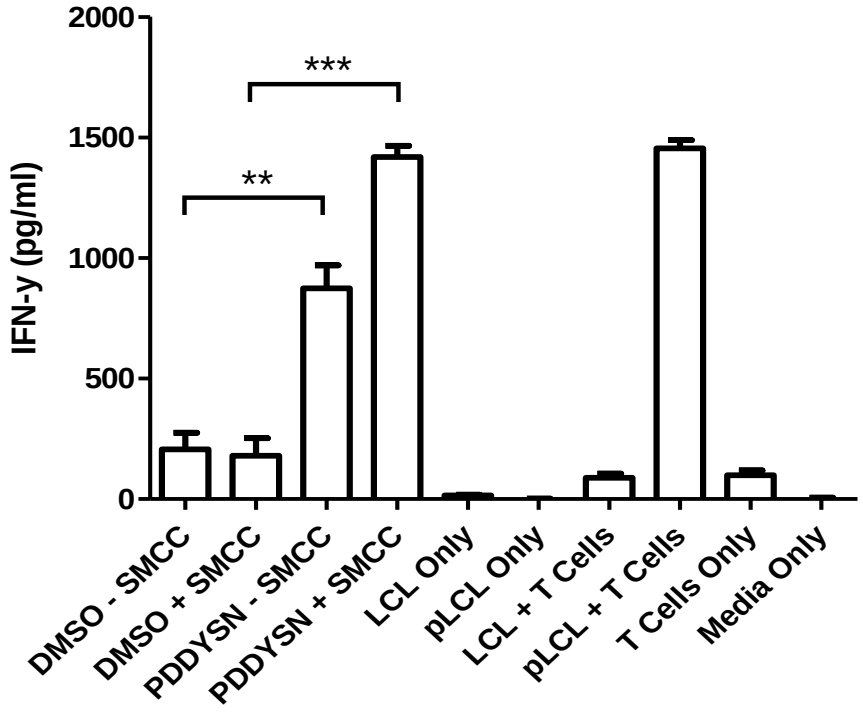


Figure 5.7 IFN-γ recognition of target cells stained with HLA-class II peptide conjugated BU69 (anti-CD70) in the presence and absence of sulfo-SMCC

The class II peptide PDDYSN was conjugated to antibodies in the presence and absence of sulfo-SMCC. BU69 conjugated with PDDYSN (PDDYSN) or unconjugated (DMSO) was used to stain target cells for 30 minutes. After washing, the target cells were incubated overnight. The cells were then cultured with peptide-specific T cells (E:T ratio 1:5) overnight and the supernatant assayed for IFN-γ production by ELISA. Control wells containing peptide pulsed target cells without T cells (pLCL only), unpulsed target cells (LCL only), T cells cultured alone (T cells only) and target cells cultured with peptide-specific T cells (LCL + T cells) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide specific T cells (pLCL + T cells) were used to demonstrate the ability of the target cells to present the DYSN peptide Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance .** $P<0.01$ and *** $P<0.001$.

5.6 Selective affinity chromatography purification

In order to purify the antibody and remove the excess peptides present, the antibodies were purified using more selective affinity chromatography using protein G columns instead of the desalting columns. HLA class-II peptides were conjugated to BU69 in the presence or absence of SMCC and purified using protein G columns. The peptide-antibody conjugates were then used to label target cells. The IFN-γ release by T cells cultured with the peptide conjugated to PDDYS in the presence of SMCC was increased in comparison with the peptide conjugated to antibody in the absence of SMCC which was similar to the background level (Figure 5.8). The cells incubated with BU69 either conjugated to an irrelevant peptide or unconjugated produced very low levels of IFN-γ (~100pg/ml). These results demonstrate that the production of IFN-γ is specific only for peptide conjugated to BU69 and the IFN-γ response is not due to excess peptide binding to HLA class-II on the surface of the B cells and being recognised by the T cells. This demonstrates that the purification of the peptide-antibody conjugates is very efficient when using the Protein G chromatographic methods. As a direct result of this, all subsequent conjugations of peptide to antibody were purified using the protein G columns.

5.7 Purified BU69 as a model of antibody-antigen processing

The above results seen with purification of class-II peptides conjugated to BU69 appears to cast doubt over the initial results seen with BU69 conjugated with the class-I peptides (Figure 5.5). Therefore, class-I peptides were again conjugated to BU69 but purified using the protein G column instead of the desalting columns. The previous experiment was repeated, labelling the LCLs with the new peptide-antibody conjugates and culturing them with the T cells. There was no increase in IFN-γ

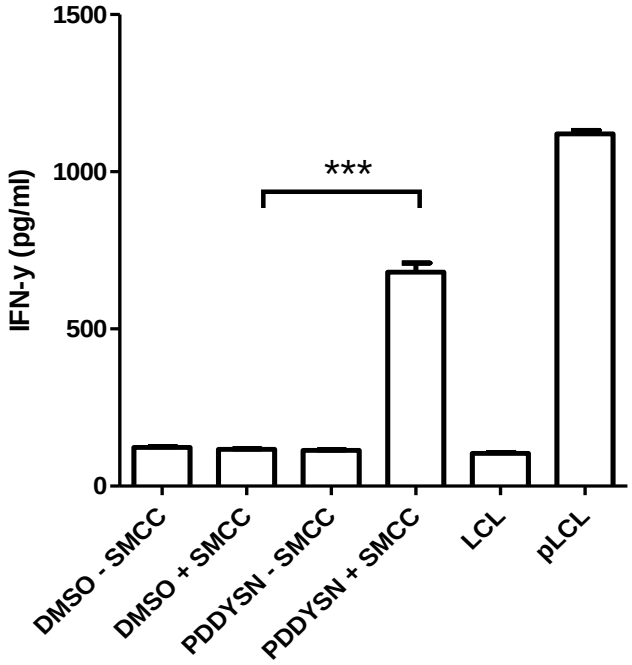


Figure 5.8 IFN-γ recognition of target cells stained with HLA-class II peptide conjugated BU69 (anti-CD70) purified using protein-A columns

Peptides were conjugated to antibodies in the presence and absence of sulfo-SMCC and excess peptide was removed using protein A columns. Target cells were stained with the BU69 conjugated to class II peptide (PDDYSN) or unconjugated (DMSO) for 30 minutes, washed and the cells incubated overnight. Peptide-specific T cells were added to the stained cells (E:T ratio - 1:5) and the cells incubated for 6 hours before the supernatant was taken to test for IFN-γ by ELISA. Control wells containing unpulsed target cells (LCL), were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide spefcific T cells (pLCL) were used to demonstrate the ability of the target cells to present the DYSN peptide. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance *** $P<0.001$.

production after 6 hours by CD8 T cells towards any of the cells incubated with the peptide-antibody conjugates (Figure 5.9). These data suggest that the HLA class-I peptides are not efficiently processed and presented by the B cell following antibody internalisation and that the previous results were due to excess peptide carry-through when using the desalting columns and highlights the importance of employing the correct downstream purification steps in separating free peptide from antibody-peptide complex. The CD8 T cells were functional as they did strongly recognise and produce IFN-γ in response to the exogenously peptide-pulsed LCLs. These results suggest that the HLA class-II restricted epitopes can be cleaved from the antibody and processed as a normal exogenous protein whereas the HLA class-I peptides, which may need to cross from the endosome to the cytosol, cannot be processed.

All of the previous results suggest that the class-II peptides conjugated to BU69 are processed by the target cell and presented to the peptide-specific CD4 T cells. However, in order to confirm that the target cells are processing the peptide, the antigen processing pathway of incubated cells was investigated using various antigen processing inhibitors to try to determine the pathway by which the peptide is processed.

5.8 Inhibitory analysis of antigen processing and presentation

The antigen processing of the class-II peptide conjugated to an antibody leads to presentation of the peptide on the surface of the target cell. It is yet to be ascertained how the peptide conjugated to the antibody is processed and therefore the hypothesis tested was that the class-II peptide is processed in the classical class-II processing pathway after binding to target cells. Labelled cells were cultured with 3-

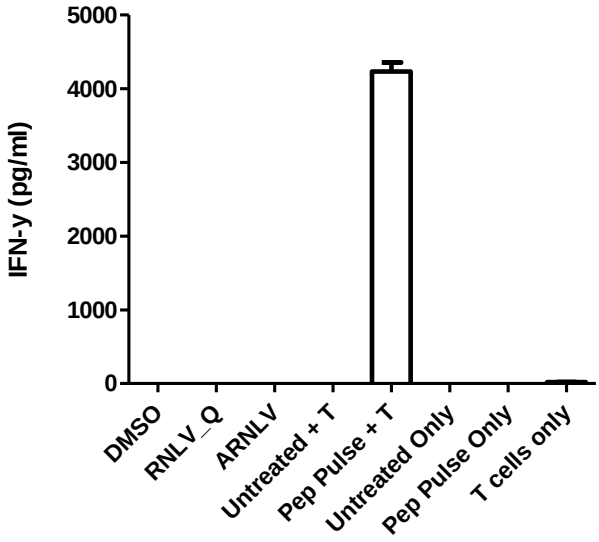


Figure 5.9 IFN-γ Recognition of target cells stained with HLA-class I peptide conjugated BU69

HLA class I peptides were conjugated to BU69 and purified using the protein G column to remove excess peptide. Target LCLs were stained with BU69 conjugated with class I peptides (RNLV_Q & ARNLV) or unconjugated BU69 for 30 minutes and washed before being incubated overnight. The target cells were then cultured with peptide-specific T cells (E:T ratio 1:5) and the supernatant assayed for IFN-γ production after 6 hours by ELISA. Control wells containing peptide pulsed target cells without T cells (Pep pulse only), unpulsed target cells (Untreated only), and target cells cultured with peptide-specific T cells (Untreated + T) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide spefcific T cells (Pep pulsed + T) were used to demonstrate the ability of the target cells to present the NLV peptide. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance.

methyladenine (an inhibitor of autophagy), chloroquine (inhibits lysosomal acidification), pepstatin (an inhibitor of aspartyl proteases), lactacystin (a proteasome inhibitor), leupeptin (a thiol protease inhibitor) and monensin (disrupts the golgi complex and inhibits transport to the plasma membrane) overnight before addition of peptide-specific T cells. The inhibitors were washed off prior to addition of the T cells as there was a possibility that the inhibitors may affect the actions of the T cells as well as those of the target cells. If this did occur, there may have been a reduction in IFN-γ production due to the inability of the T cells to release IFN-γ and not because antigen processing in the target cells is being inhibited. The media was assayed after 6 and 24 hours to determine if there was a reduction in IFN-γ production after culture with any of the inhibitors.

Similar to previous results, there was a large increase in IFN-γ production after 6 hours when the target cells were incubated with the BU69 conjugated to the class-II peptide whereas there was no IFN-γ production with the unconjugated BU69 (Figure 5.10 A). When target cells were incubated overnight in media supplemented with pepstatin, 3-methyl adenine or lactacystin there was no difference in the production of IFN-γ compared with the untreated cells. This would suggest that the processing of the peptide-antibody conjugate is not autophagy, aspartyl proteases or proteasome dependant. There was a small decrease in the production of IFN-γ when the target cells were cultured with leupeptin, demonstrating that the antigen processing of peptide-conjugated BU69 is partly dependent on thiol proteases. After cells were cultured with media supplemented with chloroquine, there was a significant decrease in the production of IFN-γ compared with the untreated cells. This result demonstrates

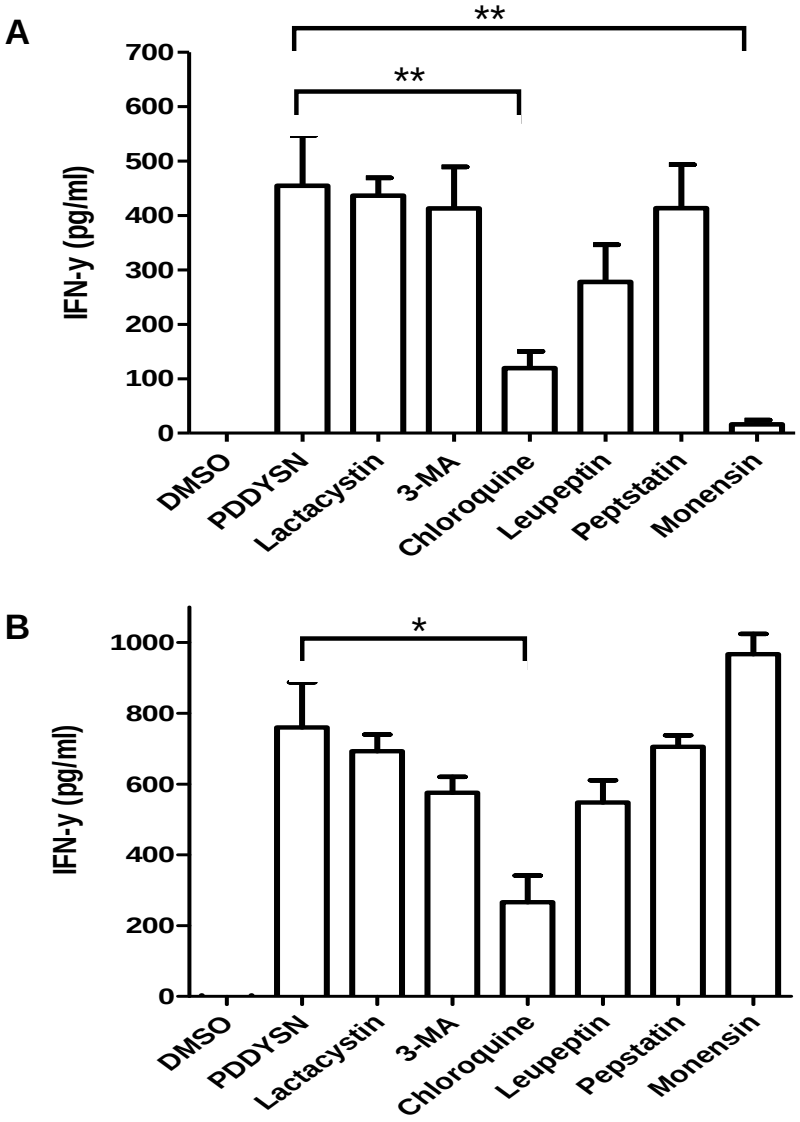


Figure 5.10 IFN-γ production by T cells cultured with target cells stained with PDDYS-conjugated BU69 and cultured with antigen processing inhibitors

LCLs were stained with either PDDYS-conjugated BU69 or unconjugated BU69, washed and cultured overnight in the presence of antigen processing inhibitors. After washing, the cells were cultured with peptide-specific T cells (E:T ratio - 1:5) for 6 hours (A) and 24 hours (B) before the supernatant was taken to assay for IFN-γ production. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance ** $P<0.01$ and * $P<0.05$.

the antigen processing of the peptide-antibody conjugate is dependent on lysosomal function. There was also a significant decrease in the production of IFN-γ when the cells were cultured with monensin demonstrating that the processing of the antibody-peptide conjugate is reliant on passage through the golgi complex to the cell surface.

After 24 hours, the cells incubated with peptide-conjugated BU69 and cultured without any antigen processing inhibitors produced IFN-γ compared with those cells incubated with the unconjugated BU69 (Figure 5.10 B). When the cells were treated with either lactacystin or pepstatin there was only a small reduction in T cell recognition. There was a larger inhibitory effect using leupeptin and 3-methyl adenine but the largest decrease was observed using chloroquine. Paradoxically, when the cells were cultured in the presence of monensin, there was an increase in T cell recognition.

It is important to stress that for both the 6 hour and 24 hour studies, after addition of T cells the rest of the experiment was performed in the absence of the inhibitors. These results suggest that the activity of either chloroquine or leupeptin is not fully reversible as the effects are still seen 24 hours after removal of the inhibitor. However, the activity of monensin is reversible as there is an increased production of IFN-γ at 24 hours compared with that after 6 hours. Interestingly, the increased production at 24 hours is increased compared with the untreated control suggesting an accumulation of antigenic cargo in the golgi rather than removal.

5.9 Response to antibody complex using blocking antibody

The previous results have demonstrated T cell recognition of target cells which have been incubated with peptide-conjugated antibody. In order to confirm that the peptides are presented on HLA class-II molecules at the cell surface, the cells were also incubated with an antibody to block all HLA-DR molecules on the surface of the target cell. The blocking antibody is specific for all HLA-DR alleles and therefore will block HLA-DR7 (the allele which presents the DYSN peptide) as well as the other HLA-DR allele present in the target cells. If there is a reduction in the amount of IFN-γ produced when the blocking antibody is used, this would suggest that the peptide is presented by HLA-DR. However, if there is no difference in the amount of IFN-γ produced, this would suggest that the peptide is being presented by another HLA class-II allele. Target cells incubated with anti-HLA-DR blocking antibody demonstrated ~60% decrease in IFN-γ production (Figure 5.11) confirming that the peptide is being presented by HLA-DR molecules on the surface of the target cell most likely HLA-DR7 as the T cells used are known to be HLA-DR7 restricted.

5.10 Targeted lysis of peptide-antibody incubated target cells

5.10.1 CD107 mobilisation assay

Having demonstrated T cell recognition of peptide-conjugated antibody complexes it was important to determine whether they could also kill target cells. Data supporting antibody-mediated cytoxicity would provide direct evidence that antibody antigenicity could cause tissue cytotoxicity. To test this hypothesis, T cells were analysed for recent degranulation using CD107 mobilisation assay. CD107a/b (LAMP1/LAMP2) is found within specialised endosomes containing perforin and granzymes which,

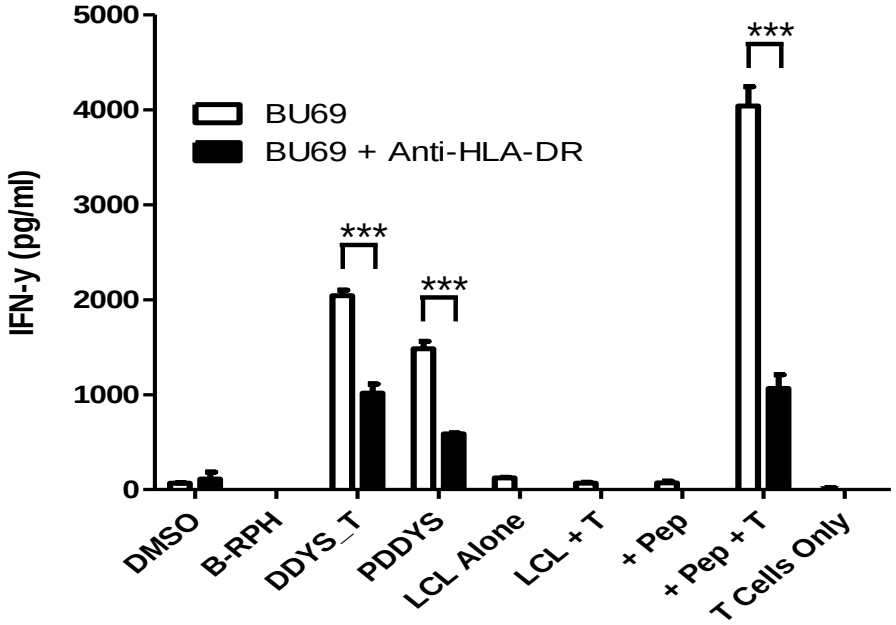


Figure 5.11 IFN-γ recognition of target cells after blocking with anti-HLA-DR antibody

LCLs were stained with BU69 conjugated with class II peptides (DDYS_T & PDDYS), control peptide (B-RPH) or without peptide (DMSO). After 30 minutes, cells were washed and cultured in the presence or absence of 2µg of anti-HLA-DR blocking antibody overnight. The blocking antibody was washed off before addition of peptide-specific T cells (E:T ratio - 1:5) and the supernatant was assayed for IFN-γ after 6 hours by ELISA. Control wells containing peptide pulsed target cells without T cells (+ pep), unpulsed target cells (LCL alone), and target cells cultured with peptide-specific T cells (LCL + T) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide specific T cells (+ pep + T) were used to demonstrate the ability of the target cells to present the DYSN peptide. Error bars demonstrate mean + standard error mean, n=3 Data was analysed using unpaired t-test to determine statistical significance *** P<0.001.

when released by the T cell, kill the target cell (Trapani & Smyth, 2002) Once the cytotoxic granules degranulate, CD107 is subsequently located on the surface membrane of the responding T cell and has been demonstrated to be a reliable proxy for cell cytotoxicity (Betts et al., 2003). However, CD107 is internalised after cells have released their granules and therefore for greater assay performance anti-CD107 antibodies are added to the cells shortly after the antigen has been added to the T cells (Betts & Koup, 2004).

After culturing the T cells with the antibody incubated target cells, the T cells were incubated with both CD107a (LAMP1) and CD107b (LAMP 2) in order to see if they had recently undergone degranulation. The percentage of cells expressing CD107a/b at the surface of the T cells when target cells were incubated with antibody either unconjugated or conjugated with an irrelevant peptide was very low, less than 2% (Figure 5.12). This confirms that T cells do not recognise and release cytotoxic granules towards target cells not expressing the specific peptide. When the target cells were incubated with BU69 antibody conjugated with class-II peptides, there was an increase in the percentage of T cells positive for CD107 specific for the peptide, which had been conjugated to the antibody (Figure 5.12). The naturally processed minimal class-II peptide epitope DYSNTHSTRYV conjugated to the antibody does not elicit an IFN-γ response and after labelling with CD107 antibodies and there is almost no CD107 labelling on the CD4 T cells either, suggesting that the CD107 assay is representative of the IFN-γ recognition assay. There is a large increase in CD107 labelling, between 10-15%, when target cells were incubated with the antibody conjugated with the extended peptides DDYSNTHSTRYVT, PDDYSNTHSTRYV, IPDDYSNTHSTRYV and LIPDDYSNTHSTRYV, cultured at

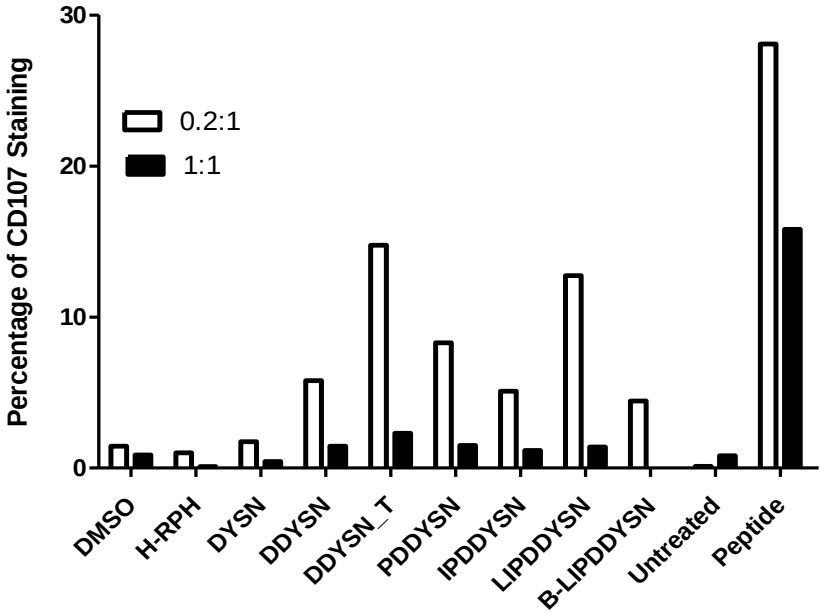


Figure 5.12 Determination of cytotoxic function of peptide-specific CD4 T cells using CD107 degranulation assay

Cells were stained with BU69 conjugated with HLA class II peptides DYSN - LIPDDYSN), control peptide (B-RPH) or unconjugated (DMSO) for 30 minutes, washed and incubated overnight. The cells were cultured with T cells (without monensin), at two different E:T ratios (0.2:1 and 1:1) for 6 hours. After 6 hours the cells were stained with anti-CD107a and anti-CD107b antibodies for 30 minutes before being washed and analysed on the flow cytometer. Untreated target cells (Untreated) were used as a negative control to show that there was no degranulation towards the target cells. Peptide pulsed target cells (Peptide) were used as positive control to demonstrate the peptide specificity of the effector T cells.

the lower E:T ratio. This is slightly lower than the CD107 labelling seen with T cells cultured with peptide-pulsed target cells as a positive control, but it is still increased in comparison with the background CD107 labelling. Degranulation frequency is similar when targets are incubated with T cells at a higher E:T ratio (1:1) but they are all decreased compared to the lower E:T ratio. This suggests that the CD107 expressed at the cell surface has been internalised and degraded before labelling has occurred. These results are contradictory to previous results using a similar model of anti-CD19 antibody conjugated with tetanus toxin peptides (Eberl et al., 1998) where lysis of target cells was shown to be mediated by Fas:FasL and there was an absence of perforin-mediated lysis. This may be due to the use of different antibody clones or possibly due to the differential effects of using different peptides.

These results correlate with T cell recognition data with the same peptides; they give an indication that, as well as being recognised by the $CD4^+$ T cells and those specific T cells release IFN-γ at a very high concentration, the target cells can also be killed by specific T cell granular cytotoxicity. These results reinforce the view that the slightly elongated peptides at both the amino and carboxy termini are better processed by the cell than the peptides that are shorter and closer to the naturally processed peptide antigen. Although the results suggest that labelling with the antibody-peptide conjugate could specifically target cells for CD4 T cell mediated lysis, the results need to be repeated using another cytotoxicity assay to validate this data and confirm killing. Importantly though, these results demonstrate that an antibody conjugated with class-II peptides could potentially target LCLs for specific lysis by the $CD4^+$ T cells and, if this is true, means that peptide-conjugated antibodies could be used to target B cell lymphoma cells.

5.10.2 Novel Flow cytometric cell targeting assay

In an attempt to confirm cytotoxicity, a novel flow cytometric based assay developed in our laboratory was employed as a readout of cell targeting. By using the same target cell with peptide (stained with FITC) or without peptide (stained with APC), it was possible to determine peptide-specific responses using the decrease in FITC-stained cells using the flow cytometer. This novel method also removes the possibility that responses seen are directed towards peptides naturally expressed by the target cells as there should be no decrease in the APC-stained cells. Target cells were separated into 2 tubes, differentially labelled with either FITC or APC conjugated anti-CD45 antibodies and analysed using the flow cytometer (Figure 5.13 A-B) and the results used for the cell targeting assay (as described in materials and methods 2.7.3).

Addition of exogenous peptide caused a decrease in the percentage of target LCLs (CD45-FITC labelled) without decreasing the percentage of non-target LCLs (CD45-APC labelled) (5.13 A). The principal of this assay is that it combines differentially labelled cell populations in the same well, with one cell population serving as a control and another population(s) serving as the target; killing is calculated by the reduction of target cells against the number of surviving control cells.

There was little killing of non-target LCLs cultured without peptide after 6 hours demonstrating that there was little non-specific cell targeting of the untreated target cells (Figure 5.13 B-C). Addition of BU69 without a peptide or with an irrelevant peptide (biotin-RPH) did not decrease cell viability demonstrating that the addition of

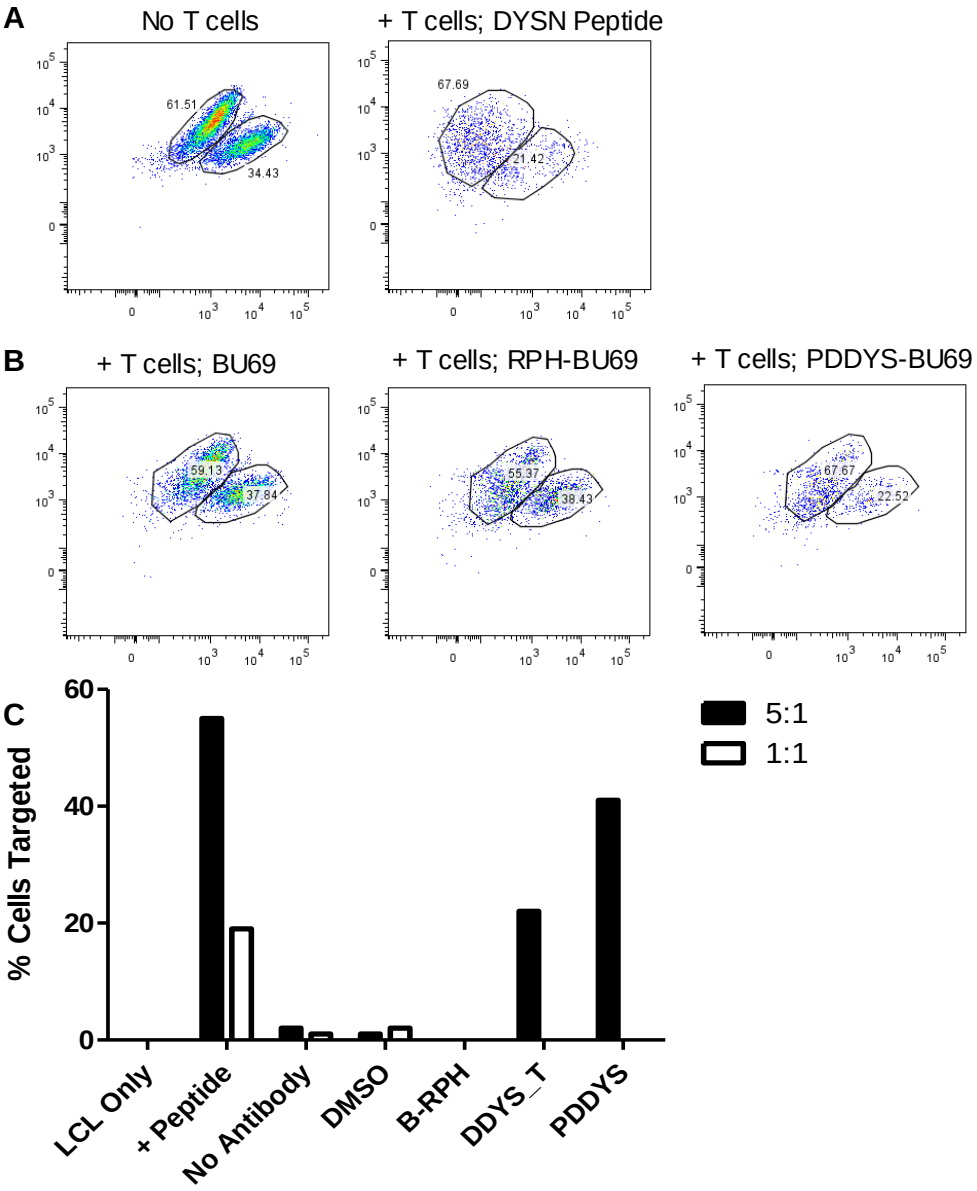


Figure 5.13 Flow cytometry based cell targeting assay following staining with BU69 conjugated to HLA class II peptides

Target LCLs were stained with one of two different anti-CD45 antibodies, conjugated with FITC or APC-H7, for 30 minutes and washed. Target cells stained with CD45-FITC were then stained with BU69 conjugated to HLA class II peptides, and the target cells stained with CD45-APC-H7 were left untreated. Cells were washed and after overnight incubation, the LCLs were washed again and cultured with T cells at two E:T ratios, 5:1 and 1:1. After 6 hour co-culture, the cells were analysed on the flow cytometer (A-B) to determine the reduction in cell number between the BU69 and FITC stained cells and the untreated APC-H7 stained cells. The results show the percentage of cells targeted after incubation with T cells for 6 hours (C).

the antibody did not cause non-specific targeting by the CD4 T cells (Figure 5.13 B-C). After labelling target cells with the DDYS_T conjugated BU69, there was an increased percentage of target cells killed at E:T ratio of 5:1 whereas there was no killing seen at an E:T ratio of 1:1. The untreated target cells (CD45 APC) remained at a similar percentage in all samples tested demonstrating that the CD4 T cells were specific for the BU69 incubated cells only. When labelling target cells with the PDDYS conjugated BU69, there was an increase in cell targeting to 45% at 5:1 E:T ratio (Figure 5.13 B-C) and again there was no cell targeting when the E:T ratio was 1:1. Similar to the other cultures analysed, there was no difference in the percentage of untreated target cells, demonstrating the peptide specificity of the T cells. These results demonstrate the potential cytotoxic activity of the peptide specific CD4 T cells which correlated with the previous degranulation assay (Figure 5.12) and also the IFN-γ recognition data.

5.11 EBV as a model of antibody-antigen presentation

The previous results have shown that there is a strong IFN-γ response to the HLA-DRβ1*07 restricted peptide DYSN. However, all of the results shown above only use a single, CMV-derived peptide and therefore a single HLA allele. It was therefore important to ascertain whether the results are only seen using this peptide or using the HLA-DR7 allele. In order to prove that the previous results seen are not specific to a single peptide, a CD4 T cell clone (kind gift from Dr. H Long, University of Birmingham) specific for an HLA-DR52b-restricted, EBV-derived peptide PRSPTVFYNIPPMPLPPSQL (Long et al., 2005) was cultured. This peptide was synthesised with PDSPE- and -PPPAA elongations, at the amino and carboxy termini respectively, and a terminal cysteine residue at the carboxy terminus. The peptide was

conjugated to the BU69 antibody, as previously described, and conjugation confirmed as previously described using the biotinylated peptide.

HLA-matched LCLs incubated with the peptide-conjugated BU69 were cultured with peptide-specific $CD4^+$ T cells for 6 hours. There is an increase in IFN-γ production when target cells were pulsed with the viral peptide compared with the unpulsed target cells demonstrating that the T cells were peptide-specific and did not recognise target cells non-specifically (Figure 5.14). There was also an increase in IFN-γ when target cells were incubated with the peptide-conjugated antibody compared with the unlabelled target cells. These results confirm that both T cell recognition and antigen processing of antibody complexes are not specific for either the CMV-derived peptide or HLA-DR allele meaning that other peptides and HLA class-II alleles could be utilised in further studies. Future therapy using this approach could utilise CD4 T cell responses to many different viruses. This would allow treatment of an increased number of patients as EBV is more prevalent in the population than CMV.

Previous results suggest that conjugation of HLA class-II peptides to primary antibodies allows the peptide to be taken into the cell, processed and presented to $CD4^+$ T cells in complex with HLA class-II. The positive results recorded with the BU69 antibody used LCLs from a lab donor and could be construed as a potential model for B cell lymphomas. However, CD70 itself is present on activated T cells (Brugnoni et al., 1997) and therefore, targeting this antigen may compromise the CD4 T cells used for the recognition of the peptide. As a result, attempts were made to try and find a different antibody that would specifically target B cells.

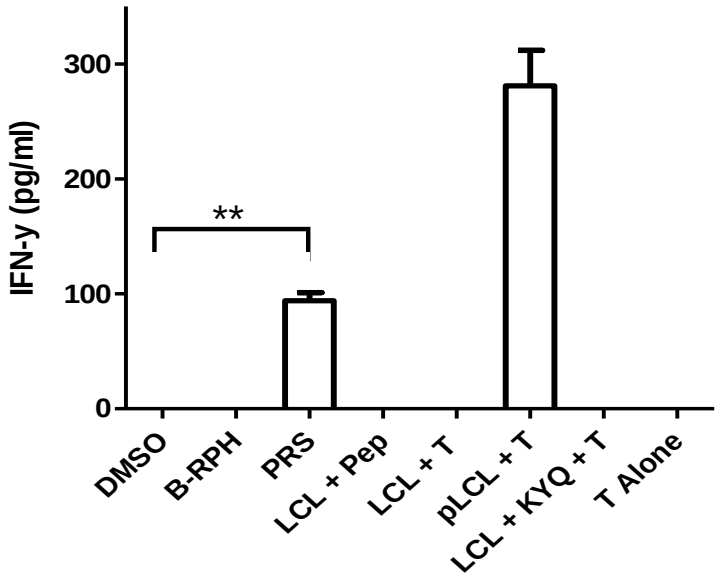


Figure 5.14 IFN-γ response to target cells stained with BU69 conjugated with EBV-derived epitope

HLA-matched LCLs were stained with BU69 conjugated with the EBV-derived peptide PRS or irrelevant peptide (B-RPH) or without peptide (DMSO). After 30 minutes, cells were washed and incubated overnight. Peptide-specific T cells (E:T ratio - 1:5) were added and incubated for 6 hours before taking the supernatant to assay for IFN-γ by ELISA. Control wells containing peptide pulsed target cells without T cells (LCL + pep), target cells were pulsed with an irrelevant peptide and incubated with T cells (LCL + KYQ + T) and target cells cultured with peptide-specific T cells (LCL + T) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide spefcific T cells (pLCL + T) were used to demonstrate the ability of the target cells to present the DYSN peptide. Error bars demonstrate mean + standard error mean, n=3 Data was analysed using unpaired t-test to determine statistical significance ** P<0.01.

5.12 Generation of a model to screen large number of antibodies

Peptide conjugation is not a laborious process and it would be possible to conjugate peptides to many antibodies. However, after the negative results from Rituximab conjugation, a method was developed that could rapidly assess many different antibodies in a single assay by conjugating HLA class-I and class-II peptides to a secondary antibody. Antibodies which gave a positive result could then be conjugated with peptides directly in an attempt to define antibodies useful for this model. Previously, the best results had been seen using the slightly elongated class-II peptides DDYSNTHSTRYVT and PDDYSNTHSTRYV and as a result, it was decided to conjugate only PDDYSN to the secondary antibody. To test the ability of the target cells to present the class-I peptides, the peptide with the similar elongations ARNLVPMVATV was also used. The antibodies of interest were specific for surface proteins expressed exclusively on B cells. After labelling the cells with the unconjugated antibodies (BU12 (CD19), BU33 (CD21), Rituximab (CD20), BU69 (CD70), A57H (IgG) and R8Z1E9 (IgG)), they were incubated with the peptide-conjugated secondary antibodies. There was no IFN-γ response when the secondary antibody was conjugated with either DMSO or ARLNVPMVATV (Figure 5.15 A), suggesting that the class-I peptides are not able to enter the class-I processing pathway after cross-linked antibodies are internalised by B cells. This confirms the results from previous figures showing that the class-I peptides are not processed after they have been conjugated to the antibody. However, there was a strong IFN-γ response when cells, incubated with BU69 (CD70), BU12 (CD19) and BU33 (CD21), were incubated with the PDDYS-conjugated secondary antibody and a weaker IFN-γ response when cells were incubated with Rituximab. The positive IFN-γ response to BU69 is consistent with the previous results and therefore suggests that this method of

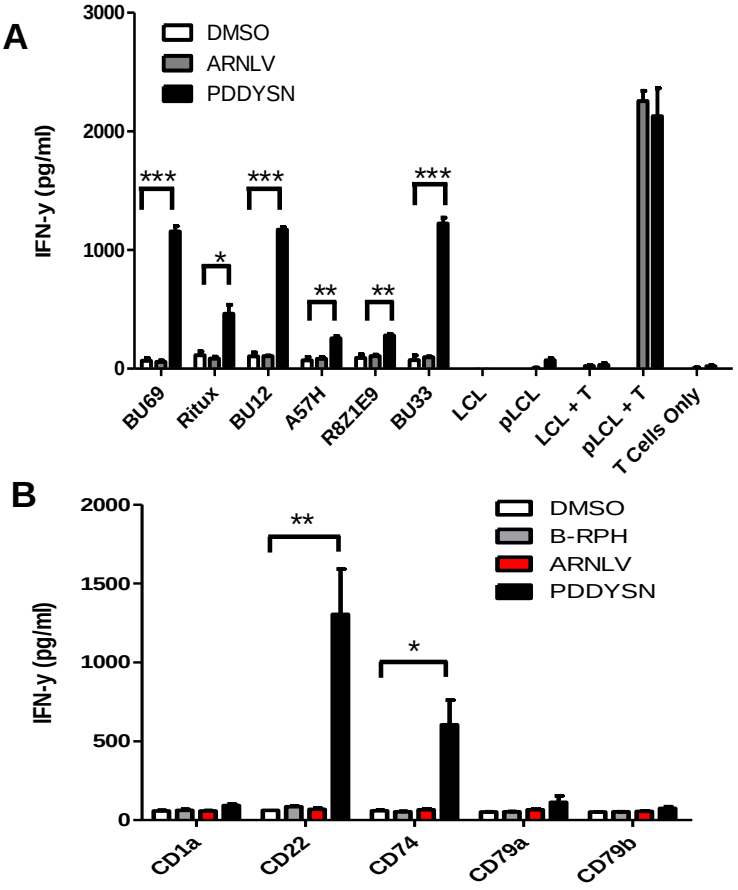


Figure 5.15 IFN-γ recognition of target cells stained with primary antibodies and a peptide-conjugated secondary antibody

Cells were stained with unconjugated primary antibodies (BU69 (anti-CD70), Rituximab (AntiCD20), BU12 (Anti-CD19), A57H, R8Z1E9 (both anti-IgG) and BU33 (Anti-CD21)) (A) or (anti-CD1a, anti-CD22, antiCD74, anti-CD79a and anti-CD79b) (B) for 30 minutes and washed before staining with a secondary antibody which had been conjugated with either HLA class I (ARNLV), class II peptide (PDDYSN), an irrelevant peptide (B-RPH) or unconjugated (DMSO) (A-B). After 30 minutes, the secondary antibody was washed off and the cells were incubated overnight. After washing, peptide-specific T cells (E:T ratio - 1:5) were added and the cells were cultured together for 6 hours before taking the supernatant to assay for IFN-γ production by ELISA. Control wells containing peptide pulsed target cells without T cells (pLCL), unpulsed target cells (LCL), and target cells cultured with peptide-specific T cells (LCL + T) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide spefcific T cells (pLCL + T) were used to demonstrate the ability of the target cells to present the DYSN peptide. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance * P<0.05, ** P<0.01 and *** P<0.001.

using a peptide-conjugated secondary antibody and looking for responses in cells incubated with an unconjugated primary antibody could be used for analysing a large number of antibodies quickly and easily. Another panel of antibodies were investigated using the HLA class-II peptide conjugated secondary antibody in order to determine if the previous results were similar with further B cell specific antibodies. Target LCLs were incubated with antibodies against CD1a (a transmembrane protein, structurally related to the MHC family not usually found on B cells) as a negative control, CD22 (belongs to a family of lectins and functions as an inhibitory receptor for surface Ig signalling), CD74 (the HLA Class-II invariant chain, functions to stabilise the HLA class-II molecule until peptide loading occurs) and CD79α and β (forms a complex with the BCR and generates a signal following activation of B cells through BCR engagement). There was no IFN-γ production when the cells were incubated with the secondary antibody containing the irrelevant peptide or without a peptide (Figure 5.15 B) demonstrating that there was very little background IFN-γ production throughout the assay. There was also very little IFN-γ production above the background when the secondary antibody conjugated to ARNLV was used either, further demonstrating that the class-I peptides cannot be processed in this way. Using the CD1a primary antibody as a negative control, there was a slight increase in IFN-γ production over background. The production of IFN-γ after labelling with CD79α and β was very similar to that seen after labelling with CD1a suggesting that there is no processing and presentation of the class-II peptide conjugated to these antibodies. When cells were incubated with the anti-CD74 antibody, there was a dramatic increase in IFN-γ production compared with the CD1a labelling. There was also a larger increase in IFN-γ production when cells had been incubated with the anti-CD22 antibody. These results suggest that antibodies against CD22 and CD74 may be useful

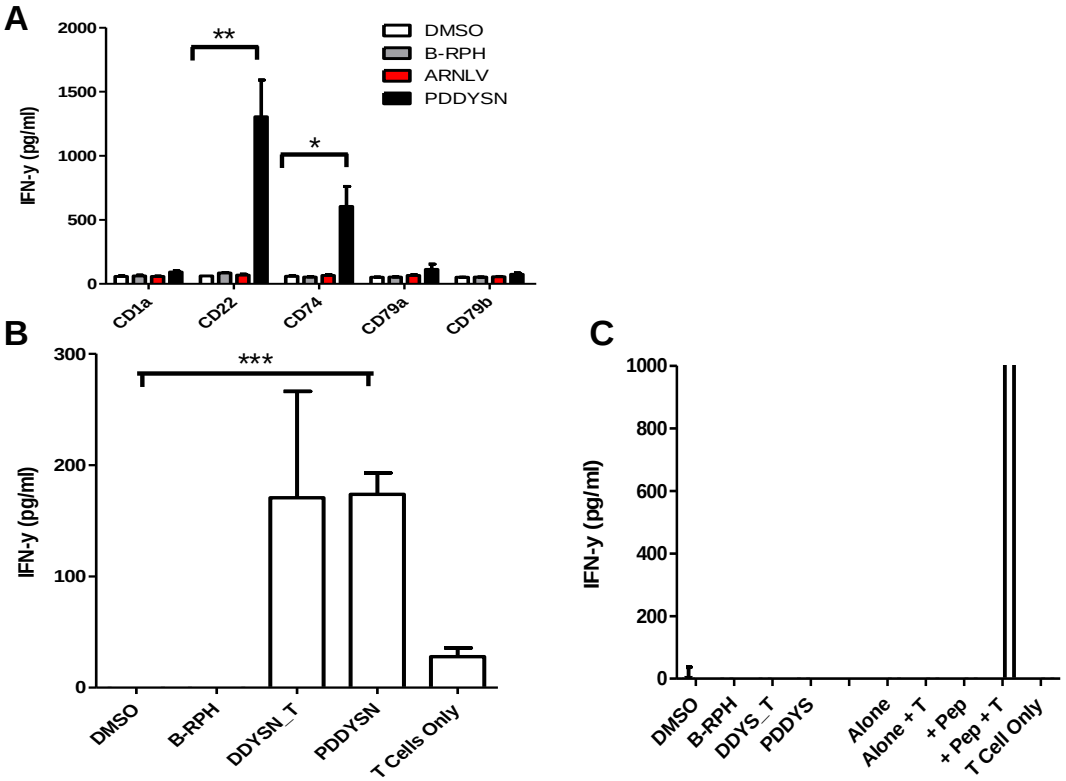


Figure 5.16 IFN-γ recognition of target cells stained with further primary antibodies and a peptide-conjugatged secondary antibody

Target LCLs were stained with primary antibodies (anti-CD1a, anti-CD22, antiCD74, anti-CD79a and anti-CD79b) for 30 minutes (A), washed and then stained again with a secondary antibody conjugated with either an irrelevant peptide (B-RPH), ARNLVPMVATC or PDDYSNTHSTRYV. Cells were cultured overnight before the addition of peptide-specific T cells (E:T ratio - 1:5). Cells were then cultured for 6 hours and the supernatant taken to assay for IFN-γ production by ELISA.

Target LCLs were stained with anti-CD74 conjugated with class II peptides (DDYSN_T and PDDYSN), control peptide (B-RPH) or no peptide (DMSO). After 30 minutes, cells were washed and incubated overnight (B). The cells were then cultured with peptide-specific T cells (E:T ratio - 1:5) and the supernatant taken to assay for IFN-γ production after 6 hours by ELISA. T cells alone were culture to determine spontaneous IFN-γ release. Error bars demonstrate mean + standard error mean, n=3. Target LCLs were stained with anti-CD22 conjugated with class II peptides (DDYSN_T and PDDYSN), control peptide (B-RPH) or no peptide (DMSO). After 30 minutes, cells were washed and incubated overnight (C). The cells were then cultured with peptide-specific T cells (E:T ratio - 1:5) and the supernatant taken to assay for IFN-γ production after 6 hours by ELISA. Control wells containing peptide pulsed target cells without T cells (+ pep), unpulsed target cells (Alone), and target cells cultured with peptide-specific T cells (Alone + T) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide spefcific T cells (+ pep + T) were used to demonstrate the ability of the target cells to present the DYSN peptide. Error bars demonstrate mean + standard error mean, n=3 Data was analysed using unpaired t-test to determine statistical significance *** P<0.005 ** P<0.01 and * P<0.05.

targets of as they are both expressed on B cells although CD74 is expressed on all cells expressing MHC class-II (Stein et al., 2007).

5.13 Direct conjugation of antibodies with positive responses

The previous data demonstrates that using a secondary antibody conjugated with peptides can induce T cell activation. However, in order to determine whether the positive results seen with the secondary antibody can translate to direct conjugation, peptides were conjugated to BU12, BU33 and BU32 (another anti-CD21 antibody). There was no recognition of the LCLs incubated with BU12 (anti-CD19) conjugated to any of the HLA class-II peptides by the DYSN-specific T cells (Figure 5.16 A). The same results were seen for both BU32 and BU33 antibodies which had also been conjugated with the HLA class-II peptides. These results demonstrate that the peptide had not been presented by the cells, possibly due to lack of internalisation or lack of access to the MHC class-II processing pathway. These results are contradictory to previous results using anti-CD19 antibody conjugated with tetanus toxin peptides (Eberl et al., 1998) where there was a response seen with the anti-CD19 antibody.

There are further studies published that show antibodies bound to CD21 (Tessier et al, 2007) and CD19 (Ingle et al., 2008 & Gerber et al., 2009) are internalised after binding. However, the results shown in these previous studies demonstrate lack of CD19 internalisation when there is high surface expression of CD21. This may explain the negative results seen here as the target cells were strongly positive for both CD19 (90%) and CD21 less so (45%) (Figure 5.17), meaning that the lack of internalisation using BU12 (anti-CD19) may be due to expression of CD21. Despite the findings reported, the lack of T cell recognition may be due to lack of

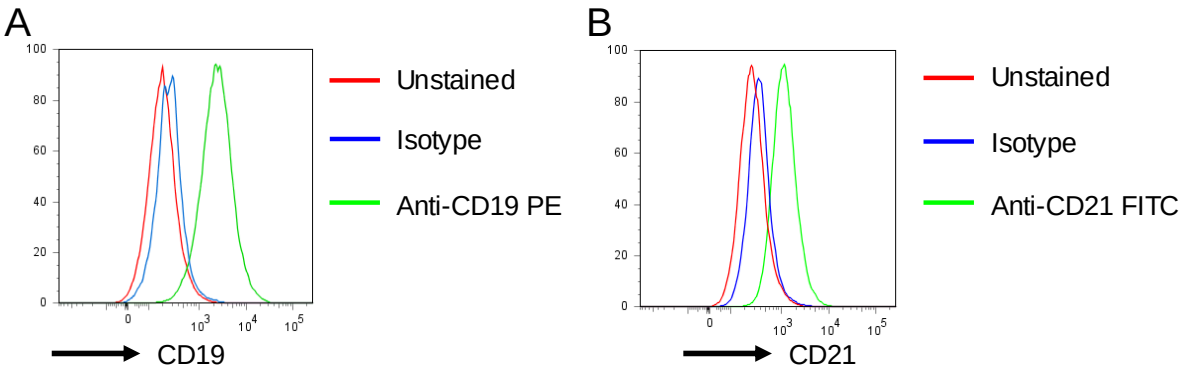


Figure 5.17 CD19 and CD21 staining of LCLs

LCLs were stained left unstained (A-B Red), stained with isotype control antibodies (A-B Blue) or with antibodies against CD19 conjugated to PE (A Green) and CD21 conjugated to FITC (B Green). After 30 minutes, the antibodies were washed off and the cells were analysed on the flow cytometer.

internalisation or failure to enter the class-II antigen processing pathway possibly because the antibodies used for this experiment are from different clones and therefore could elicit a different biological response after binding. Once the HLA class-II peptides DDYS_T and PDDYS had been conjugated to anti-CD22 and anti-CD74 as well as the control peptide biotin-RPH and DMSO, target LCLs were incubated with these antibodies to determine whether they produce a similar response. Cells incubated with anti-CD74 conjugated without a peptide or with an irrelevant peptide produced very little IFN-γ (Figure 5.16 B). However, cells incubated with anti-CD74 conjugated to both of the class-II peptides demonstrated a large increase in IFN-γ production after incubation with DYS-specific T cells. This suggests that the anti-CD74 antibody is efficiently internalised after binding to the surface CD74 and processed in the same way as BU69 allowing presentation of the peptides at the surface which can then be recognised by the peptide-specific $CD4^+$ T cells. Cells were incubated with the anti-CD22 antibody conjugated with the class-II peptides as well as the irrelevant peptide and DMSO. There was very little IFN-γ produced when cells were incubated with the anti-CD22 antibody conjugated with any of the peptides, including the HLA class-II peptides (Figure 5.16 C). This suggests that the anti-CD22 antibody behaves differently to the anti-CD74 antibody and either does not internalise after binding or is excluded from the class-II processing pathway therefore denying the conjugated peptides access to HLA class-II molecules. These data would suggest that cross-linking is required for CD22 ligands to be directed toward the antigen processing machinery.

5.14 Targeted depletion of B cells in peripheral blood.

Previous results have demonstrated that B cells incubated with antibody-peptide conjugates can be targeted by peptide-specific CD4 T cells when the target cells are the only cell present. However, I wanted to determine whether the peptide-specific T cells could lyse the target B cells in PBMCs using the FACS-based targeting assay. The frequency of peptide-specific T cells in fresh PBMCs from the donor chosen was <1% and as a result of this, autologous peptide-specific T cells were added to increase the E:T ratio. After labelling, the concentration of B cells in the untreated PBMCs was ~50% of the total $CD3^-$ cells and the addition of 50,000 extra peptide-specific $CD4^+$ T cells did not alter the percentage of the B cells suggesting there was little non-specific targeting of the PBMCs by the newly introduced T cells (Figure 5.18). Upon addition of the peptide-conjugated secondary antibody only (anti-mouse IgG), there was a decrease in the percentage of B cells (36%) suggesting that they were being targeted by CD4 T cells. This decrease was surprising as the antibody should not be able to bind to the cells as it recognises mouse IgG and not human IgG. However, cells were not washed and it is possible that the secondary antibody could have been taken up by Fc receptors present on the surface of cells. Therefore, the peptides would then be processed and presented by these cells and this could account for the decrease in the percentage of B cells incubated only with the secondary antibody. When the cells were incubated with the specific primary antibodies BU12 (CD19) or A57H (Human IgG) and then the peptide-conjugated secondary antibody, there was a further decrease in the percentage of B cells (70-76%) (Figure 5.18). This potentially demonstrates an increase in B cell lysis by the peptide-specific CD4 T cells when there was a primary antibody specific for B cells present and that within an environment where there are many different types of leukocytes, the antibodies bind

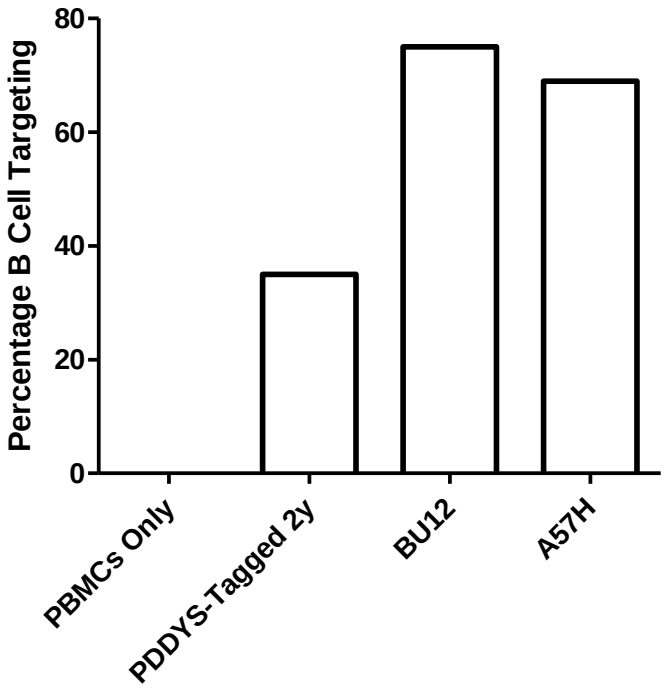


Figure 5.18 B cell targeting in PBMCs spiked with peptide specific T cells using secondary antibody conjugated with HLA class II peptides

PBMCs were stained with either BU12 (anti-CD19) or A57H (anti-IgG), washed and then stained a second time with a peptide-conjugated (PDDYSNTHSTRYV) anti-mouse IgG antibody. The primary antibody would bind to the cell and the peptide-conjugated secondary antibody would bind to the primary antibody effectively coating the cell in peptide-conjugated antibody. The frequency of DYSN-specific T cells in the donor PBMCs was very low and therefore cultured DYSN-specific T cells were added to the PBMCs to increase the E:T ratio. After washing, the PBMCs with the added DYSN-specific T cells were left to incubate for 6 hours. The cells were stained using antibodies against CD3, CD4 and CD19 and analysed on the FACS to determine the frequency of B cells present in each culture. The percentage of B cells present in the PBMCs was calculated as a percentage of CD3 negative cells as the DYSN-specific T cells added would alter the percentage of T cells in the culture and alter the percentage of B cells as a result. Therefore, the T cells were removed from the equation and the B cells calculated as a percentage of non-T cells.

to their targets and direct an immune response against the targeted cells where previously only a single cell type was used. Furthermore, this would suggest that if this work was to be moved into an animal model, it could potentially target a B cell tumour within all the other cells present in the haematopoietic system, although it must be remembered that a peptide-conjugated secondary antibody was used and as a result it is not a direct targeting assay. Ideally, this work would use a single antibody instead of having to use a primary antibody and a peptide-conjugated secondary antibody. Future work will attempt to address this and use an antibody specific for B cells which has been directly conjugated with a viral peptide to determine if the same results are seen with this model.

5.15 Testing Therapeutic Antibodies with Peptide-Conjugation

All of the previous results have used LCLs as target cells and although they are a transformed B cell line, they are viewed as an lymphoma cell line and as a result of this, another cell line was sought which would provide a more accurate model for lymphoma in order to test another hypothesis; peptides conjugated to antibodies already used in therapy for patients of various cancers (including breast and non small cell lung carcinoma) and heamato-lymphoid malignancies (including chronic lymphocytic leukaemia (CLL) and acute myeloid leukaemia (AML)), could target malignant cells. The potential for peptides conjugated to the primary antibody to produce a positive IFN-γ response has been shown in previous results. Many of these cancers and leukaemias already have antibody therapies approved (Table 5.2) and it is possible that using the peptide conjugated antibodies could enhance the efficacy of such therapies and therefore work began to focus on either antibodies that have been approved for immunotherapy or those that could target surface proteins on malignant B cells.

Name	Approval Date	Ab Type	Target	Treatment
Muromonab-CD3	1986	Murine	TCR:CD3	Transplant rejection
Abciximab	1994	Chimeric	Glycoprotein 2a/3b	Cardiovascular disease
Daclizumab	1997	Humanised	CD25	Transplant rejection
Rituximab	1997	Chimeric	CD20	Non-hodgkin lymphoma
Basiliximab	1998	Chimeric	CD25	Transplant rejection
Infliximab	1998	Chimeric	TNF-α signalling	Autoimmune disorder
Palivizumab	1998	Humanised	RSV Protein Epitope	Respiratory syncitial virus
Trastuzumab	1998	Humanised	ErbB2	Breast cnacer
Gemtuzumab	2000	Humanised	CD33	Acute myeloid leukemia
Alemtuzumab	2001	Humanised	CD52	Chronic lymphocytic leukemia
Adalimumab	2002	Human	TNF-α signalling	Autoimmune disorder
Efalizumab	2002	Humanised	CD11a	Psoriasis
Ibritumomab tiuxetan	2002	Murine	CD20	Non-hodgkin lymphoma
Tositumomab	2003	Murine	CD20	Non-hodgkin lymphoma
Bevacizumab	2004	Humanised	VEGF	Colorectal cancer
Cetuximab	2004	Chimeric	EGF receptor	Head and neck cancer
Natalizumab	2006	Humanised	Alpha-4 integrin	Multiple sclerosis
Panitumumab	2006	Human	EGF receptor	Colorectal cancer
Ranibizumab	2006	Humanised	VEGF	Macular degeneration
Certolizumab pegol	2008	Humanised	TNF-α signalling	Crohn's disease

TNF-α: Tumour necrosis factor alpha; VEGF: Vascular endothelial growth factor; EGF: Epidermal growth factor.

Table 5.2 Current antibodies approved for immunotherapy

Shows the FDA-approved list of monoclonal antibodies for treatment in various indications. Table contents taken from Waldmann, TA. 2003.

5.15.1 Labelling Burkitt's Lymphoma Cells with BU69-Peptide

The results shown so far use BU69 conjugated with peptides and LCLs as the target cells to demonstrate the principle of the work. Ramos cell line is a human Burkitt's lymphoma cell line which is HLA-$A2^-$ and more importantly HLA-$DR7^+$ meaning that the cells could be targeted using the HLA class-II peptides. Firstly, the Ramos cell line was incubated with a commercial anti-CD70 antibody to determine whether the BU69 antibody could be used with these cells. After analysing Ramos cells using flow cytometery, they were strongly positive for surface CD70 expression (Figure 5.19 A) and therefore the cells could be incubated with BU69 conjugated with class-II peptides to determine whether they could process and present the conjugated peptide in the same manner as previously shown. The class-II peptide-conjugated anti-CD74 was also used to stain the Ramos cells. After labelling, Ramos cells were cultured with the peptide-specific CD4 T cells and the supernatant taken to assay the production of IFN-γ after 6 hours. The Ramos cells pulsed with exogenous DYSN peptide were strongly recognised by the peptide-specific CD4 T cells (Figure 5.19 B) whereas the untreated Ramos cells were not recognised by the T cells. This demonstrates the ability of Ramos cells to present the DYSN peptide to the T cells and that the T cells do not recognise the Ramos cells non-specifically. After labelling with either BU69 or anti-CD74 conjugated with the class-II peptides, there was no production of IFN-γ by the T cells. This demonstrated that there was no presentation of the peptide at the surface of the Ramos cells. This may be due to the inability of the Ramos cells to internalise the antibody although there may be a difference in the antigen processing by the Ramos cells that has prevented the expression of the peptide at the surface of the cell.

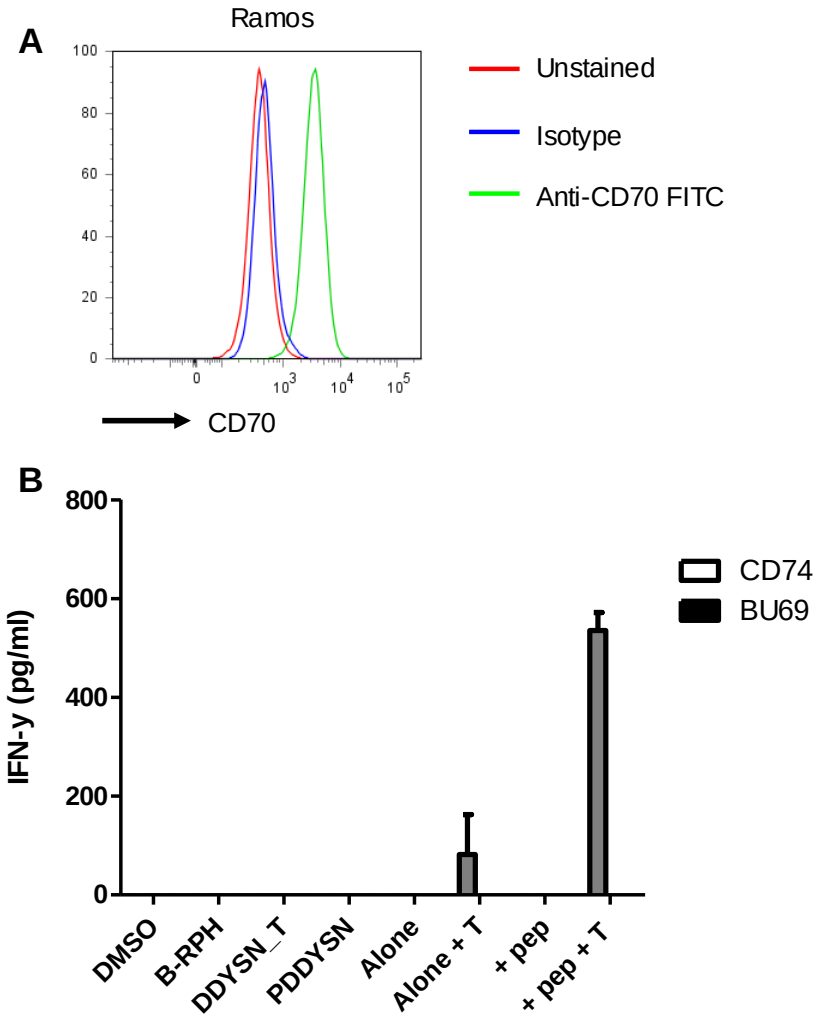


Figure 5.19 CD70 staining and IFN-γ release by Ramos cells stained with BU69 or anti-CD74

Ramos cells were left unstained (A Red), stained with isotype control (A Blue) or stained with commercial anti-CD70-FITC antibody (A Green) for 30 minutes. Cells were washed and analysed on the flow cytometer. Ramos cells were stained with BU69 alone (DMSO) or peptide-conjugated BU69 or peptide conjugated anti-CD74 (B-RPH (control), DDYSN_T and PDDYSN) for 30 minutes, washed and incubated overnight. Peptide-specific T cells were added (E:T ratio - 1:5) for 6 hours before the supernatant was taken to assay for IFN-γ production by ELISA (B). Target cells alone (Alone) and with T cells (Alone + T) demonstrate little IFN-γ production towards untreated target cells. Addition of DYSN peptide (+ pep) did not increase the IFN-γ production until peptide-specific T cells were added to the culture (+ pep + T). Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance.

5.15.2 Targeting Breast Cancer Cells with Herceptin-Peptide

As well as trying to target B cell lymphomas, there was the potential to target breast cancer as well. Herceptin is an antibody therapy for breast cancer which targets the Her2/neu receptor on breast cells and prevents growth factor binding, effectively starving the cancerous cell and preventing tumour growth (Goldenberg, 1999 and Hudis, 2007). The MCF-7 cell line used as a model for breast cancer was HLA-$A2^+$ but HLA-$DR7^-$, therefore the class-I peptides but not the class-II peptides could be investigated. MHC class-I peptides were conjugated to Herceptin and used to stain MCF-7 cells. Assaying for IFN-γ showed that there was no recognition of the untreated MCF-7 cells (Figure 5.20), demonstrating that the T cells used in the assay did not recognise MCF-7 cells in a peptide independent manner. The NLV-specific $CD8^+$ T cells can recognise MCF-7 cells pulsed with exogenous peptide demonstrating that the MCF-7 cells can present the peptide at the surface of the cells in complex with HLA-A2. However, there was no production of IFN-γ seen when cells were incubated with Herceptin conjugated with any of the HLA class-I peptides (<50pg/ml). There are previous publications suggesting Herceptin is quickly internalised after binding to the Her2/neu receptor (Mittendorf et al, 2006). Therefore, the lack of IFN-γ response to the antibody-peptide conjugate incubated cells is probably not due to the lack of internalisation.

In order to test the class-II peptides in a breast cancer model, another cell line MDA.MB.231 was cultured which is HLA-$A2^+$ and HLA-$DR7^+$. The MDA.MB.231 cells do not naturally express HLA class-II molecules at the surface but cells can be induced to express them by addition of IFN-γ to the culture media (Basham &

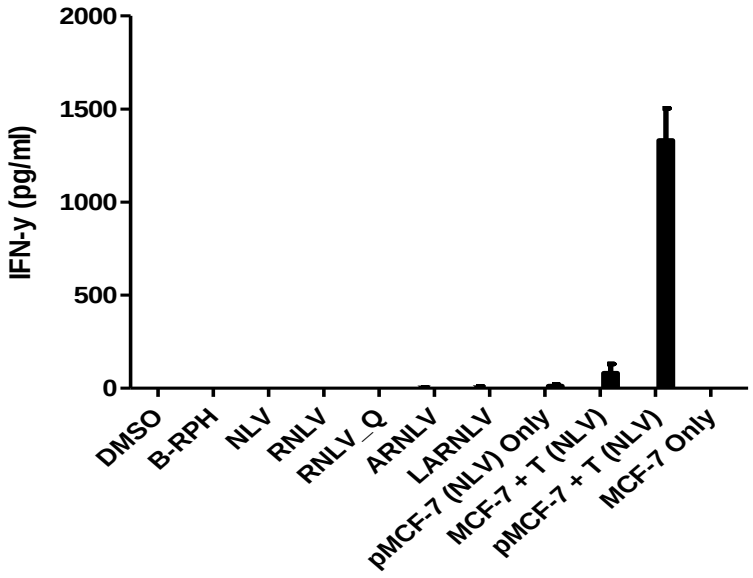


Figure 5.20 IFN-γ recognition of MCF-7 breast cancer cells stained with peptide-conjugatged Herceptin

Breast Cancer cells, MCF-7, were stained herceptin unconjugated (DMSO) or conjugated to class I peptides (B-RPH, NLV, RNLV or RNLV_Q). After 30 minutes, cells were washed and incubated overnight. Peptide-specific T cells were added (E:T ratio - 1:5) and the supernatant assayed for IFN-γ production after 6 hours by ELISA. Control wells containing peptide pulsed MCF-7 cells without T cells (pMCF-7 (NLV) only), unpulsed MCF-7 cells (MCF-7 only), and MCF-7 cells cultured with peptide-specific T cells (MCF-7 + T (NLV) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed MCF-7 cells cultured with peptide spefcific T cells (pMCF-7 + T (NLV)) were used to demonstrate the ability of the target cells to present the NLV peptide. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance.

Merigan, 1983, Collins et al., 1984 & Gosselin et al, 1993). After treatment with IFN-γ for 18 hours, to induce the expression of HLA class-II, cells cultured with T cells in the absence of peptide were not recognised. However, when the treated cells are pulsed with exogenous DYSN peptide, there is an increase in cell recognition demonstrating that the IFN-γ treatment of cells has upregulated HLA class-II expression on the surface. However, when the IFN-γ treated cells are incubated with Herceptin, which has been tagged with HLA class-II peptides, there is no T cell response (Figure 5.21) above the background (Herceptin conjugated with SMCC but no peptide (DMSO)). This data suggests that there is no expression of the peptides at the cell surface. Since the cell line can express the HLA class-II peptide, and as $CD4^+$ T cells can recognise cells pulsed with peptide, this means that the cell line can be induced to express HLA class-II at the surface but it cannot process and present the peptide conjugated to Herceptin. The HLA class-I peptide conjugated Herceptin labelling was repeated using the MDA.MB.231 cell line but the results were similar to those seen with the MCF-7 cell line. Taken together, these results mean that the potential of this peptide-antibody therapy for breast cancer using Herceptin is low and as a result no further work was undertaken.

5.16 Engineering Antibodies Encoding Viral Peptide Epitopes

Previous results show peptides conjugated to antibodies can target B cells after processing and presentation of the viral peptide. I set out to test the hypothesis that B cells can process endogenous peptides derived from the CDR3 of immunoglobulin and present these peptides on the surface of the cell in complex with MHC molecules. By introducing a known viral peptide into the CDR3, I could determine the processing capability of B cells to present the CDR3-derived viral peptide. By doing so, the B

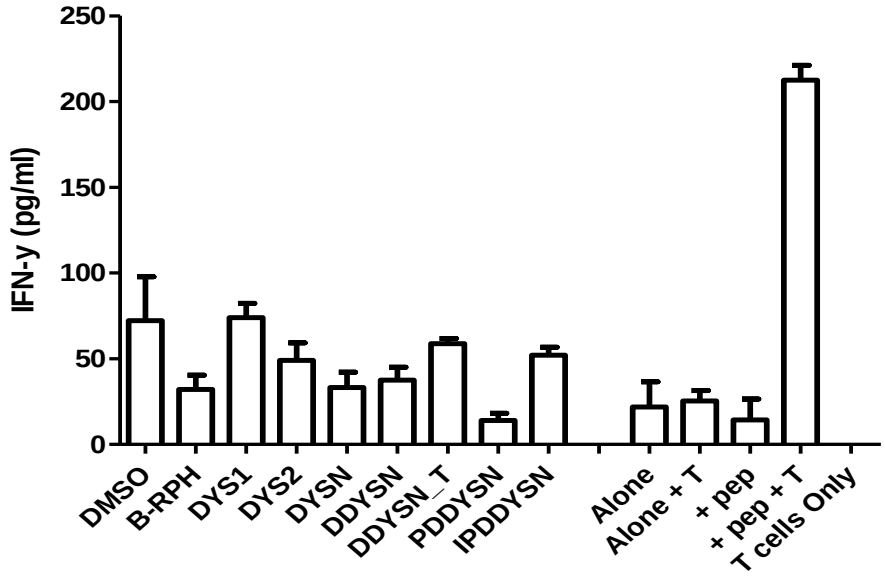


Figure 5.21 IFN-γ recognition of MDA-MB-231 cells stained with peptide conjugated Herceptin

MDA-MB-231 cells were stained with class II peptide conjugated herceptin (B-RPH - IPDDYSN) or unconjugated herceptin (DMSO), washed and incubated overnight. Peptide-specific T cells were added (E:T ratio - 1:5) and the supernatant assayed for IFN-γ production after 6 hours by ELISA. Control wells containing peptide pulsed target cells without T cells (+ pep), unpulsed target cells (alone), and target cells cultured with peptide-specific T cells (Alone + T) were used to demonstrate that there was no spontaneous or non-specific release of IFN-γ. Peptide pulsed target cells cultured with peptide specific T cells (+ pep + T) were used to demonstrate the ability of the target cells to present the NLV peptide. Error bars demonstrate mean + standard error mean, n=3. Data was analysed using unpaired t-test to determine statistical significance.

cell would become a target for CMV-specific T cells. The key question being: can CDR3-derived peptides be processed and presented by HLA molecules at the surface of the paternal B cell or are these peptides excluded from the HLA class I and II processing pathways in order to avoid being targeted.

5.16.1 Mutating the CDR3 of Rituximab

Rituximab, a chimeric antibody specific for CD20, was chosen as it is an approved therapeutic antibody which is routinely used to treat patients with B cell malignancies. The CMV-derived $pp65_{495-503}$, HLA-A2 restricted peptide NLVPMVATV (Wills et al., 1996) was inserted into the CDR3 of the Rituximab sequence (Figure 5.22), labelled Rituximab-NLV. The assumption behind this work was that the default pathway for antibodies bound to a cell surface and internalised would be the class II processing pathway.

5.16.2 Production of Rituximab-NLV

Three different methods were employed to generate Rituximab-NLV, as discussed in materials and methods 2.6.3.4. Briefly, packaging cells in a single flask were transfected with both heavy and light chain in one tube with transfection reagent (A), packaging cells in a single flask transfected with heavy chain and light chain separately mixed with transfection reagent (B) and two flasks of packaging cells transfected with either the heavy or light chain and the media from the two flasks swapped so each cell line is transduced with both chains (C). Once transduced, media taken from the flasks Rituximab-NLV A, B and C was purified and assayed for production of antibody by ELISA (Figure 5.23 A). There is no response seen using PBS as negative control and a large response seen when using Rituximab as positive

A

QAYLQQSGAELVRPGASVKMSCKASGYTFTSYNMHWVKQTPRQGLEWIGAIYPGNGDT
SYNQKFKGKATLTVDKSSSTAYMQLSSLTSEDSAVYF**CARVVYYSNSYWYFDVWGTGTT**
VTVSGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKAEPKSCDKTHTCPPCPAPELLGG
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

B

QAYLQQSGAELVRPGASVKMSCKASGYTFTSYNMHWVKQTPRQGLEWIGAIYPGNGDT
SYNQKFKGKATLTVDKSSSTAYMQLSSLTSEDSAVYF**CNLVPMVATVYWYFDVWGTGTT**
VTVSGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSS
GLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKAEPKSCDKTHTCPPCPAPELLGG
PSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYN
STYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL
TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW
QQGNVFSCSVMHEALHNHYTQKSLSLSPGK

Figure 5.22 Sequence of the heavy chain of Rituximab containing viral epitope

The sequence of Rituximab (A) (taken from www.drugbank.ca) with the CDR3 underlined and the sequence of Rituximab-NLV (B) with the viral epitope NLVPMATV in the CDR3.

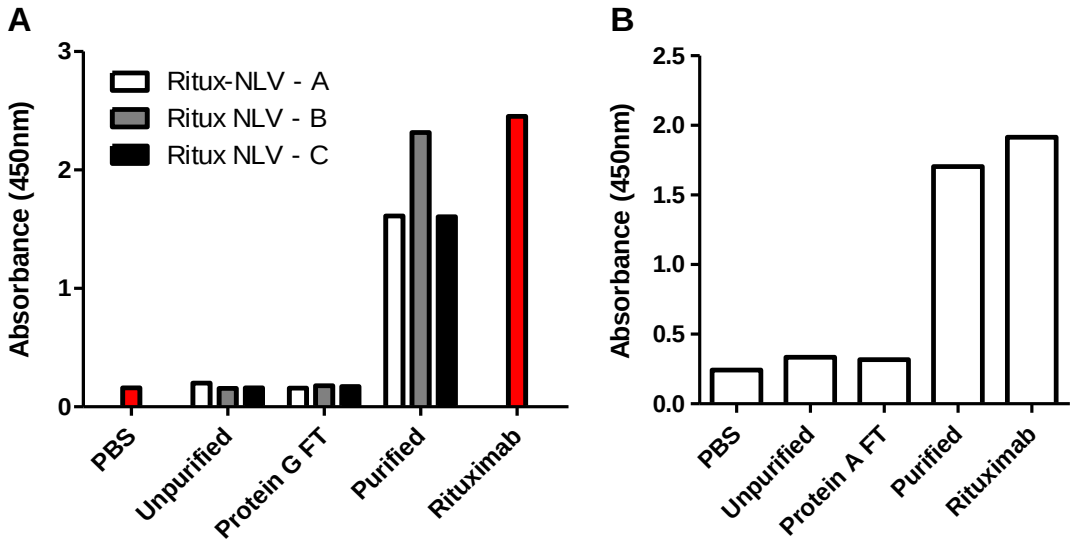


Figure 5.23 Production and purification of Rituximab-NLV

Media from transfected lenti-X 293T cells was purified using a protein G column as previously described (A). Transfected lenti-X 293T cells were grown in media containing IgG depleted FCS. The media was purified using a protein A column (B). The purified antibody was coated onto a 96 well plate and incubated for 60 minutes at 37°C. The plate was washed and a secondary antibody, mouse anti-human IgG was added for 60 minutes at room temperature. The plate was washed and a third antibody was added, anti-mouse-HRP, for 60 minutes at room temperature. The plate was washed and TMB added to each well for 10-15 minutes at room temperature. Addition of 10% HCl stopped the reaction and the wells were read at absorbance of 450nm using the GloMax reader. (FT - Column wash flow through).

control. There was no positive response seen against samples taken at different stages of the purification process suggesting that no antibody has been lost during the purification process. Although there is no response seen in the unpurified samples, there is a large response seen when the purified Rituximab-NLV was assayed from all 3 flasks. After demonstrating that Rituximab-NLV had been purified successfully, the concentration of antibody was determined using the Qubit fluorimeter. The concentration of antibody contained in Rituximab-NLV B was slightly increased (350μg/ml) compared with the concentration from flasks A and C (300μg/ml) suggesting purification of the antibody had been successful. However, the protein concentration of Rituximab-NLV may be wrong due to the presence of bovine IgG in the media from the FCS used to culture the transfected cells. Therefore to remove this contaminant, the transfected cells were grown in media supplemented with FCS depleted of bovine IgG (kind gift from Dr M Goodall).

After 5 days growth at 37°C, media from all 3 flasks was pooled and purified using a protein A column and tested using ELISA (Figure 5.23 B). The results are similar to the previous ELISA with no antibody lost during the purification and no response seen to the unpurified sample. There is a strong response seen from the purified sample similar to the positive control (Rituximab). The protein concentration from the sample was 100μg/ml, slightly decreased compared with previous results. This may be due to the removal of bovine IgG but may be due to decreased protein production by the transduced cells. The purified Rituximab-NLV was stored at 4°C until required for further experiments.

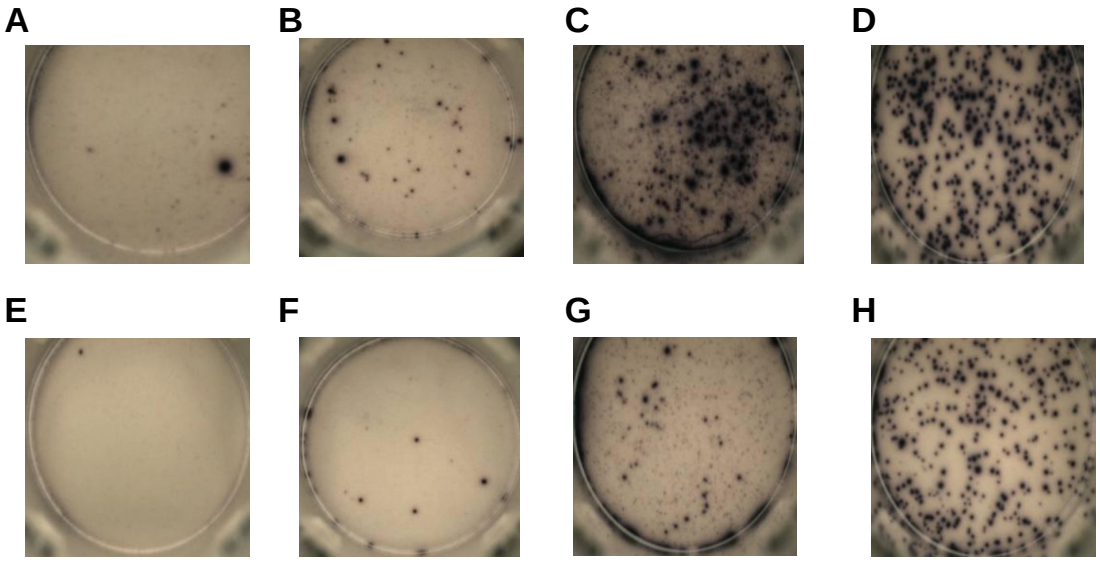


Figure 5.24 IFN-γ ELISPot of PBMCs stained with immune complexes of Rituximab and Rituximab-NLV formed with anti-kappa antibody

PBMCs were kept unfractionated (A-D) or depleted of B cells (E-H) using magnetic selection. Immune complexes were formed of Rituximab (C and G) and Rituximab-NLV (B and F) antibodies using mouse anti-kappa for 30 minutes at room temperature. The complexed antibodies were added to $1x10^5$ PBMCs previously plated in ELISpot wells. The cells were incubated overnight at 37°C without washing. Cells were removed and the wells washed 5x in PBS and the secondary antibody (7-B6-1) added to each well. The wells were washed and the substrate added for 10-12 minutes before stopping the reaction with excess water. The wells were read in an ELISpot plate reader and the number of spots recorded. The background response to PBS (A and E) was determined and the response to NLV peptide (D and H) was recorded as positive control.

5.16.3 Processing and Presentation of Rituximab-NLV

After mutating Rituximab, producing Rituximab-NLV, the antibody no longer binds to CD20 and therefore cannot be taken into the cell to access the antigen processing pathway. As a result of this, Rituximab-NLV had to be taken into the cell via the Fc region and to facilitate this, it had to be made into an immune complex (see materials and methods 2.6.3.5). The immune complexes produced either by heating or by addition of an anti-kappa antibody were added to unfractionated PBMCs or PBMCs depleted of B cells and production of IFN-γ assayed by ELISpot.

Using an anti-kappa antibody to form immune complexes of Rituximab and Rituximab-NLV, there was a strong IFN-γ response towards complexed Rituximab in whole PBMCs which was reduced significantly in the B cell depleted PBMCs (Figure 5.24 C & G). Addition of immune complexed Rituximab-NLV to whole PBMCs also produced an IFN-γ response (B) which was somewhat weaker than the response to unmutated Rituximab. There was still a response seen when complexed Rituximab-NLV was added to B cell depleted PBMCs (F). There was strong response seen to the NLV peptide-pulsed PBMCs with and without B cells (D & H) and very little response to untreated cells (A & E). After heating Rituximab-NLV, there were spots seen in wells containing both unfractionated PBMCs and B cell depleted PBMCs (Figure 3.25 B & E) although there were fewer spots seen in the B cell depleted PBMCs. Although not conclusive, these results suggest that there may be antigen processing of the Rituximab-NLV but further results are needed to confirm this.

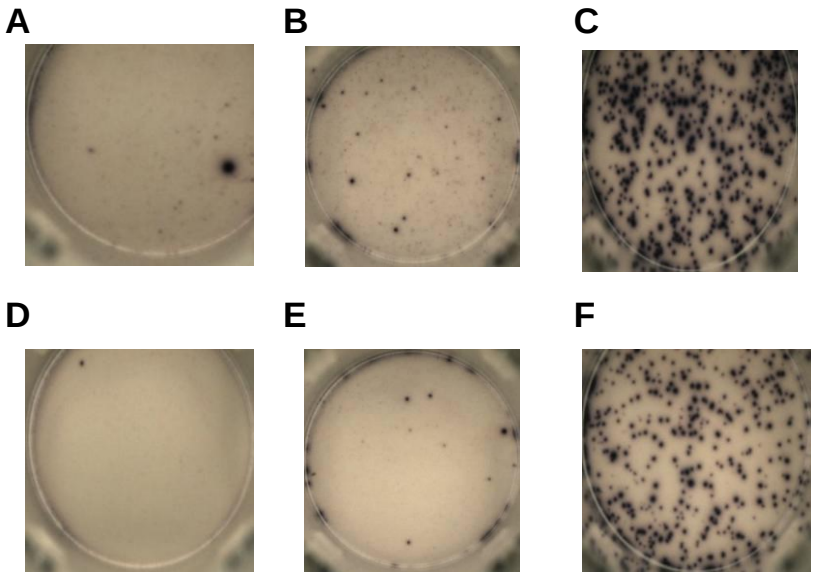


Figure 5.25 IFN-γ ELISPot of PBMCs stained with immune complexes of Rituximab and Rituximab-NLV formed by heating

PBMCs were kept unfractionated (A-C) or depleted of B cells (D-F) using magnetic selection. Immune complexes were formed of Rituximab-NLV (B and E) by heating at 65°C for 30 minutes. The complexed antibody was added to $1x10^5$ PBMCs previously plated in ELISpot wells. The cells were incubated overnight at 37°C without washing. Cells were removed and the wells washed 5x in PBS and the secondary antibody (7-B6-1) added to each well. The wells were washed and the substrate added for 10-12 minutes before stopping the reaction with excess water. The wells were read in an ELISpot plate reader and the number of spots recorded. The background response to PBS (A and D) was determined and the response to NLV peptide (C and F) was recorded as positive control.

5.17 Discussion

In this chapter, an attempt was made to generate an antibody which was conjugated to a viral peptide to determine whether this conjugate could be used to deliver the peptide into the antigen processing pathway of the target cell. The hypothesis tested was: the peptide conjugated to the antibody would be processed by cellular machinery and presented at the surface of the target cell. This would allow targeted killing of cells bound by the antibody as peptide-specific CD8 or CD4 T cells would then recognise the peptide which had originally been conjugated to the antibody.

After confirming that the conjugation of peptide to Rituximab was successful, cells were incubated with MHC class-I peptide and MHC class-II peptide-conjugated Rituximab. There was no response seen when target cells were incubated suggesting that there was no presentation of the peptide at the surface of the cell in complex with MHC molecules suggesting that the hypothesis is false. However, it was difficult to determine whether the lack of response was due to the peptide not entering the antigen processing pathway or due to lack of internalisation of Rituximab. Studies suggest that Rituximab is not internalised after binding to CD20 (Einfeld et al, 1988). Although there have been reports of a small percentage of Rituximab internalised after binding (Michel & Mattes, 2002 & Jilani et al., 2003), the lack of internalisation or inability to enter the antigen processing pathway are the most likely reasons for the lack of response. I believe that in order for the peptides to enter either of the HLA processing pathways, the antibody must first be internalised by the cell whereupon the peptide can be cleaved from the antibody and processed. As a result of this, the hypothesis was re-tested using a different antibody, BU69, which had been shown to

be internalised quickly after binding to CD70 (Israel et al., 2005 and Adam et al., 2006).

After confirming that peptides have been conjugated to BU69, target cells were incubated with BU69 conjugated with either class-I or class-II peptides. There was a response seen when target cells were incubated with both class-I and class-II peptides suggesting that the hypothesis is true. These results suggest that target cells take up the antibody, cleave the peptide and process both class-I and II peptides for presentation that T cells can then recognise. The results also appear to suggest that amino acids flanking the naturally processed peptide sequence are important as the strongest T cell response is seen when the antibody has been conjugated with peptides elongated at both the carboxy and amino termini. Consequently, some of the weakest responses are seen with the shorter peptides and the naturally processed peptide. However, further experiments demonstrated that the positive initial results observed were as a result of excess, unconjugated free peptide binding to surface MHC on the target cells and therefore there was doubt as to whether there was antigen processing of the peptide conjugated to BU69. These results suggest that the hypothesis may not be true as first thought and that the response seen was a result of the unconjugated peptides.

After removal of excess unconjugated peptides, a T cell response was seen using MHC class-II peptides only. The results shown here support previous results using similar peptide-conjugation technology where CD4 T cells in mice (Casten et al, 1988) and humans (Eberl et al., 1998) could recognise target cells incubated with anti-idiotype antibodies conjugated with antigenic peptides from either the tobacco

hornworm moth or tetanus toxin. These data suggest that the peptide conjugated antibody can gain access into the class-II processing pathway but not the class-I processing pathway. Uptake of extracellular protein is largely sequestered by the class-II processing pathway and the results shown here suggest that it can enter the class-II but not the class-I processing pathway. However, It may be possible that the antigen processing of the class-II pathway cleaves the bond between the antibody and the peptide whereas the class I processing pathway cannot break the bond. This would mean that the peptide-antibody complex could enter the class-I processing pathway but the conjugation technique may prevent correct processing. If this is the case, altering the conjugation could allow correct processing of the antibody-peptide conjugate and presentation of the class-I peptide on the cell surface.

After showing that the class-II peptides were presented at the cell surface, further work was undertaken to determine the processing that was needed to present the peptide using inhibitors of antigen processing. The results showed that lactacystin and pepstatin had little effect on the production of IFN-γ suggesting that the peptide is not processed utilising either the proteasome or aspartyl proteases. However, the processing is partly due to the activity of thiol proteases as there was a small reduction in response when leupeptin was added. Proteases are found in the compartment associated with class-II processing and break down polypeptide chains at low pH. Therefore, the expectation would be that inhibition of proteases would have an effect on the antigen processing although this is not the case. It may be due to the presence and activity of other proteases that are not inhibited by either pepstatin or leupeptin. The response seen in the presence of 3-methyl adenine suggests that the autophagy pathway is not utilised by the antibody-peptide conjugate.

There is a decreased response when cells were cultured with cholroquine, suggesting that the antibody-peptide complex is taken up into a phagolysosome after internalisation and degraded using proteases (although not aspartyl proteases). The addition of chloroquine disrupts the pH in the lysosome preventing acidification and subsequently prevents protease activity. The activity of cholorquine appears to be irreversible as there is still a decreased response 24 hours after removal of the inhibitor. Addition of monensin to the media results in a decreased response after 6 hours which is reversible and leads to an increased response after 24 hours. This may be explained by the fact that monensin is an inhibitor of the golgi complex and prevents transport of molecules to the surface of the cell. If this was to occur, there may be a backlog of HLA class-II molecules waiting to be transported to the surface. When the monensin was removed and the presentation pathway cleared, this backlog of HLA class-II molecules would then be expressed at the surface of the cell alongside the newly synthesised HLA class-II molecules. Therefore, the target cells would then have an increased expression of HLA class-II molecules, expressing the DYSN peptide, at the surface of the cell which the peptide-specific CD4 T cells can recognise and this could lead to an increase in IFN-γ production. These results demonstrate that the peptide-conjugated antibodies are processed and presented in the classical MHC class-II antigen processing pathway. Using an antibody which binds to HLA-DR, the results demonstrate a decreased response when HLA-DR is blocked, preventing T cells from accessing the peptide-MHC on the cell surface. This demonstrates that the peptide is being presented on the surface in complex with HLA-DR, as it has been shown to do so in previous studies. Taken together, these results demonstrate that the antibody-peptide complex is processed in the classical class-II

processing pathway before presentation on the correct MHC class-II allele at the cell surface.

A question that arose after these results was: Can a different peptide, from a different virus, elicit a similar response or is the response to peptide-conjugated antibody peptide specific? Conjugation of the EBV-derived, HLA-DR52b restricted peptide PRS (Long et al., 2005) to BU69 produced similar responses as that seen with the CMV derived peptide HLA-DR7 restricted peptide. This suggests that conjugation of any viral class-II peptide could elicit an immune response in this model and there is no restriction on where peptides are derived from. This means that if this is to be used in an *in vivo* setting, the peptide conjugated to the antibody could be generated after determining the immune response to different viruses from each patient, essentially tailoring the therapy to their immune system.

After the failure with Rituximab and subsequent success seen with BU69, it is clear that different antibody targets will act differently and not all will be amenable to this system. Using a secondary antibody conjugated with peptides would allow high-throughput testing of this system to determine which antibodies would be useful. This new system worked well with BU69 and there was a large response seen similar to the direct conjugation. Using this new system, there was also a response seen using antibodies against CD19 and CD21 as well as Rituximab. The positive result using Rituximab was unexpected as previously there was no response using the direct peptide conjugation. The positive response is possibly due to the forced internalisation due to cross-linking of the Fc region as has been published previously (Coffey et al., 2004). Therefore, this result suggests that a positive result seen with secondary

conjugation may not automatically translate to a positive result when tested with direct conjugation. Positive results were also seen with BU12 (anti-CD19) and BU33 (anti-CD21) when using secondary conjugation but when the same antibodies were tested with direct conjugation there was no response. The lack of recognition most likely reflects failure of internalisation of the antibodies and therefore the peptides are not gaining access to the class-II processing pathway within the target cell.

This confirms the previous result which shows that even if there is a positive response using secondary conjugation, it does not reveal which antibodies will be useful using direct conjugation. This suggests that the use of the secondary antibody cross-links the primary antibody, causing internalisation of the complex and therefore allowing the peptide access to the antigen processing pathway. When there is no cross-linking of the primary antibody, there is no internalisation and therefore no access to the antigen processing pathway for the conjugated peptide. Previous studies have shown that cross-linking of an antibody bound to its antigen on the surface of the cell can cause internalisation of the whole complex (Coffey et al., 2004). There is a high degree of probability that internalisation of the antibody is an important step in allowing the peptide access into the class-II antigen processing pathway.

The results suggest that the assay using the peptide-conjugated secondary antibody to screen many antibodies at once may provide some antibodies as candidates but the positive results seen do not automatically translate into a positive result once the primary antibodies are directly conjugated with the peptides. It does however rule out some antibodies as those which do not produce a response using secondary conjugation do not produce a response using direct conjugation.

Until this point, the only antibody which had shown a positive response using direct conjugation was BU69 and as a result of this, the secondary conjugation system was used again to find potential antibodies specific for B cells which could produce a positive response. Antibodies to CD22 and CD74 both showed responses using the secondary conjugation but only anti-CD74 antibody produced a positive response using direct conjugation. This result was very important as it showed that this system is not limited to BU69 and can be used with other antibodies and secondly, it allowed specific targeting of a single cell type. Interestingly, CD74 is a therapeutic target found on many B cell malignancies (Pawlak-byczkowska et al, 1989 and Burton et al., 2004) and therefore may represent a useful target for this model.

Further experiments demonstrated firstly the ability of peptide-specific CD4 T cells to degranulate (CD107 assay) in response to peptide-antibody incubated cells and secondly to lyse peptide-antibody incubated cells whilst leaving untreated cells of the same type alone. The results from the degranulation assay demonstrate an increased degranulation when there were fewer peptide-specific T cells present. CD107 is internalised and degraded quickly after degranulation and normally, target cells are treated with monensin to prevent destruction of the antibody incubated CD107. This prevents degradation of the FITC-conjugated antibody, by neutralising the pH, allowing the signal to be read by the flow cytometer. In this assay, monensin was not used as there were concerns that this may have an adverse effect on the antigen processing, which would affect the presentation of the peptides conjugated to the secondary antibody. However, this means that the CD107 may be internalised and degraded before the cells were incubated with the antibody. It is possible that the

lower CD107 labelling on the cells at the higher effector to target ratio (1:1) means that the target cells are being killed quicker and therefore the CD107 is being internalised and degraded before the anti-CD107 specific antibodies are added. If there are fewer effector cells, the target cells will be killed over a longer period of time and therefore the CD107 expressed at the surface of the T cells, which are attacking the target cells, will be present for longer as the cells degranulate over a longer period of time. Therefore, when the anti-CD107 specific antibody is added after 24 hours, there is more CD107 still on the surface of the T cells at the lower E:T ratio (0.2:1).

These results are very important and demonstrate the potential therapeutic possibilities of this system whereby specific cells can be targeted by peptide-antibody complexes and the resulting presentation of the peptide will render the target cell susceptible to peptide-specific CD4 T cells. Using this method, it may be possible to specifically target and kill malignant cells using different antibodies.

Taking this forward, various malignant cell lines were targeted using available antibodies in an attempt to show lysis of these cells using the peptide-antibody complex. Cell lines for Burkitt's lymphoma and breast cancer were targeted using peptide-antibody complexes but there was no response towards class-I or II peptides. These results suggest that the peptides are not presented at the surface of the cells possibly because the peptides do not gain access to the class-I or class-II processing pathways or because the peptides are discarded and therefore not presented at the surface. Previous studies have shown the internalisation of Herceptin (Mittendorf et al, 2006) and previous results have shown internalisation of BU69. This suggests that

the antigen processing is altered in different cell lines and the antigen processing is very important in this model. Although the initial attempts were unsuccessful, there is the possibility that malignant cells can be targeted once further investigation has been completed.

The hypothesis behind this work was based around the ability of an antibody, chemically conjugated with a peptide, to cross the plasma membrane into the cell where the peptide would be separated from the antibody by cellular enzymes (Figure 5.26). The peptide could then either pass from the endosome into the cytosol and gain access to the HLA class-I processing pathway, or it could be retained in the endosome and gain access to the HLA class-II processing pathway. This would require the cell to separate the peptide from the antibody and then process it, ready to be presented on the surface of the cell as part of a peptide-MHC complex.

As a consequence of previous results, another hypothesis was suggested: an antibody containing an HLA-A2 binding peptide embedded within the CDR3 region could process and present the peptide. Or would the antibody be prevented from entering the antigen processing pathway? In order to answer these questions, another model was set up with an antibody containing a viral peptide within the CDR3. The antibody was produced using lentiviral transduction and assessed for uptake and presentation by professional antigen presenting cells. Initial results suggest that complexing the antibody may result in the presentation of the CDR3-derived viral epitope at the cell surface although this remains to be confirmed. Rituximab-NLV did not appear to bind to B cells due to the mutated heavy chain CDR3, although this was difficult to fully determine at the time. Therefore, the responses seen in unfractionated PBMCs and B

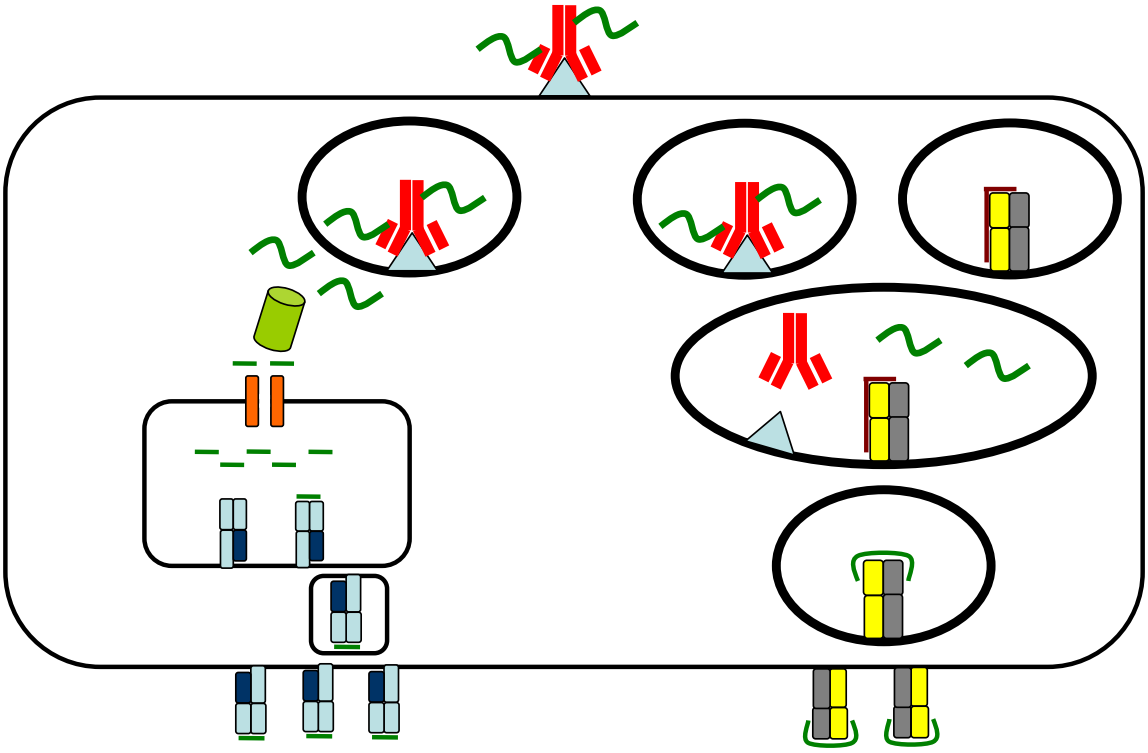


Figure 5.26 Potential antigen processing of the antibody-peptide conjugate

After internalisation, the peptide would be removed from the antibody in the endosome and passed into the cytosol. From there it would be processed by the proteasome and the peptides passed into the endoplasmic reticulum via TAP. The peptide then binds to HLA class I molecules and could then be expressed at the cell surface. The alternative would see the antibody-peptide complex broken down in the endosome. The endosome fuses with the compartment containing the MHC class II molecule folded with the invariant chain, which is removed before peptides can be loaded onto the class II molecule. After which, the peptide loaded class II molecules would be transported to the surface of the cell.

cell depleted PBMCs were assumed to be due to uptake of immune complexes via Fc receptors. The reduction in IFN-γ spots in B cell depleted PBMCs when complexed Rituximab-NLV was added may be a result of the removal of a large percentage of professional antigen presenting cells which would normally take up free antibody via their Fc receptors.

Despite the results using Rituximab-NLV, the response to Rituximab in B cell depleted PBMCs casts some doubt on the validity of the results. Rituximab binds to CD20 and therefore when there are B cells present Rituximab would bind to these cells which could then be recognised by circulating NK cells leading to release of IFN-γ by NK cells. However, using B cell depleted PBMCs, there should have been no CD20 for Rituximab to bind to and therefore there could be no NK-mediated release of IFN-γ. Even if Rituximab was taken into the cell via Fc receptors, there should not be any peptides contained in Rituximab that could elicit a T cell response. Therefore, the presence of IFN-γ spots in the B cell depleted PBMCs casts doubt on the validity of the results seen with Rituximab-NLV. However, if the results seen when Rituximab-NLV was added to PBMCs are correct, the immune complex would have been taken into the antigen presenting cell by Fc receptor uptake and the antibody processed by the cell resulting in the presentation of CDR3-derived epitopes at the cell surface. This would mean that circulating antibodies, once taken into professional antigen presenting cells, can be processed and CDR3-derived peptides presented at the surface of the cell. This would give a clear indication as to why the B cell repertoire is edited to remove B cell receptors which contain HLA-binding epitopes within their CDR3.

5.18 Conclusion

In this chapter, the hypothesis tested was that antibodies containing viral epitopes could be internalised by target cells and the epitope processed and presented at the surface in complex with MHC class-I or II. Initially, using Rituximab the results were negative but this was possibly due to the lack of internalisation of the antibody. When using BU69, which is known to be internalised, there is expression and recognition of MHC class-II peptides by peptide specific $CD4^+$ T cells but there is no expression or recognition of MHC class-I peptides. The peptide appears to enter the classical MHC class-II processing pathway before presentation at the surface of the cell. This phenomenon is not restricted to CMV-derived peptides nor is it restricted by MHC class-II allele as shown by the positive response to the EBV-derived HLA-DR52-restricted peptide also producing a response. In an attempt to find more antibodies that could lead to presentation of the conjugated peptide, only anti-CD74 produced a positive response demonstrating that it is not BU69 specific. The results shown may be very important in an immunotherapeutic setting and as a result of this, two different malignancies were targeted, breast cancer and Burkitt's lymphoma. Although initial results using both class-I and class-II peptides showed poor results, there are a lot of avenues left to explore.

6. General Discussion

There are an enormous number of potential lymphocyte receptors available to the immune system using gene segment re-arrangements, 10^{18} in T cells and 10^{16} in B cells. Despite this, the number of functional receptors present in the periphery is thought to be around $25x10^{6}$ (Arstila et al., 1999) due to the inability to pair and non-functioning of many of the estimated receptors. However, it is thought that ~98% of all lymphocytes generated are deleted during positive and negative selection in either the thymus or bone marrow (Janeway et al, 2001). Selection processes are known to be dependent on the ability of lymphocytes to recognise and react to non-self proteins only and in the case of T cells, their ability to weakly recognise MHC class-I or class-II. This study suggests that there could potentially be a 3rd selection process, dependent on CDR3 sequence, through which B cells must pass before populating the periphery. The potential process of immunoediting of B cells would be a novel selection process which has not been previously described (Figure 6.1 A-B). In this model, B cells are forced to present peptides from their CDR3 on the surface and any peptides recognised by bone-marrow resident T cells would result in the B cell being killed by the T cell, either through Fas:FasL interaction or through perforin/granzyme mediated lysis. The interaction between B and T cells at this stage of maturation may or may not be an absolute requirement for B cells to pass this stage of selection. If no interaction occurs, B cells may either die by neglect if T cell interaction is a necessity, or they could exit the bone marrow and populate the periphery if T cell interactions are not required.

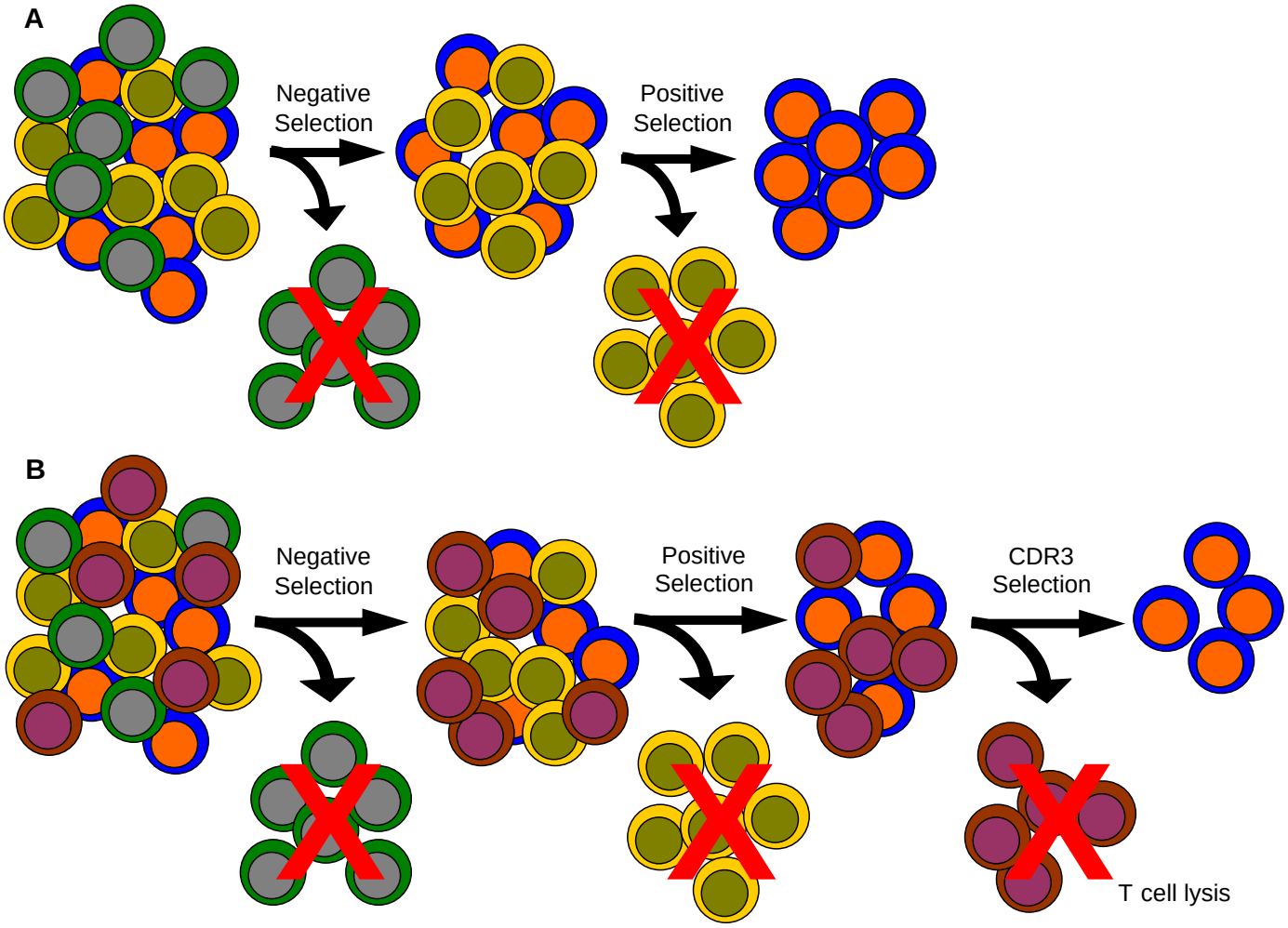


Figure 6.1 Model of B cell selection demonstrating 3rd selection step

B cells normally undergo positive and negative selection in the bone marrow to remove autoreactive B cells before they populate the periphery (A). Data previously shown suggested a 3rd selection step during B cell maturation in the bone marrow dependent on the CDR3 sequence of each B cell clone (B). B cells containing a potentially immunogenic HLA-binding peptide in the CDR3 which could be recognised by bone marrow resident T cells would be deleted in the bone marrow at this stage of maturation.

Data presented demonstrates the potential for a process of editing of the B cell repertoire in four HLA alleles suggesting that the phenomenon seen is not an artefact of HLA prediction algorithms used in the study although the results seen are not statistically significant. One of the main problems with peptide prediction analysis is that none of the algorithms available for epitope prediction are perfect and there will always be limitations associated with each. However, similar results seen with 2 algorithms across the 4 HLA alleles investigated suggest that the results seen may be valid. The results seen here were restricted to analysis of 4 different HLA types and it may be possible that editing or selection of lymphocytes occurs in an HLA-restricted manner. This may be true as the results shown for BCR and TCR were not fully consistent across all HLA types tested and this may mean that the selections observed may be restricted to certain HLA alleles or that some algorithms are more accurate than others.

There may also be a selection pressure on T cells during development based on their CDR3 sequence. However, in contrast to B cells, there appears to be immunoselection of T cells containing an HLA-binding peptide within the CDR3. This immunoselection of T cells was also shown in different HLA alleles suggesting that the results are not an artefact of the prediction algorithms.

It is attractive to propose that the observed reduction in B cell HLA-restricted CDR3-derived peptides acts as a mechanism to limit the development of auto-immune disease. However, a previously published theory of T-B interactions (João, 2007) suggests that B cells could present CDR3-derived peptides on their surface which mediate immune regulation. Presentation of these peptides would (i) shape the TCR

repertoire and (ii) 'gently' stimulate T cells and this B:T cell interaction may be required to prevent T cell death by stimulating the TCR to provide continuous survival signals. However, results shown here would appear to contradict this theory because there appears to be a lack of CDR3-derived peptides in the BCR due to editing of the B cell repertoire removing B cells bearing CDR3 with self-HLA binding peptides. In this study the cellular basis underpinning this immunoediting has not been investigated. However, it is attractive to postulate that there is interaction between B and T cells, as previously suggested, but instead of shaping the TCR repertoire in the thymus and stimulating T cells in the periphery, the interaction of B and T cells occurs in the bone marrow and shapes the BCR repertoire.

This model (Figure 6.1) may provide explanations for diseases which arise as a consequence of either a decreased number of CD8 T cells or a deficiency in CTL cytotoxicity. Firstly, patients with an unusually low number of CD8 T cells do not present with increased viral infections as may be expected but often present with bronchial problems including bronchitis, bronchiectasis and bacterial infections of the respiratory tract (Sugiyama et al, 1986; Plebani et al., 1996; de la Salle et al., 1999 & Moins-Teisserenc et al., 1999) which are classical signs of antibody deficiency. The theory postulated above may explain why a lack of T cells can lead to antibody deficiency because B cells require T cell interaction to mature fully and exit the bone marrow. This would suggest that B cells require signals from the T cell interaction to exit the bone marrow. Patients with a deficiency in CD8 T cells would therefore present with decreased numbers of B cells and consequently with bronchial problems due to antibody deficiency.

Secondly, mutations in either Fas or FasL are responsible for auto-immune lymphoproliferative syndrome (ALPS) (Rieux-Laucat et al., 1995 & Drappa et al, 1996). Patients present with hypergammaglobulinemia and increased lymphocyte counts suggesting there is a problem preventing the maturation and homeostasis of B cells. This may be explained by the inability of Fas-mediated killing of B cells which present potentially immunogenic CDR3-derived peptides by bone-marrow derived T cells. This scenario would suggest that B cells do not require T cell interactions to allow their exit from the bone marrow and it is the Fas-mediated killing deficiency in T cells which is the cause of the increased lymphocyte count and hypergammaglobulinemia.

Initial results suggested that there may be an immunoediting process in B cells of healthy donors and it would be interesting to determine if there could be a similar process in malignant B cells. There is a decrease in the frequency of CDR3-derived HLA-binding peptides in malignant B cells and therefore potentially a process of immunoediting of the B cell repertoire. If this is true, it would remove potentially tumour-specific T cell targets and may provide a reason as to why many previous results studying the T cell immune response towards the idiotype from B cell malignancies have been poor (Timmerman et al., 2002 & Levy et al, 2008). These results would suggest that targeting B cell malignancies with anti-idiotypic T cells would prove to be very difficult and would fail to elicit a response in the majority of patients.

Furthermore, it has been suggested that T cells require constant stimulation through TCR engagement to prevent apoptosis (Tanchot et al, 1997). Contradictory to the

published hypothesis (João, 2007) which suggests B cell CDR3-derived peptides provide this stimulation, the lack of T cell epitopes found within the CDR3 of the BCR suggests that the published hypothesis of shaping the TCR repertoire may not be true. A second competing theory can be suggested using the results shown here. There is an apparent stage of immunoselection of T cells bearing a TCR containing CDR3-derived peptides and these may be presented on the surface of the T cell, as shown previously. This may provide the interaction needed to (i) shape the TCR repertoire and (ii) keep T cells alive in the periphery. T cells would be selected in the thymus if they presented a CDR3-derived peptide recognised by other T cells present in the thymus and in this way, the TCR repertoire could be shaped. The presentation of the CDR3-derived peptide by memory T cells in the periphery would be recognised by CDR3-peptide specific T cells and this interaction could gently stimulate the T cells (as described in the published hypothesis) to prevent the onset of apoptosis.

Many previous studies have investigated immune responses towards B cell idiotypic peptides (Hsu et al., 1997, Berger et al., 1998 & King et al., 1998). One of these studies, Berger et al., 1998, attempted to prove the presence of an idiotypic peptide on the surface of the T cell with encouraging results. However, there have been no previous studies investigating the processing and presentation of CDR3-derived peptides in B or T cells. As part of this report, attempts were made to generate models of antigen processing and presentation of CDR3-derived epitopes in both B and T cells. As part of these models, functional TCRs and BCRs were transduced into target cells using bicistronic lentivirus vectors. Previously, similar bicistronic lentivirus vectors had been used to express TCRs in T cells (Joseph et al., 2008) with good transduction efficiency. Whilst every attempt was made to make both models as

physiologically relevant as possible, there are issues with the generation of the models. Firstly, the cells are being infected with lentivirus which may alter the repertoire of peptides expressed at the cell surface. Secondly, after synthesis of the introduced TCR inside the cell, the protein may be broken down immediately and presented at the cell surface, therefore not entering the normal pathway of a TCR protein chain. Despite these potential problems, these models may provide an interesting insight into the antigen processing and presentation capabilities of (i) T cells in presenting TCR CDR3-derived peptides and (ii) various cell types in presenting Ig CDR3-derived peptides. Results shown here are similar to previous studies which suggest that CDR3-derived peptides are presented at the surface of T cells and B cells. However, further investigation of the antigen processing and presentation of these proteins may provide useful data for future idiotype studies.

The knowledge that there is immunoediting of B cells led to the hypothesis that B cells expressing a BCR containing potentially immunogenic CDR3-derived peptides are removed to prevent the initiation of an auto-immune reaction after cellular uptake of antibody. Previously, the results suggested that CDR3-derived peptides could be processed and presented on the surface of the cells which can be recognised by peptide-specific T cells. The expression of these peptides appears to result in the production of IFN-γ by T cells suggesting that there is a T cell response towards the peptide, thus, showing that there can be a strong immunogenic response towards peptides derived from the CDR3. The above hypothesis was therefore reversed to suggest that antibodies encoding immunogenic epitopes could be used to specifically target cells for lysis by CD4 or CD8 T cells. Initial results shown here suggest that this system could be extremely useful in targeting malignant cells using antibodies

already available. Despite previous studies utilising this technique (Casten et al, 1988 & Eberl et al., 1998), there has not been any studies in the past 10 years using this approach to target malignant cells. Whether this is due to the lack of positive results seen using malignant cells or lack of useful antibodies is impossible to ascertain. Initial results using peptide-conjugated BU69 staining Ramos cells produced negative results suggesting that these malignant cells differ in their antigen processing and possibly presentation pathway compared to the positive results seen when using LCLs. This has previously been shown to happen for the antigen processing of MHC class I peptides in melanoma (Kageshita et al, 1999), renal cells (Seliger et al., 1996) and in other cell types (Cordon-Cardo et al., 1991). Although these reports show deficiencies only in MHC class I processing, the results shown here suggest that malignant B cells also alter the MHC class II antigen processing capabilities apparently preventing processing of the T cell epitope.

The results produced demonstrate that conjugation using MHC class I peptides does not result in recognition by peptide-specific CD8 T cells. This is most probably because the peptide-conjugated antibody is an extracellular antigen and will therefore naturally enter the MHC class II processing pathway. Therefore, in order to present the MHC class I peptide, the antibody complex would need to be cross-presented to allow the peptide to move from the endosome into the cytosol/endoplasmic reticulum and enter the MHC class I processing pathway. There are very few cells which have the ability to cross-present antigens and it has been shown that LCLs cannot cross-present exogenous antigen (Münz et al., 2000). However, one cell type which has shown the ability to cross-present exogenous antigen is dendritic cells (Lizée et al., 2003). Therefore, by using DCs loaded with class I peptide conjugated antibody, it

may be possible to demonstrate the presentation of class I peptides at the cell surface and ascertain if cross-presentation is required for this. If this is the case, the potential for class I peptide-conjugated antibodies in treating malignancies may be reduced. However, the use of class II peptide-conjugated antibodies in treating B cell malignancies is a very attractive option and it may be possible to treat other malignancies.

The results shown here may also provide an explanation for the onset of adverse events including fever, chills and myalgia after administration of antibody therapies such as Rituximab (Maloney et al., 1997) and intravenous immunoglobulin (IvIg) (Duhem et al, 1994). There have been previous suggestions that the adverse effects are due to complement after infusion (van der Kolk et al, 2001). However, these adverse effects only occur in a small subset of patients treated and therefore, although there is an increase in complement proteins after treatment, it may not completely solve the issue. An interesting theory regarding the adverse effects is the role of CDR3-derived peptides and the T cell responses directed towards them. Data presented possibly demonstrates the ability of cells to process and present peptides from within the CDR3 region at their surface. This may also occur when antibodies are administered to patients whereby the peptides from the CDR3 could potentially be presented at the surface of the cell. It could offer an explanation as to why the adverse effects are seen in only a small subset of patients because the potentially immunogenic peptides present could only be presented by a restricted number of HLA alleles. Therefore, the adverse effects seen may be due to HLA restriction and patients could be HLA typed before the antibody was administered to determine if they might present with complications following therapy. It may be possible to investigate this in

the future by HLA typing patients known to have presented with adverse effects and to determine if there are common HLA alleles between patients. By doing so, patients could be given alternative therapy that would prevent the onset of any adverse events.

The data shown suggests that there could be an overall scenario whereby B cells undergo editing in the bone marrow before populating the periphery (Figure 6.2 A). T cells do not appear to undergo a selection process during maturation in the thymus. If a B cell containing a CDR3-derived peptide reaches the periphery, it could become activated and produce antibodies containing the CDR3-derived peptide. Other cell types could take up this antibody and present the CDR3- derived peptide at the cell surface for recognition by T cells (Figure 6.2 B) leading to the initiation of an immune reaction against the original B cell. However, this finding can be used to target specific cells for T cell mediated lysis by conjugating a viral peptide to an antibody. This technology could be a very important immunotherapeutic tool in the future.

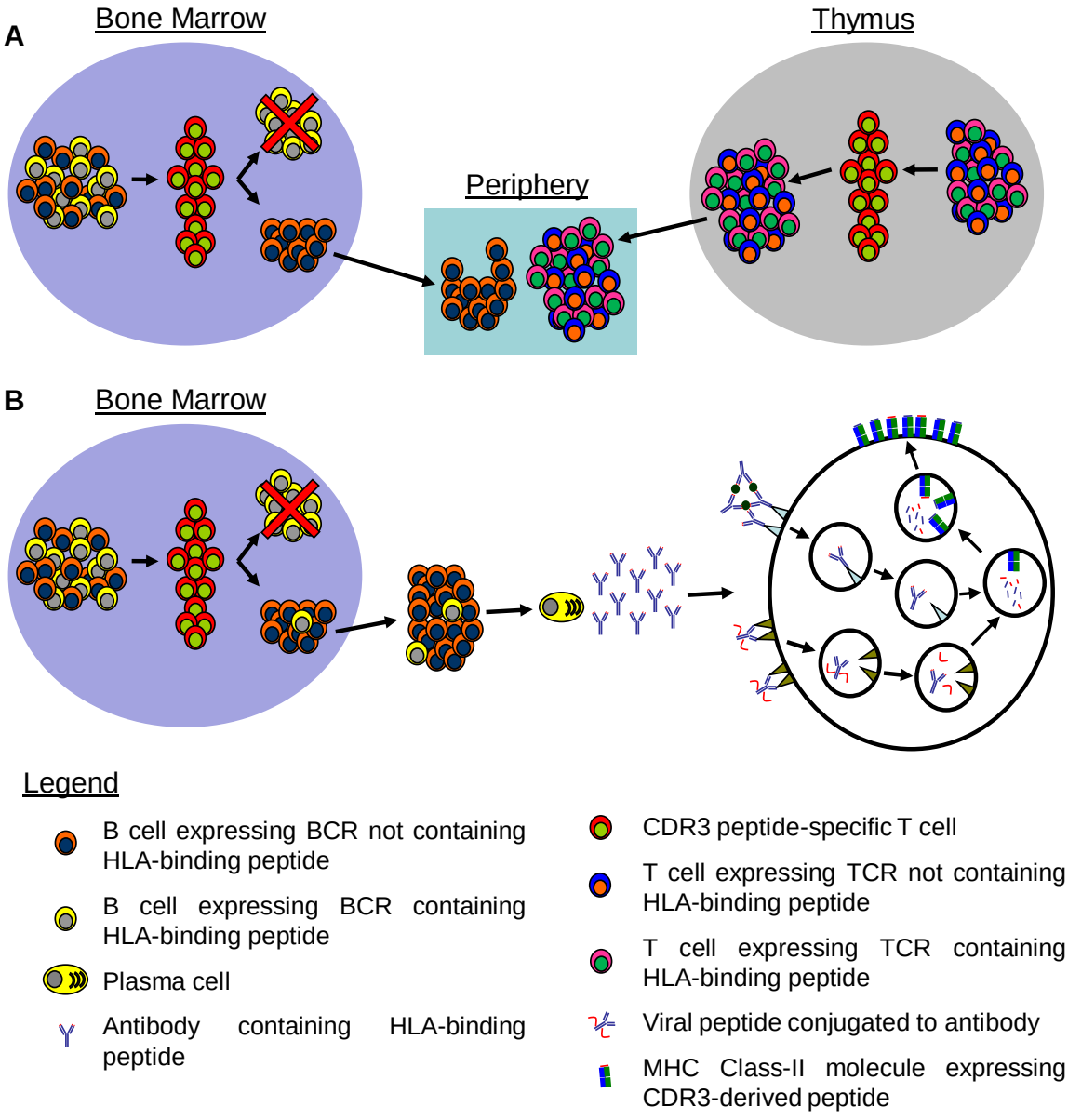


Figure 6.2 Overview of CDR3-derived sequence lymphocyte selection and CDR3 peptide antigen processing

B cells undergo immunoediting in the bone marrow and B cells without HLA-binding CDR3-derived peptides populate the periphery (A). T cells may undergo a process of enrichment of HLA-binding CDR3-derived peptides and there is no immunoediting in the T cell repertoire (A). B cells which contain an HLA-binding CDR3-derived peptide could produce antibodies which are bound by Fc receptors, internalised, processed and presented by the cell, leading to the potential initiation of an immune response (B).

7. References

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Appendix - A1

Sequences of Immunoglobulin Heavy Chain CDR3 from Healthy Donors

Ig Heavy Chain Sequence	V Region	J Region
ADDTAVYYCARQVYCGGDCFSTDSEFDLWGQGTVVTVSSASPT	3-11*01	3*01
RAEDTAVYYCARSTVDTAMAFDYWGQGTLVTVSSASTKG	3-21*01	4*02
TAADTAVYYCARNARTPDIRGRGTLVTVSSASPTSPKV	2-23*01	2*01
TAADTAVYYCARDPGGGGSGSYFDYWGQGTLVTVSSASPTSP	4-61*01	4*02
VEDTAVYFCARTQYDRSGYYYWYFDHWGRGTLVTVSSASPTSP	3-53*01	2*01
AADTAVYYCARAPRMYGDNTHREFDHWGQGTLVTVSSASTKG	3-7*01	5*02
RSDDTAVYYCARDLDLTGAYYGMDVWGQGTTVTVSSASTKGP	1-2*02	6*02
RIEDTAVYYCVISSGYYNAAGWGQGALVTVSSASTKGPSVFPL	3-30*01/07	4*02
ADTAVYYCARDREDSSGYYPEGFDYWGQGTLVTVSSASTKGPS	4-59*02	4*02
VTAADTAVYYCARVVAAAANFDYWGQGTLVTVSSGSASAPT	4-31*03	4*02
TAADTAMYFCVRQMVRDGYDFWGQGTLVTVSSASTKGPSVF	4-39*01	4*02
RAEDTAVYYCARDSESFGPYYSGMDVWGQGTSVTVSSASTKGP	3-33*01	6*02
RAEDTAVYYCEAVGRGYSFDYWGQGTLVTVSSASTKGPSVFP	3-30*01	4*02
RAEDTGVYYCARDRSSWYQYYYGMDVWGQGTTVTVSSASTK	3-21*01	6*02
AADTAVYYCVRHNDIAVAFRRGYLDYWGPGTQVTVSSASTKGP	4-39*02	4*02
ASDTAMYYCARQGGTATTLGGVWFDPWGQGTLVTVSSASTKG	5-51*01	5*02
KPEDTGVYYCTTGPMRCSGDNCVFDYWGQGTLVTVSSASPTSP	3-15*07	4*02
DTAVYYCTRDPSRCSGGSCYNYYGMDVWGQGTTVTVSSGSAS	3-49*04	6*02
TAMYKCVREPAVVPAAFRPKFSEDWYFDLWGRGTLVTVSSAST	3-30*04	2*01
DTAIYFCVRQRCEARDCYIGNNFYYMDVWGTGTTVTVSSASPTS	1-69*03	6*03
TAADTAVYYCARIKYTHGWFGFDPWGQGTLVTVSSASTKGPSV	4-59*01	5*02
TAADSATYFCARGFTYGPDLDSWGQGIQVTVSSASPTSPKVFP	4-31*03	4*02
ADTAVYYCARLIAAAGPGAGTRGRFDPWGQGTLVTVSSASTKG	4-59*01	5*02
LKTEDTAVYYCTRVGVRFVTSGWGRGTVVTVSSGSASAPTL	3-49*04	4*02
KTEDTGVYYCSTDRDFWRGWNGLDVWGQGTTVIVSSASTKGP	3-15*01	6*02
TAADTAVYYCARERDAFDIWGQGTMVTVSLGSASAPTLFP	4-59*01	3*02
TAADTAVYYCARDSGYRPFDYWGQGTLVTVSSGSASAPTLF	4-61*01	4*02
RAEDTAVYYCVRDRDGVAGVYWGQGTLVTVSSGSASAPT	3-7*01	4*02
KTEDTAVYYCTTVPYYDSGGLDYWGQGTLVTVSPGSASAPT	3-15*01	4*02
DTAMYFCARQEADKDMVEQYYFPYWGQGTLVTVSSASTKGP	5-a*03	4*02
TAVYYCARWRGYYDSSGYYDNWFDPWGQGTLVTVSSGSASA	4-59*01	5*02

TAADTAVYYCARRRVLGGLDPWGPGILVTVSSASTKGPSVFPK	4-4*07	5*02
RAEDTAVYYCARDLLVVGSRGGFDSWGQGTLVTVSSGSASAP	3-7*01	4*02
TATDTAVYYCARTLSASYAQADFWGQGTLVTVSSGSASAPTLFP	4-59*01	4*02
RAEDTAVYYCASKSDWFDLWGQGTLVTVSSGSASAPTLFP	3-23*01/04	4*02
RAEDTAVYYCARDLEDYGGILDYWGQGTLVTVSSGSASAPTL	3-21*01	4*02
TAADTAVYYCARGSGGRSTNWFDPWGQGTLVTVSSASPTSPK	4-59*01	5*02
TAADTAVYFCVRRRASHGGVWFDSWGQGNLVTVSSASPTSP	4-59*04	5*01
RAEDTAIYYCAKAVGDSGYFQPLNWWGQGTLVTVSSASTKGP	3-23*01/04	4*02
VTAADTAVYYCAISSYSSRYYYYYMDVWGKGTTVTVSSGSAS	4-59*01	6*03
TAADTAVYYCARDSSGYYYVERWGQGTLVTVSSGSASAPTL	4-61*01	4*02
QASDTALYYCGRHLYGPVSPLDDWGQGTQVTVSAASPTSPKV	5-51*01	4*02
RAEDTAVYYCARDKATMVRHIWFDPWGQGTLVTVSSGSASA	3-48*03	5*02
LRAEDTAVYYCASGAIYRAFDYWGQGTLVTVSSGSASAPTLFP	3-33*01	3*02
TAVYYCARDVELNYDSSGYLGAFDIWGQGTMVTVSSGSASAP	3-48*03	4*02
TAADTAVYYCARDDSSGYSNWFDPWGQGTLVTVSSGSASAP	4-39*01	5*02
RSDDTAVYYCARGYSSSWSLRAEYFQHWGQGTLVTVSSGSAS	1-2*02	1*01
RAEDTAIYYCARPQKAMVNWGYFDYWGQGTLVAVSSGSASA	3-7*01	4*02
DTAMYYCAICLSCDFWSGYPYYFDYWGQGSLVSVSSASTKGP	5-51*03	4*02
TAVYYCARDPGEYSSSSGVYYYYGMDVWGQGTTVTVSSGSAS	1-69*06	6*02
TAVYYCAREQTYSSGWYLAMVTNQNDLDVWGQGTTVTVSSAS	3-30*09	6*02
DTAVYYCARILLFSPFGEPPDYYYGMDVWGQGTTVTVSSGSAS	4-39*01	6*02
RDDDTAVYYCARDYGFKLDYWGKGTPVTVSSGSASAPTLFP	3-7*01	4*02
TAADTAVYYCARDSSGYYYVERWGQGTLVTVSSGSASAPTLFP	4-61*01	4*02
RAEDTAVYYCAKVGGYCTNGVCIDYWGQGTLVTVSSGSASAP	3-30*18	4*02
AEDTAVYYCAKVMVEMATIGNYFDYWGQGTLVTVSSASTKG	3-30*03	4*02
ADDTAVYFCGKDAFSYDLSSHLYHIKDWGQGTLVTVSSASTKG	3-23*01	4*02
LTVDDTGVYFCARGLSSGFYYRYGLDVWGQGTTVTVSSASPT	3-21*01	6*02
LRAEDTAIYYCARAHGSGWHTDFFDYWGQGTLVTVSSASTKG	3-23*01/04	4*02
LRAEDTAVYYCAKVRRGGDCFDYWGQGTLVSVSSGSASAP	3-23*04	4*02
LRVDDTGVYYCARGDGYNWGLFRYWGQGTPVTVSSASPTSP	3-48*03	4*02
LRAEDTAVYYCARDPGGRYINNWFDPWGQGTLVTVSSGSAS	3-33*01	5*02
LTVDDTGIYFCARGLSSGFYYRYGLDVWGQGTTVTVSSASP	3-21*02	6*02
ADTAVYYCARAPRMYGDNTHREFDHWGQGTLVTVSSASTKG	3-7*02	5*02
ISLRDDDAAVYFCARGDNNWAEGKYWGQGTRVTVSSGSASA	3-7*01	4*02
EDTAVYYCARDGAVVTPNWVAFDIWGQGTMVTVSSGSASAP	3-33*01	3*02
LRAEDTAVYYCARDYYGGLDSWGQGTLVTVSSASPTSPKVF	3-30*01	5*02

LRAEDTAVYYCAKGRGGYDEDDAFDVWGQGTMVTVSSASPTS	3-23*01	3*01
VAPADTAFYYCARVRGDIDAFDIWGQGAMVTVSSASTKGPSV	4-59*01	3*02
VTAADTAAYYCARGGYDILTGYAKVFDYWGQGTLVTVSSGSAS	4-39*01	4*02
LRVEDTAVYYCAREAVVVPGRPDYWGQGTLVTVSSASPTSP	3-7*01	4*02
LRTEDTALYYCAKRADYGTNSGAFDIWGQGTLVTVSSASTKGP	3-23*01/04	3*02
EDTAVYYCARVPCGGVGSCKFRWFDPWGQGTLVTVSSASTKGP	3-11*01	5*02
LRAEDTAVYYCAKRRIVGGTVFDYWGQGTLVTVSSASPTSPK	3-23*/0104	4*02
LRAEDTAVYYCAREYYGNYYWGQGTLVTVSPASPTSPKVFP	3-48*01	4*02
LRAEDAAIYYCARSGTDRPNLLDIWGQGTMVTVSSASTKGPS	3-74*01	3*02
LRAEDTALYYCAKRRGGNSGPFDYWGQGTLVTVSSASTKGPS	3-23*04	4*02
LRSEDMAVYYCVREFYGDLMWGQGTLVTVSSASPTSPKVF	3-30*09	5*02
DTAIYFCARDLATDFDWSLGPAAGFDIWGQGTMVTVSSTSPTSP	3-7*01	3*02
LRAEDTALYYCARVGSSWKFDYWGQGTLVTVSSASTKGPSVFP	3-23*01	4*02
SAVYYCTRGGLTSEKFSVYHGGMDVWGQGTTVTVSSASTKG	4-61*02	6*02
AVYYCARGSDYSNPQTPRDRYNYFYGMGVWGPGTTVTVSSAS	4-61*02	6*02
LRADDTGLYYCANWDLRSDYWGQGTLVTVSSASPTSPKVFP	3-11*01	4*02
LRAEDTAVYFCVSTLTVRPEAGAFHYWGHGTLVTVSSASTKG	3-21*01	4*01
LRAEDTAIYYCAKFRYDYGDYYGIDYWGQGTLVTVSSASPTSP	3-23*01	4*02
LRVEDTAVYYCAREAIVVPGRPDYWGQGTLVTVSSASTKGPS	3-7*01	4*02
LRVEDAAIYYCVRDFYGGKSDYLDYWGQGTLVTVSSASTKG	3-30-3*01	4*02
VRPDDTARYFCAKPGSGYEGAFDTWGQGALVTVSSAPTKGPS	3-23*01/04	4*02
LRAEDTAVYYCAREYYGNYYWGQGTLVTVSPASPTSPKVFP	3-48*01	4*02
LTAADTAVYFCARDVRSNYFDNWGQGIAVSVSSASPTSPKVFP	4-61*02	4*02
LKPEDTAVYYCISSGYCSDGRCYRYWGPGTLVTVASASPASPKV	3-49*03	4*02
VTAADTAVYYCARTIGSGSYIDFWGQGALVTVSSASTKGPSVFP	4-4*02	4*02
IEDTAIYYCASNWGSGSPRVHEGAFDIWGQGTKVSVSSASTKG	3-15*01	3*02
LRAEDTALYYCARVGSSWKFDYWGQGTLVTVSSASTKGPSVF	3-23*04	4*02
LRVEDTAVYYCARDGPNDSWPLDHWGQGTLVTVSSASTKGPS	3-30*14	4*02
LKAEDTAVYYCAKIRYCGADCYESFEIWGQGTMVTVSSASPT	3-23*01/04	3*02
LTVDDTALYYCAREKPAGLVTLDPWGQGTLVTVSSASPTSP	3-7*01	4*02
LKSEDTVVYYCMSYESREYFDGFDRWGQGTVVTVSSASPTS	3-15*07	3*02
VTAADTAVYFCARESGIAAAGTLDSWGQGILVTVSSASPTS	4-59*01	4*02
EDTAVYYCARDFIAARPALARISFDYWGQGTLVTVSSASTKG	3-33*03	4*02
LSTEDTAMYYCARGSRGQPCDYWGQGTLVTVSSGSASAPTL	3-72*01	4*02
VTAADTAVYYCARHLSGRDYWGQGTLVTVSSGSASAPTLFP	4-39*01	4*02

TAIYYCARHVSDLRVPTDLGDYYYAMDFWGQGTTVTVSSAS	5-a*01	6*02
LRGEDTAMYYCAKWDGYGSDWGQGTLVTVSSASPTSPKVFP	3-23*04	4*02
LKASDTAMYYCARRSSGWYGTGFDPWGQGTLVTVSSASTKG	5-a*01	5*02
AADTAVYYCARRYTSGWYQDWYFDLWGRGTLVTVPSASPT	4-59*01	2*01
VTAADTAVYFCARAYGDYENWFDPWGQGTLVTVSSASTKGP	4-30-2*01	5*02
LRASDTAMYYCARVTSYSSSFYFDYWGQGTLLTVSSGSASAP	5-a*01	4*02
RTDDAGVYYCTRGSILWFGTSGWYYDLWGRGSLVTVSSASPT	3-74*01	2*01
LKTEDTAVFYCTTDYYDSTGSYLMDYWGQGTLVTVSSASPTS	3-15*01	4*02
LRAEDTAVYYCARATREADYWGQGTLVTVSSGSASAPTLF	3-11*03	4*02
VTAADTAMYFCARPRRTLPNTEPFIFDVWGQGTMVTVSSAS	4-61*01	3*01
LRAEDTAVYYCARGGATAYYYYYYMDVWGKGTTVTVSSA	3-74*01	6*03
VTAADTAVYFCARGSGWYDYWGQGTLVTVSSASPTSPKVFP	4-59*01	4*02
LRVEDTAVYYCARDYYGALEPAACSDYWGQGTLVTVSSPST	3-7*01	4*02
LASEDTAVYYCARAQSLYGAGSFAGYWGQGSLVTVSSASPTSP	1-46*01	4*02
LRAEDTAVYYCARDLYCRTGTCNHYPDYWGQGTLVTVSSAS	3-21*01	4*02
LRGEDTAVYYCARDLNTSGDYWGQGTLVTVSSGSASAPTLFP	3-48*01	4*02
LRAEDTAVYYCATNSGWRFEYWGQGTLVTVSSASTKGPSVFP	3-7*01	4*02
LRVEDTAVYYCAKEKGLHLPFDYWGQGTLVTVSSGSASAPTLFP	3-23*01	4*02
LKASDTAMYYCARHRFEQQLGYYFDYWGQGNLVTVSSRSASA	5-51*03	4*02
VTAADTAVYYCARADTASRHWDVWGQGTTVTVSSASTKGPSV	4-4*07	4*03
LRAEDTAVYYCARQSSFDYWGQGTLVTVSSASTKGPSVFP	3-33*01	4*02
SDDTAVYYCARVGGLCGGGSCRHYFDFWGQGTLVTVFSGSA	1-2*02	4*02
LRAEDTAVYYCARGQTMIVVAPITSFDYWGQGTLVTVSSGSA	3-23*01/04	4*02
VTAADTAVYYCARDFSGSYRKTAFDVWGQGTMVTVSSGSAS	4-39*07	3*01
DTAVYYCARGGDHADPFNHRHTAPGGFDYWGQGTLVTVSSG	4-59*01	4*02
TALYYCARDARPPYCSEGICRPDAFGIWGQGTMVTVSSGSA	4-61*02	3*02
LRADDTAVYYCARWRISYHSSGFYAHLDSWGQGSLVTVSSAS	3-21*01	4*02
LRADDTALYYCARVSIPFPDWFDPWGQGTLVTVSSASTKGP	3-23*01	5*02
LRAEDTAVYYCTRDLIIGSGSNDFWGQGTLVTVSSASPTSPKVF	3-74*01	4*02
VTAADTALYYCARATSGSYSPGYFDARGRGTMVTVSSASPT	4-61*01	3*01
LRAEDTAVYYCAKDGGGVNGYYYGMDVWGQGTTVTVSSGS	3-23*04	6*02
LRAEDTAVYYCAKSVAPDFFGSEDYFDYWGQGTLVTVSSTS	3-30*03	4*02
LRGEDTAVCYCSFTLDFWGQGALVTVSSASTKGPS	3-7*01	4*02
LRVEDTAIYYCTRDGPAQSPWGQGTLVTVSSASTKGPSVF	3-53*01	5*02
LRAEDTAVYYCASVSSNGWGFDYWGQGTLVTVSSASPTSPK	3-74*02	4*02
LGAEDSAVYYCAKWPSDLLRQGSFDYWGQGALVTVSSAST	3-23*01/04	4*02

LRSDDTAVYYCAKDLGSSNAFHIWGQGTMVTVSSASPTSPKVF	1-2*02	3*02
DTAVYYCAKDYYDSSGPQSYYYYGMDVWGQGTTVTVSSGSA	3-48*02	6*02
LRAEDTAIYYCAKRRGLEVPDWSFDLWGRGTLVTVSSASTKG	3-23*01/04	2*01
VTAADTAVYYCARAMITSRAFDFWGQGTLVTVSSASPTSPKVF	4-4*07	4*02
EDTAVYYCSSTYTTMVRGVIRRAFDYWGQGTLVTVSSASTK	3-15*01	4*02
LKIEDTAVYYCTTGASDGSYYKTDSWGQGTLVTVSSASTKGP	3-15*01	4*02
VTAADTAVYYCARLMAVAGRLDVWGQGTTVTVSSGSASAP	4-59*01	3*01
LRVEDTAVYYCARGQWLVFRGQGALVTVSSGSASAPTLF	3-7*01	4*02
TAVYYCAIEYRPPPMARGGTGYSYGMDVWGQGTTVTVSSGSA	3-23*01/04	6*02
LRAEDTAVYYCTKTLVAAAGQSWGQGTLVTVTSASPTSPKVFP	3-7*01	1-1*01
LRSEDTAVFYCARLVGSSEFFAFDIWGQGTMVTVSSASTKGP	1-69*06	3*02
LRAEDTAVYYCARLFQEGDSRDRHFDLWGRGTLVTVSSAST	3-7*01	2*01
LRAEDTAVYYCVRADDGDYQTLNWGQGTLVTVSSASTKGPS	3-74*01/02	4*02
LRAEDTAVYYCAKGDLPMVRAPAGYWGQGTLVTVSSGSAS	3-23*01	4*02
VTAADTAVYYCAGVPMGGSSSGAFDIWGQGTMVIVSSGSAS	4-59*01/03	3*02
SDTAMYYCARHREGEDQIYYYYGLDVWGQGTTVTVSSASTK	5-a*03	6*02
ADTAVYYCARLHRDKRDYYYYYGMDVWGQGTTVTVSSGSA	4-39*01	6*02
VTAADTAVYYCAGRFFVRSEPGMDVWGQGTTVIVSSASPTSP	4-4*02	6*02
VTAADTAVYYCARNFYYSMDVWGQGTTVTVSSASTKGPSVFP	4-4*02	6*02
LTPDDTAMYYCVSFSGMGEGTLVTVSSGSASAPTLFP	1-2*02	5*02
VTAADTAVYYCARERLREEGLFDPWGQGILVTVSSASTKGPS	4-4*02	5*02
LRDEDAAVYFCARESAATGTRYFDYWGQGTLVTVSSGSASA	3-74*01/02	4*02
LRAEDTAVYSCARELGGDDVFDIWGQGTVVTVSSGSASAPTLF	3-33*01	3*02
LKIEDTALYYCTTDWGSGTNPKIAFDLWGQGTMVTVSSASPT	3-15*01	3*01
LKTEDTAVYYCTRRVGRARRLNWFDPWGQGTLVTVSSASTKG	3-49*04	5*02
LRVEDTAVYFCAKDLRPGSVVTQLDHWGQGTLVTVSSASTKG	3-30*03/18	4*02
LRVEDTALYYCASHKGVSYYGAFDFWGRGTAVSVSSASTKG	3-30*04	3*02
LRAEDTAVYYCVRELFDYTGPWGYWGQGTLVAVSSASPTSP	3-7*01	4*02
VTAADTAVYYCARGNPTPTMYGLYYWGQGTLVTVSSASTKG	4-4*02	4*02
DTARYFCARAGYYDFWSQFLKGMDVWGQGTTVTVSSASTKG	4-b*01	6*02
LRLEDTALYYCVKSSRQQGLAYYDYWGRGTLVTVSSASTKG	3-9*01	4*02
LRAEDTAVYYCAKLVYSGSYHFDYWGQGTLVTVSSASTKG	3-23*01/04	4*02
LTAEDTAVYYCAREWRAFDIWGQGTRVTVSSASPTSPKVFP	3-21*01	3*02
VTAADTAVYYCTRGSGYGADAGTFDPWGQGTLVTVSSGSASA	4-61*01	5*02
VTAADTAVYYCAREYYSGGWLRTDYWGQGILVTVSSASTKG	4-59*01	4*02

DTAMYYCARHEGERGLWFGELPYYFDYWGQGTLVTVSSGSAS	5-51*01	5*02
LKTEDTAVYYCNTWVAVGGLMFDYWGQGTLVTVSSASTKG	3-15*01	4*02
VTAADTALYYCARIIDGYIMVPFDYWGQGILVTVSSASPTSPK	4-61*01	4*02
VTAADTAVYYCASRDGHSIDYWGQGVLVTVSSASPTSPKVFP	4-4*06	4*02
LRAEDTAVYYCVRSGYGPRYYFDCWGQGTLVTVSSAPTKG	3-7*01	4*02
YYCARDMVVFGGINVDTPMITLLPGWFDPWGQGARVTVSSAST	4-59*01	5*02
LRLEDTALYYCVKSSRQQGLAYYDYWGRGTLVTVSSASTKG	3-9*01	4*02
VTPEDTAVYYCARDNYGMDVWGQGTSVTVSSGSASAPTLF	6-1*01	6*02
ATAADTAVYYCARDRGGVGASDAFDIWGQGTMVTVSSASPTS	4-59*01	3*02
LRAEDTAVYYCARDRNDIWSGYLLGWFDLWGRGTLVTVSSASP	3-7*01	2*01
LRAEDTAVYYCARDRGEDNPIDYWGQGTLVTVSSASPTSPKVF	3-33*01	4*02
VTAADTAVYYCVRDCGWLVSGDAFDTWGQGTMVTVSSASTK	4-b*02	3*02
LRAEDTAVYYCVRGGVLGATDYWGQGTLVTVSSASTKGPSVFP	3-74*01/02	4*02
AEDTAIYYCAREVGYVSPSSWAIDAFDVWGQGTMVTVSSASTK	3-48*03	3*01
LRSEDTAVYYCARGRRDGYNPFDYWGQGTLVTVSSGSASAP	1-69*01	4*02
LTAEDTATYYCARLSVTYGWEFLDYWGRGTLVTVSSASPTSP	3-48*03	4*02
LRAEDTALYYCAVDVSSSPTLWGQGTLVTVSSGSASAPTLFP	3-23*04	4*02
VTAADTAVYYCARLPPPGSGSYFDPWGQGVLVTVSSGSASAP	4-59*03	5*02
ADTAVYYCARAEYCTGGVCYGPRHLDNWGQGTLVTVSSASPTS	4-61*01	4*02
VTAADTAVYYCATYYIGLTGLHWYFDVWGLGSLVTVSSPSPTS	4-61*01/08	2*01
ADTAVYYCARRKYYFDSSGLILDTFDIWGQGTMVTVSSGSASA	4-b*01	3*02
LRAEDTAVYYCASVRLALSSTALDYWGQGTLVTVSSASTKG	3-48*03	4*02
LQASDSAMYFCARSPIPWGNLWSGAQLDYWGQGTLVTVSSASP	5-51*01	4*02
VTAADTAVYYCARGKPTTVFDYWGQGTLITVSSASTKGPSVFP	4-4*02	4*02
VTAADTAVYYCGTDSTGWLGYGIDVWGPGTTVTVSSASPTSP	4-4*02	6*02
VTAADTAIYMCARVIRTGEGGRGYFDLWGRGTLVTVSSASPTS	4-4*02 or 4-61*03	2*01
VRPDDTARYFCAKPGSGYEGAFDTWGQGALVTVSSAPTKGPSV	3-23*04	4*02
LRAEDTAIYFCARVFYDGRGSHHYYFDFWGQGTLVTVSSASTK	3-11*03	4*02
VTPADTAVYYCARDDFWSGTHWFDPWGQGTLVTVSSASPTSPK	4-61*08	5*02
LRAEDTAVYYCAKNYDFWSTYYFDYWGQGTLVTVSSGSASAP	3-23*01/04	4*02
ADTAVYYCARGLYYYGSGSLGYCYDMDVWGQGTTVTVSSGSA	4-59*01	6*02
LTVADTAVYYCVRVRKERGGSLDDWGQGTTVTVSSASTKGPS	4-4*02	4*03
VTAADTAVYYCARGPGRGYADYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
EDTAVYYCAREGDPSGSGTYLDAYDIWGQGTMVTVSSGSASAP	3-48*03	3*02

VTAADTAIYYCVQGDSLVAFDVWGQGTLVTVSSASPTSPKVFP	4-4*07	3*01
TGVYFCAREGIYSYSSGSIWGSRTYNGMDAWGKGTTVIVAPAST	3-33*01	6*04
LTAEDTAMYYCARRGANRDFAFDVWGPGTMVTVSSASPTSPK	3-11*03	3*01
LKASDTAMYYCARHKSGVTTVTTLDYWGQGTLVTVSSGSASA	5-a*01/03	4*02
LRPEDTATYFCAKGDFGSSQYYFDYLGQGTLVTVSSASPTSPK	3-23*04	4*02
VTAADTATYYCMRSGYSGGGHWGQGKLVTVSSASTKGPSVFP	4-31*06	5*02
LTAEDTALYYCAKDLVGEHTYWGQGTLVTVSSASPTSPKVFP	3-23*01/04	4*02
VTAADTAVYYCARADGATVTYWYFDLWGRGTLVTVSSGSASA	4-59*01	2*01
VTAADTAVYYCARGSNSWSFDYWGQGTLVTVSSGSASAPTLFP	4-59*01	4*02
VTAADTAVYYCARLRGNYFPDVWGKGTTVTVSSGSASAPTLFP	4-59*02 or 4-61*08	6*03
VTAADTAVYYCARHGSHWTFDSWGQGTLVTVSSGSASAPTLFP	4-59*01	4*02
LRAEDTAVYYCASGYSAYALGYWGQGTLVTVSSGSASAPTLFP	3-74*02	4*02
VTAADTAVYYCARYGSGSYHFDYWGQGILVTVSSASPTSPKVFP	4-59*01	4*02
LRAEDTAVYYCARTSNFGDYPIDYWGQGTQVTVSSASPTSPKVF	3-21*02	4*02
VTAADSATYFCARGFTYGPDLDSWGQGIQVTVSSASPTSPKVFP	4-31*03	4*02
VTAADTAVYYCARDLGYSYSYYFDSWGQGTLVTVSSGSASAP	4-61*01	4*02
VTAADTAMYFCARDHTYSGRGGMDVWGPGTTVAVSSASPTSP	4-31*06	6*02
AADTAVYYCARQTYYYDSSGNYYLDYWGQGTLVTVSSGSAS	4-59*01	4*02
VTAADTAVYYCAREKWELHTRAFDIWGQGTMVTVSSGSASAPT	4-59*01	3*02
VTAADTATYYCMRSGYSGGGHWGQGKLVTVSSASTKGPSVFP	4-31*06	5*02
VTAADSATYFCARGFTYGPDLDSWGQGTQVTVSSASPTSPKVFP	4-31*03	4*02

Sequences from data set were compared with known Ig sequences to determine variable region and joining region usage (Imgt.cines.fr - version 3.2.16 (Accessed 24 September 2010) (Brochet, X et al, 2008)).

Appendix – A2

Table of Immunoglobulin CDR3-Derived Peptides Predicted to bind to HLA Alleles from SYFPEITHI and BIMAS

HLA-A1^{+}	HLA-A1^{-}	HLA-A2^{+}	HLA-A2^{-}
YYDSGGLDY	SYDLSSHLY	SLRDDDAAV	GLDPWGPGI
LRDEDTAVY	LRDDDAAVY	YQYYYGMDV	DILTGYAKV
LRDEDAAVY	PTDLGDYYY	DLWGRGTLV	TLNWGQGTL
RTEDTALYY	RSDDTAVYY	AMYYCAICL	HLYGPVSPL
KTEDTAVYY	DSESFGPYY	QTYSSGWYL	DLMWGQGTL
KTEDTGVYY	SGDNCVFDY	KVMVEMATI	LMWGQGTLV
RSEDMAVYY	KTEDTAVYY	DLWGRGSLV	RIIDGYIMV
RADDTGLYY	RSEDTAVYY	TLPNTEPFI	ALYYCAVDV
CSDGRCYRY	TVDDTALYY	SLYGAGSFA	TLWGQGTLV
RSEDTAVFY	KSEDTVVYY	GIWGQGTMV	GLILDTFDI
RLEDTALYY	STEDTAMYY	ALYYCAKDL	RLALSSTAL
TSEDTAIYF	RTDDAGVYY	TVTYWYFDL	YIGNNFYYM
RAEDTAVYY	KTEDTAVFY		DLWGQGTLV
MVEQYYFPY	ASEDTAVYY		AIYYCAKAV
RAEDTAIYY	TSDDTAVYY		LATDFDWSL
RVEDTAVYY	RADDTALYY		GLTSEKFSV
RPEDTAVYY	RAEDTAVYY		GVWGPGTTV
RAEDAAIYY	GADDTAVYY		DLWGQGTMV
RAEDTALYY	DLDLTGAYY		RVHEGAFDI
RAEDTAMYY	RIEDTAVYY		WLVFRGQGA
YGDYYGIDY	YCEAVGRGY		YCARGQWLV
RVEDAAIYY	RAEDTGVYY		LVGSSEFFA
KPEDTAVYY	KPEDTGVYY		GLYYWGQGT
TIEDTAIYY	RAEDTAIYY		SQFLKGMDV
RAEDTAIYY	FGEPPDYYY		AVYYCNTWV
KIEDTAVYY	RVDDTGVYY		YVSPSSWAI
EGEDQIYYY	TVDDTGIYF		TLWGQGTLV
TPDDTAMYY	RPDDTAIYY		YMCARVIRT
KIEDTALYY	RVEDTAVYY		

RVEDTALYY	KAEDTAVYY		
TAEDTAVYY	RGEDTAMYY		
RLEDTALYY	NTEPFIFDV		
TPEDTAVYY	RGEDTAVYY		
	HTAPGGFDY		
	RADDTAVYY		
	RGEDTAVCY		
	RVEDTAIYY		
	GAEDSAVYY		
	TAEDTATYY		
	RAEDTALYY		
	GVEDTAVYY		
	TAEDTAMYY		
	TAEDTALYY		
	RADGATVTY		

HLA-B7^{+}	HLA-B7^{-}	HLA-B8^{+}	HLA-B8^{-}
APRMYGDNT	DPSGSGTYL	RERLREEGL	CAKVRRGGD
AVYYCARDL	RPGSVVTQL	CARLRGNYF	CAKGRGGYD
AAGWGQGAL	GVYWGQGTL	CAREKWELH	CAKRRIVGG
AVAFRRGYL	VPYYDSGGL	ELHTRAFDI	CAKRRGGNS
AVYYCARIL	FPYWGQGTL	CAKEKGLHL	CAKFRYDYG
EPPDYYYGM	AVYYCARDL	CAKWPSDLL	SPRVHEGAF
CARDYGFKL	AVYYCARTL	CAKRRGLEV	CAKIRYCGA
GVYFCARGL	CAREKPAGL	DSRDRHFDL	CAREKPAGL
CARDYYGGL	AARPALARI	CARDKATMV	TPRDRYNYF
CARGGYDIL	AVYYCARHL	DARGRGTMV	VRKERGGSL
CVREFYGDL	RPRRTLPNT	CARDRNDIW	DIRGRGTLV
AVYFCVSTL	CARDYYGAL	CARDRGEDN	CARDREDSS
EVPDWSFDL	AVYYCTRDL	CARHKSGVT	CARDRSSWY
LVFRGQGAL	AVCYCSFTL		CARDYGFKL
AVYYCTKTL	CAKWPSDLL		CARDYYGGL

DSRDRHFDL	AVYYCAKDL		CARGGYDIL
CARNFYYSM	AVYYCVREL		CARDYYGAL
AVYSCAREL	NPTPTMYGL		CARGKPTTV
SVTYGWEFL	TVTYWYFDL		
GVCYGPRHL			
AVYYCARGL			

Key

Peptides predicted to bind to: SYFPEITHI
Both Algorithms
BIMAS

Appendix – A3

Sequences of immunoglobulin heavy chain CDR3 from CD27$^-$ B cell receptors from one healthy donor

Sequence	V Region	J Region
RVEDTAVYYCAKGGWLENWGQGTLVTVSSGSASAPTLF	3-23*01/04	4*02
AEDTAVYYCAKDSPGGSSSWYVGYYYGMDVWGQGTTVTVSSGSAS	3-23*01/04	6*02
SSEDTAVYYCAREDPDSYYDDYWGQGTLVTVSSWSASAPTLF	1-46*01/03	4*02
TAADTAVYYCARERDNDGMDVWGQGTTVTVSSGSASAPTLF	4-59*01	6*02
RSDDTAVYYCARTFGHRHDAFDIWGQGTMVTVSSGSASAPTLF	1-18*01	3*02
TAADTAVYYCARVERTITVVKFDYWGQGTLVTVSSGSASAPTLF	4-31*03	4*02
TAADTAVYYCARDSSELWPTGWFDPWGQGTLVTVSSGSASAPTLF	4-59*01	5*02
RAEDTAVYYCAKVSLRAPRSWVWFDPWGQGTLVTVSSGSASAPTLF	3-30*03/18	5*02
EDTAVYYCARDNVVEYQLPQSLDYYYGMDVWGQGTTVTVSSGSAS	1-69*01	6*02
TAADTAVYYCARRGQWLVKGWFDPWGQGTLVTVSSGSASAPTLF	4-4*02	5*02
PEDTAVYYCARYIAVAEFDYWGQGTLVTVSSGSASAPSLF	6-1*01	6*02
TAADTAVYYCARDSGSGYSSGWYDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARHFKQQRPNWFDPWGQGTLVTVSSGSASAPTLF	4-39*01	5*02
TATDTAIYYCSRHGSWLQPQNGFDVWGQGTLVTVSSGSASAPTLF	4-39*01	3*01
TAADTAVYYCARARGYEPFDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
RAEDTAVYYCAKFGLAGITVAGNDYWGQGTLVTVSSGSASAPTLF	3-30*03/18	4*02
TAADTAVYYCARDRYGDYGAVNWFDPWGQGTLVTVSSGSASAPTL	4-61*02	6*02
TAADTAVYYCARVNVRGWKIYYGMDVWGQGTTVTVSSGSASAPTL	4-59*01	6*02
TAADTAVYFCARISGYDIDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCATRRIAAAGIVYWGQGTLVTVSSGSASAPTLF	4-31*03	4*02
TATDTAMYYCARHGSWLQAQIGFDIWGQGTMVSVSSASPTSPTLF	4-39*01	3*02
TAADTAVYYCARARGGYYDSGEFDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARTGRAYSSGWIDYWGQGTLVTVSSGSASAPTLF	4-31*03	4*02
TAADTAVYYCARRLGGSFSTLYYFDYWGQGTLVTVSSGSASAPTLF	4-39*01	4*02
TAADTAVYYCARGLAATPVDFDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARGRVPAVVGQGTLVTVSSGSASAPTLF	4-59*01	5*02
TAADTAVYYCARREGTDAFDIWGQGTMVTVSSGSASAPTLF	4-39*01	3*02
TAADTAVYYCARVSDMTFDPWGQGTLVTVSSGSASAPTLF	4-4*02	5*02
TAADTAVYYCAREGGAATLSFDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARDPAAAGRFAPSGFDYWGQGTLVTVSSGSASAPTL	4-61*01	4*02

TAADTAVYYCARDRGGNLLDMHYWGQGTLVTVSSGSASAPTLF	4-61*01	4*02
TAADTAVYYCARAPQLWLIDPWGQGTLVTVSSGSASAPTLF	4-59*01	5*02
DTAVYYCARGGVGYCSGGSCQTDYYYGMDVWGQGTTVTVSSGSAS	4-59*01	6*02
TAADTAVYYCARVGLERQYYYYYGMDVWGQGTTVTVSSGSASAPT	4-59*01	6*02
TAADTAVYYCARGGAYNWNDVNFDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARYYVWGSYHDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARDRRVEMATIIYYYGMDVWGQGTTVTVSSGSASAP	4-61*01	6*02
TAADTAVYYCARASIAAAGVSDIWGQGTMVTVSSGSASAPTLF	4-59*01	3*02
TAADTAVYYCARDGARYYYGMDVWGQGTTVTVSSGSASAPTLF	4-59*01	6*02
TAADTAVYYCARAKRGGAARLVWFDPWGQGTLVTVSSGSASAPTLF	4-59*01	5*02
TAADTAVYYCARGGSGWYYFDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARVKLLMDYSNYGHWFDPWGQGTLVTVSSGSASAP	4-59*01	5*02
TAADTAVYYCARVSFGSSLYYFDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
RAEDTAVYYCARGGGGRCLRMDVWGQGTTVTVSSGSASAPTLF	3-11*01	6*02
TAADTAVYYCAREVRYGSGSFSMDVWGQGTTVTVSSGSASAPTLF	4-59*01	6*02
TAADTAVYYCARVGASSGADYWGQGTLVTVSSGSASAPTLF	4-4*02	4*02
TAADTAVYYCARGMGSSSPIDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARGPRFDPWGQGTLVTVSSGSASAPTLF	4-59*08	5*02
TAADTAVYYCARTITIFGVPPPGWFDPWGQGTLVTVSSGSASAPTLF	4-59*01	5*02
RAEDTAVYYCARDVGAKLFDYWGQGTLVTVSSGSASAPTLF	3-74*01/02	4*02
RAEDTAVYYCAKDQGELHDAFDIWGQGTMVTVSSGSASAPTLF	3-23*01/04	3*02
RAEDTAVYYCARGKSYYDSIGVGTLVTVSSGSASAPTLF	3-11*01	4*02
TAADTAVYYCARDRDIFTWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TPEDTAVYYCARDRFGELLRPYYYYGMDVWGQGTTVTVSSGSASAP	6-1*01	6*02
RAEDTAVYYCASSYDYVWGSYRSFAPEDYWGQGTLVTVSSGSASAP	3-74*01/02	4*02
TAADTAVYYCARVGSSWYSRWFDPWGQGTLVTVSSGSASAPTLF	4-59*01	5*02
TAADTAVYYCAAGGSYGVGPANWFDPWGQGTLVTVSSGSASAPTLF	4-30-4*01	5*02
TAADTAVYYCARQVITARHSDYWGQGTLVTVSSGSASAPTLF	4-39*01	4*02
RAEDTAVYYCARVSRFLEWLPSGSIDYWGQGTLVTVSSGSASAPTLF	3-74*01	4*02
KTEDTAVYYCTRVRDIAVAGPVDYWGQGTLVTVSSGSASAPTLF	3-49*04	4*02
TAADTAVYYCARGDLTLDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCAREKVYYGMDVWGQGTTVTVSSGSASAPTLF	4-59*01	6*02
RSEDTAVYYCARAPRNIVVVIAMPSGAFDIWGQGTMVTVSSGSASAP	1-8*01	3*02
TAADTAVYYCVRAMLGVPAFDYWGQGTLVTVSSGSASAPTLF	4-4*02	4*02
TAADTAVYYCARALPIDYYGSGSYIDYWGQGTLVTVSSGSASAPTLF	4-30-4*01	4*02

RSEDTAVYYCARDLWFLENLYYFDYWGQGTLVTVSSGSASAPTLF	1-46*01/03	4*02
TAADTAVYYCARSGYFPGGFDYWGQGTLVTVSSGSASAPTLF	4-4*02	4*02
TAADTAVYYCARHAPDLVVVVDNWFDPWGQGTLVTVSSGSASAPTL	4-39*01	5*02
TAADTAVYYCARNRAVAGTKGFDYWGQGTLVTVSSGSASAPTLF	4-4*02	4*02
RSEDTAVYYCARAPLGAGYFVDYWGQGTLVTVSSGSASAPTLF	1-69*01	4*02
TAADTAVYYCARGSKWELLLDWGQGTLVTVSSGSASAPTLF	4-39*01	4*02
TAADTAVYYCARDMFFGVAPDAFDIWGQGTMVTVSSGSASAPTLF	4-59*01	3*02
TAADTAVYYCARAEAAGMVHNDAFDIWGQGTMVSVSSGSASAPTLF	4-61*02	3*02
TAADTAVYYCARAPIRPHPFDLWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARDSGSGYSSGWYDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARANYILEWLIDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
ADTAVYYCARYHYYYDSSGYYPETNWFDPWGQGTLVTVSSGSASA	4-31*03	5*02
TAADTAVYYCARDIERFLRPWGQGTLVTVSSGSASAPTLF	4-4*02	5*02
TAADTAVYYCARARGYEPFDYWGQGTLVTVSSGSASAPTLF	4-59*01	4*02
TAADTAVYYCARERIYKNAGGLLSDWGQGTLVTVSSGSASAPTLF	4-59*01	1*01
TAADTAVYYCARAGATDIAVAGYYFDYWGQGTLVTVSSGSASAPTL	4-4*02	4*02
TAADTAMYYCARVGSYGYHDWGQGALVTVSSGSASAPTLF	4-59*01	4*02
LRAEDTAVYYCAKVMVEMATIGNYFDYWGQGTLVTVSSASAPTLF	4-59*01	6*02

Sequences from data set were compared with known Ig sequences to determine variable region and joining region usage (Imgt.cines.fr - version 3.2.16 (Accessed 24 September 2010) (Brochet, X et al, 2008)).

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Appendix – A4

Sequences of immunoglobulin heavy chain CDR3 from CD27+ B cells from healthy donors

Sequence	V Region	J Region	IgA/G
RPEDTAVYFCAKAPGGNFGDYSEALDVWGQGTTVIVSSASTKGPS	3-30*03/18	3*01	IgG
TAADTALHYCARDDISDPSFDLWGRGTLVTVSSASTKGPS	4-39*01	2*01	IgG
TAADTAVYFCARGPRAYCRGGNCLVPLRWGQGTLVTVSSASPTS	4-31*01	4*02	IgA
SDTAMYYCVRHRRIELSNGDNLFHGMDLWGQGTTLTVSAASPTS	5-a*01	6*02	IgA
AADTAVYYCTRTPRHGYCSSTSCYGEFDYWGQGTPVTVSSASTKG	4-61*01	4*02	IgG
RAEDTAVYYCARDGWIATSKYRLDSWGQGTLVTVSSASTKGPSV	3-30*15	4*02	IgG
KASDTAMYYCARYHNTWYGDSWGQGTLVTVSSASTKGPSVF	5-51*01	4*02	IgG
TAADTAVYYCARGRDYGEFDSWGQGTLVTVSSASTKGPSV	4-31*01	4*02	IgG
TAADTAVYYCARAALMVRGHAFEIWGQGTLVAVSSASPTSPSV	4-4*07	3*02	IgA
RAEDTAVYYCARDLSWQLFDYWGQGALVTVSSASTKGPSV	3-74*01/02	4*02	IgG
TDEDTAVYFCARGHPGYTFAYWGQGTPVTVSSASTKGPS	4-59*02	4*02	IgG
TAADTAIYYCARAIVAIGVVGDWFDPWGRGTLLIVSSASTKGPS	4-61*01/03	5*02	IgG
TAEDTAVYSCARGRRVGALSYFDSWGQGSLVTVSSASTKGPS	3-23*01/04	4*02	IgG
AADTAVYYCARDPRAYYYDSSASSGFDSWGQGTLVTVSSASTKG	4-59*01	5*01/02	IgG
TAADTAVYYCARGLGYCIDGVCHLFDYWGQGALVTVSSASPTSP	4-59*01	4*02	IgA
RTEDTAVYYCARDYGGNLDLWGQGIVVTVSSASTKGPK	3-30*01	4*02	IgG
TAADTAVYYCARRSDIWHAFDPWGPGTMVTVSSASTKGPS	4-39*01	3*01/02	IgG
TAADTAVYYCAGSGNYYYLQYWAQGSQVTVSSASPTSPS	4-59*01 or 4-61*01	1*01	IgA
TAADTAVYYCARDHSLGWWYVWGQGTLVTVSSASTKGPS	4-61*01/03	5*02	IgG
RAEDTAVYYCAKLGEHSSLGLDVWGQGTTVTVSSASTKGPS	3-74*01/02	6*02	IgG
RAEDTAIYYCAKGSHSSWSFFDFWGQGTLVTVASTSPTSPS	3-23*04	4*02	IgA
TAADTAVYYCARGQWETWFDLWGQGTLVTVSSASTKGPS	4-b*01	5*02	IgG
TAADTAFYYCARIHCGVGICYWDWGQGTLVTVSSASTKGPS	4-b*02	5*02	IgG
TAADTAVYYCARGDLRGGSFYGGYDHWGQGILVTVSSASTKGP	4-30-4*03	5*02	IgG
TTADTAVYFCARGQLMGPWGRGTLVTVSSASTKGPS	4-59*01	4*02	IgG
GAEDTADYYCARGKLNDYGLDVWGQGTTVTVSSASTKGPS	3-74*01/02	6*02	IgG
TAADTAVYYCARRSDIWHAFDPWGPGTMVTVSSASTKGPS	4-39*01	3*01/02	IgG
AEDTAVYYCARCDCSGSCYLRAHCGMDVWGQGTTVIVSSASPTS	3-21*01	6*02	IgA
TAADTAVYYCARDYGGYWGQGTLVTVSSASTKGPS	4-59*01	4*02	IgG

RAEDTAVYYCIMMTVGWGHGTLVTVSSASTKGPS	3-74*01	5*01/02	IgG
AADTAVYYCARFRVGATATLGDYYFDSWGQGTLVTVSSASPTSP	4-61*01/08	4*02	IgA
NAADTAVYFCARGPRAYCRGGNCLVPLRWGQGTLVTVSSASTKG	4-31*03	4*02	IgG
TAADTAVYFCARGPRLMVYARFDNWGPGTLVTVSSASTKGPS	4-b*02	4*02/03	IgG
ADTAIYYCARDFRQLDTRYKHHYYGMDAWGQGITVTVSSASPTS	4-4*07	6*02	IgA
RAEDTAVYYCARVPLGQWLVGADWGQGTLVTVSLASTKGPS	3-74*01/03	4*02	IgG
RAEDTAVYFCARGAYGKFYFDYWGQGTLVTVSSASTKGPS	3-74*01/02	4*02	IgG
TALYYCVKDRGVGGVLRYYDWLHSFDCWGQGTLVTVSSASTKG	3-9*01	4*02	IgG
RAEDTAVYYCAKKYDNRSPSFDYWGQGSLVTVSSASTKGPS	3-23*04	4*02	IgG
KTEDTAVYYCARAPCSNDICHLLNWGQGTLVTVSSASTKGPS	3-72*01	4*02	IgG
TTADTAVYFCARGQLMGPWGRGTLVTVSSASTKGPS	4-59*01	4*02	IgG
RAEDTAVYYCARDERGYTTSCIDYWGQGTLVTVSSASTKGPS	3-21*01	4*02	IgG
RAEDTAVYYCARGLISGSQSAGGMDVWGQGTTVTVSSASPTSPS	3-48*03	6*02	IgA
RPEDTAVYFCAKAPGGNFGDYSEALDVWGQGTTVIVSSASTKGPS	3-30*03/18	3*01	IgG
TSEDTAVYYCARGRDGYIRGLFDYWGQGILVTVSSASTKGPS	1-69*01	4*02	IgG
ADTAVYFCARGGASRESSGWNQVHGLDVWGQGTSVIVSSASPTS	4-4*07	6*02	IgA
TAADTAVYYCARDRATNSRYFYGLDVWGQGTTVIVSSASTKGPS	4-61*01	6*02	IgG
TAADTAVYFCARGPRAYCRGGNCLVPLRWGQGTLVTVSSASTKG	4-31*04	4*02	IgG
RAEDTAVYYCAKDQGSGWGGDAFDIWGQGTMVTVSSASTKGPS	3-30*03/18	3*02	IgG
DTAVYYCGRETEPSSDYFSAQPGWPYFDHWGQGALVTVSSASTK	3-33*05	4*02	IgG
RAEDTAVYYCARDLSWQLFDYWGQGALVTVSSASTKGPS	3-74*02	4*02	IgG
TVADTAVYFCARGKEWLPDNWFDPWGQGILVTVSSASTKGPS	4-4*07	5*02	IgG
RSEDTAVYYCARASMLLDWGQGTLVAVSSASTKGPS	1-8*01	4*02	IgG
TAADTAVYYCARGDLRGGSFYGGYDHWGQGILVTVSSASTKGPS	4-30-4*03	5*02	IgG
TAADTAVYYCAVTTTSTGASEIWGPGTMVTVSSASPTSPS	4-4*07	3*02	IgA
RAEDTAVYFCARGAYGKFYLDYWGQGTLVTVSSASTKGPS	3-74*01/03	4*02	IgG
ADTAVYYCAKIAIFQHVNLPYYYYAMDDWGQGTKVTVSSASTKG	4-4*07	6*02	IgG
TAADTAVYYCARGRDYGEFDSWGQGTLVTVSSASTKGPS	4-31*01	4*02	IgG
TAADTAVYYCARDRRPGGSPHYDYWGQGTLVTVSSASTKGPS	4-61*01	4*02/03	IgG
TAEDTAVYSCARGRRVGALSYFDSWGQGSLVTVSSASPTSPS	3-23*04	4*02	IgA
TAADTAVYYCARRSDIWHAFDPWGPGTMVTVSSASPTSPS	4-39*01	3*01/02	IgA
TAVYYCANNYYDFRRGYPDPTYYGMDVWGQGTTVTVSSASTKG	1-69*01	6*02	IgG
RAEDTAVYFCAKARTTVTTWDYWGQGTLVTVSSASTKGPS	3-23*04	4*02	IgG
TAADTAVYFCARAESAGYYYYDFWGQGTLVTVSSASTKGPS	4-4*07	4*02	IgG
AVYYCARDPKQSVDGSDFYSYHYGMDVWGQGTTVTVASASPTS	3-48*01/02	6*02	IgA

EDTAVYYCALQPTHSSMGTRTISGRITDYWGQGTLVTVSSASTKG	3-7*01	4*02	IgG
RAEDTAVYYCARDLSWQLFDYWGQGALVTVSSASTKGPS	3-74*01/03	4*02	IgG
TTEDTAVYYCTTITGWDRTKFDTWGRGTLVTVSSASTKGPS	3-15*01	4*02	IgG
TVADTAVYFCARGKEWLPDNWFDPWGQGILVTVSSASPTSPS	4-4*07	5*02	IgA
AVYYCARGLRTRGPAIFGVVMPLRLHFGLDVWGQGTTVTVSSAST	1-8*01	6*02	IgG
GAEDTAVYYCVKCHVVAAGWCNFFDPWGQGTLVTVSSASPTSPS	3-23*01/04	5*02	IgA
RAEDTAVYYCARAFQGVILDYWGQGTLVTVSSASTKGPS	3-74*03	4*02	IgG
TAADTAIYYCARGKSVVGATDDYFDYWGQGALVTVTSASTKGPS	4-61*08	4*02	IgG
RSEDTAVYYCARYSGTYYPSYFDQWGQGTLVTVSSASTKGPS	1-69*01	4*02	IgG
TAADTAVYYCARRSDIWHAFDPWGPGTMVTVSSASPTSPS	4-39*01	3*01/02	IgA
TAVYYCANNYYDFRRGYPDPTYYGMDVWGQGTTVTVSSASTKG	1-69*01	6*02	IgG
TAADTAVYYCAVTTTSTGASEIWGPGTMVTVSSASPTSPS	4-4*07	3*02	IgA
TTADTAVYFCARGQLMGPWGRGTLVTVSSASTKGPS	4-61*07	1*01	IgG
KSEDTAVYYCATKHCGGGNCHFQIFDFWGQGVLVSVSSASPTSPS	3-15*01	4*02	IgA
DTAVYYCARDGMAEGVGRYHYYYGVDVWGQGTTVTVSSASTK	4-59*01	6*02	IgG
RVEDTGVYYCARGLRYLYGLDVWGQGTTVTVSSASTKGPS	3-74*01/02	6*02	IgG
TAADTAVYYCARGGMLIQEWSNLYSWGQGTPVIVSSASTKGPS	4-59*01	4*02	IgG
TAADTAVYYCARGERSIDWYFDLWGRGTLVTVSSASTKGPKV	4-59*01	2*01	IgG
TAADTAVYYCARQDTTGVFAYWYFDVWGRGTLVTVSSASPTSPS	4-59*01/03	2*01	IgA
RAEDTAVYYCARDDFGDQSLLDYWGQGTLVTVSSASTKGPS	3-21*01	4*02	IgG
RVEDTGTYYCARGGGQTWGQGVLVTVSSASPTSPS	3-74*01/03	5*02	IgA
TAADTAVYYCARDYSANSRYFDYWGQGTLVAVSSASTKGPS	4-59*01	4*02	IgG
RASDTATYYCARHLVPDLVREFYYYALDVWGHGTTVTVSSASTK	5-51*02	6*02	IgG
RVEDTAIYYCASDEAEQLVAYQHGMDFWGLGTTVTVSSASTKGP	3-30*03/18	6*02	IgG
RVDDTAVYYCARARPGSYGLDIWGQGIVVIVSSASPTSPS	3-74*01/03	3*02	IgA
STEDTAVYYCARPGPNGSGKEDVFDLWGQGTMVAVSSASTKGPS	3-30*01/15	3*01/02	IgG

Sequences from data set were compared with known Ig sequences to determine variable region and joining region usage (Imgt.cines.fr - version 3.2.16 (Accessed 24 September 2010) (Brochet, X et al, 2008)).

Appendix – A5

Sequences of T cell receptor β-chain CDR3 from healthy donors

Sequence	V Region	J Region
QTSVYFCASSYGARGNTEAFFGQGTRLTVVEDLNKVFPP	6-6*01	1-1*01
LGDSALYFCASSVGKSYEQYFGPGTRLTVTEDLKNVFPP	9*02	2-7*01
PSQTSVYFCASSYGDGTYEQYFGPGTRLTVTEDLKNVFPP	6-5*01	2-7*01
QPEDSALYLCASSQAGYSYNEQFFGPGTRLTVLEDLKNVFPP	4-3*01	2-1*01
PSQTSVYFCASSETSGRSDEQFFGPGTRLTVLEDLKNVFPP	6-1*01	2-1*01
ELGDSALYFCASSVTQGKSCEQYFGPGTRLTVTEDLKNVFPP	9*03	2-7*01
KLEDSAMYFCASSDRGQPQHFGDGTRLSILEDLNKVFPP	2*01	1-5*01
QQEDSAVYLCASSSRGLGSPLHFGNGTRLTVTEDLNKVFPP	7-2*01	1-6*01
ELGDSALYFCASSVEESHNEQFFGPGTRLTVLEDLKNVFPP	9*02	2-1*01
SSQTSVYFCAISETGTATDTQYFGPGTRLTVLEDLKNVFPP	10-3*02	2-3*01
ELGDSALYFCASSVGQGKSYEQYFGPGTRLTVTEDLKNVFPP	13*01	2-7*01
ELGDSALYFCASSVAPTPTRNEQFFGPGTRLTVLEDLKNVFPP	13*01	2-1*01
PSQTSVYFCASSYGDGTYEQYFGPGTRLTVTEDLKNVFPP	6-6*04	2-7*01
KLEDSAMYFCASSPGGTRQPQHFGDGTRLSILEDLNKVFPP	2*02	1-5*01
SSQTSVYFCAISEKGFEQYFGPGTRLTVTEDLKNVFPS	10-1*02	2-7*01
QQEDSAVYLCASSLQTSGVNEQYFGPGTRLTVTEDLKNVFPP	7-8*01	2-7*01
QQEDSAVYLCASSLQPSGINEQYFGPGTRLTVTEDLKNVFPP	7-2*01	2-7*01
SPEDSSIYLCSADFRGEQYFGPGTRLTVTEDLKNVFPP	29-1*01	2-7*02
QTSVYFCAISTQYFGPGTRLTVLEDLKNVFPP	10-3*03	2-3*01
TERGDSAVYLCASSPDLIYEQYFGPGTRLTVTEDLKNVFPP	7-3*05	2-7*01
QQEDSAVYLCASSPDLIYEPTFGPGTRLTVTEDLKNVFPP	7-2*01	2-1*01
QSSFYICSAVPAATTVNNYGYTFGSGTRLTVVEDLNKVF	20-1*02	1-2*01
PSQTSVYFCASSYGGDGYTFGSGTRLTVVEDLNKVFPP	6-5*01	1-2*01
EQRDSAMYRCASSLAGEGYEQYFGPGTRLTVTEDLKNVFPP	7-7*01	2-7*01
QQEDSAVYLCASSPELITEQAFGPGTRLTVTEDLKNVFPP	7-2*01	2-7*01
QQEDSAVYLCASSPGLLYTGYFGPGTRLTVLEDLKNVFPP	7-8*01	2-3*01
PSQTSVYFCASSYGGTEAFFGQGTRLTVVEDLKNVFFP	6-5*01	1-1*01
SSQTSVYFCAISAPDRVGETQYFGPGTRLLVLEDLKNVFPP	10-3*01	2-5*01
QQEDSAVYLCASSGDLIYRYFGPGTRLTVTEDLKNVFPP	7-2*03	2-7*01
PSQTSVYFCASSYGGEGTTPGSGTRLTVVEDLNKVFPP	6-1*01	1-2*01
EQRDSAMYRCASSLGQGNEQFFGPGTRLTVLEDLKNVFPP	7-6*01	2-1*01

QQEDSAVYLCASSPRGGGVGNTIYFGEGSWLTVVEDLNKVFPP	7-2*04	1-3*01
EQRDSAMYRCASSLQGTNTGELFFGEGSRLTVLEDLKNVFPP	7-7*01	2-2*01
QQEDSAVYLCASRGVGSPLHFGNGTRLTVTEDLNKVFPP	7-2*01	1-6*02
PSQTSVYFCASNSPGQGRTPLHFGNGTRLTVTEDLNKVFPP	6-1*01	1-6*01
EQRDSAMYRCASSPAGASNQPQHFGDGTRLSILEDLNKVFPP	7-7*01	1-5*01
APSQTSVYFCASSPRGYNEQFFGPGTRLTVLEDLKNVFPP	6-5*01	2-3*01
SSQTSVYFCAIKADTLTDTQYFGPGTRLTVLEDLKNVFPP	10-3*03	2-3*01
TQQEDSAVYLCAIKPDLIYEFGPGTRLTVTEDLKNVFPP	7-2*03	2-7*01
LELGDSAVYFCASSQMRERTETQYFGPGTRLLVLEDLKNVFPP	3-1*02	2-5*01
LQPEDSALYLCASSREAGYEQYFGPGTRLTVTEDLKNVFPP	4-3*01	2-7*01
PEDSSFYICSARDPREAYTGELFFGEGSRLTVLEDLKNVFPP	20-1*01	2-2*01
AELEDSGVYFCASSQYRYNEQFFGPGTRLTVLEDLKNVFPP	14*01	2-1*01
KNPTAFYLCASTRLAGQPQHFGDGTRLSILEDLNKVFPP	19*01	1-5*01
SPEDSSIYLCSVEGLAGGDEQFFGPGTRFTVLEDLKNVFPP	29-1*01	2-1*01
EDSGVYFCASSQLLAGGSIEGGRSYNEQFFGPGTRLTVLEDLK	14*01	2-1*01
APSQTSVYFCASTLTVNTGELFFGEGSRLTVLEDLKNVFPP	6-5*01	2-2*01
SEPRDSAVYFCASSLRSGGAGTGELFFGEGSRLTVLEDLKNVF	12-4*01	2-2*01
APSQTSVYFCASSEGASGANVLTFGAGSRLTVLEDLKNVFPP	6-1*01	2-6*01
LQPEDSALYLCASSPGLGGNTGELFFGEGSRLTVLEDLKNVFPP	4-3*01	2-2*01
TKLEDSAMYFCASSSDRNTEAFFGQGTRLTVVEDLNKVFPP	2*02	1-1*01
LELGDSAVYFCASSQARVTSGVSSYNEQFFGPGTRLTVLEDLN	3-1*02	2-1*01
APSQTSVYFCASSYEGTSYNEQFFGPGTRLTVLEDLKNVFPP	6-5*01	2-1*01
AELEDSGVYFCASSLQGIPYEQYFGPGTRLTVTEDLKNVFPP	14*01	2-7*01
LGDSALYFCASSLVGSGGAQEQFFGPGTRLTVLEDLKNVFPP	9*03	2-1*01
ELGDSALYFCASSVLLAGDNEQFFGPGTRLTVLEDLKNVFPP	9*01	2-1*01
TEQRDSAMYRCASRTTGYTEAFFGQGTRLTVVEDLNKVLPP	7-7*01	1-1*01
TSSQTSVYFCAIWREGDTQYFGPGTRLTVLEDLKNVFPPA	10-3*02	2-3*01
ELEDSGVYFCASSQDWLGTGPNYNSPLHFGNGTRLTVTEDL	14*01	1-6*01
LQPEDSALYLCASSPGLGARTGELFFGEGSRLTVLEDLKNVFPP	4-3*04	2-2*01
TKLEDSAMYFCASSESGLAGNTGELFFGEGSRLTVLEDLKNV	2*01	2-2*01
AQKNPTAFYLCASSIGGQIYTEAFFGQGTRLTVVEDLNKVFPP	19*01	1-1*01
SEPRDSAVYFCASSRQVNEQFFGPGTRLTVLEDLKNVFPP	12-4*01	2-1*01
ELEDSGVYFCASSQDLWTPGGNYNSPLHFGNGTRLTVTEDLN	14*01	1-6*01
ELGDSALYFCASSGGRSYEQYFGPGTRLTVTEDLKNVFPP	9*02	2-7*01

TDRWDTAVYLCASSPYSGTSSTREQFFGPGTRLTVLEDLKNVF	11-2*01	2-1*01
ELGDSALYFCASSPGTSGREVGTQYFGPGTRLTVLEDLKNVF	9*01	2-3*01
QQEDSAVYLCASSPPGQASYGYTFGSGTRLTVVEDLNKVFPP	7-2*01	1-2*01
QQEESAVYLCASSFIAGENYNEQFFGPGTRLTVLEDLKNVFPP	7-8*01	2-1*01
ELGDSALYFCASSPRDAANYGYTFGSGTRLTVVEDLNKVFPP	13*02	1-2*01
ELGDSALYFCASSVGPFSLDEQYFGPGTRLTVTEDLKNVFPP	9*02	2-7*01
APSQTSVYFCAQTPDWAGEGGYTFGSGTRLTVVEDLNKVFP	6-5*01	1-2*01
TERGDSAVYLCASSLIGGTRFTGELFFGEGSRLTVLEDLKNVF	7-3*05	2-2*01
TQQEDSAVYLCASSPPGQASYGYTFGSGTRLTVVEDLNKVFP	7-8*03	1-2*01
LQPEDSALYLCASSQEFSGYEQYFGPGTRLTVTEDLKNVFPP	4-2*01	2-7*01
APSQTSVYFCASSYTVPWTGDEQYFGPGTRLTVTEDLKNVFPP	6-5*01	2-7*01
TQQEDSAVYLCASSPPGQASYGYTFGSGTRLTVVEDLNKVFPP	7-2*04	1-2*01
VPSQTSVYFCASSGGGATDTQYFGPGTRLTVLEDLKNVFPP	6-4*01	2-3*01
TQQEDSAVYLCASSFITRLSYNEQFFGPGTRLTVLEDLKNVFP	7-2*01	2-1*01
TCSQTLVYFCAISVQGAEETQYFGPGTRLLVLEDLKNVFPP	10-3*02	2-5*01
QQEDSAVYLCASSRPGPLSQETQYFGPGTRLLVLEDLKNVFPP	7-2*02	2-5*01
TERGDSAVYLCASSPYSGTSSTREQFFGPGTRLTVLEDLKNVF	7-3*01	2-1*01
DSALYFCASSSPRGVPGANVLTFGAGSRLTVLEDLKNVFPP	9*01	2-6*01
TQQEDSAVYLCASSSGTSAEQFFGPGTRLTVLEDLKNVFPP	7-8*01	2-1*01
TSSQTSVYFCAISGPGAGSYEQYFGPGTRLTVTEDLKNVFPP	10-3*02	2-7*01
APSQTSVYFCANGVMGGYNEQFFGPGTRLTVLEDLKNVFPP	6-1*01	2-1*01
TQQEDSAVYLCASSLGISYNEQFFGPGTRLTVLEDLKNVFPP	7-2*01	2-1*01
APSQTSVYFCASSYGGSLADGYTFGSGTRLTVVEDLNKVFPP	6-5*01	1-2*01
APSQTSVYFCASSPSGGHYNEQFFGPGTRLTVLEDLKNVFPP	6-6*01	2-1*01
APSQTSVYFCASSYSGADEQFFGPGTRLTVLEDLKNVFPP	6-5*01	2-1*01
APSQTSVYFCASAPTGGGGDEQFFGPGTRLTVLEDLKNVFPP	6-5*01	2-1*01
ATRSQTSVYFCASNPLGGSGYGYTFGSGTRLTVVEDLNKVFPP	10-2*01	1-2*01
APSQTSVYFCASSYGGQGGQPQHFGDGTRLSILEDLNKVFPP	6-5*01	1-5*01
APSQTSVYFCASSYDGNTEAFFGQGTRLTVVEDLNKVFPP	6-5*01	1-1*01
APSQTSVYFCASSPVSGSGSYGYTFGSGTRLTVVEDLNKVFPP	6-5*01	1-2*01
TEQRDSAMYRCASSLGGILDNSPLHFGNGTRLTVTEDLNKVF	7-6*01	1-6*01
TEQRDSAMYRCASSLGETQYFGPGTRLLVLEDLKNVFPP	7-6*04	2-5*01
TSSQTSVYFCAIRGGGEETQYFGPGTRLLVLEDLKNVFPP	10-3*02	2-5*01
APSQTSVYFCASSYRDYSPLHFGNGTRLTVTEDLNKVFPP	6-5*01	1-6*02
TSSQTSVYFCASRPGLGPLHFGNGTRLTVTEDLNKVFPP	10-3*02	1-6*01
APSQTSVYFCASSPTSGGTTDTQYFGPGTRLTVLEDLKNVFPP	6-5*01	2-3*01

APSQTSVYFCASAPQGGYEQYFGPGTRLTVTEDLKNVFPP	6-6*01	2-7*01
ELGDSALYFCASSLEGQSYEQYFGPGTRLTVTEDLKNVFPP	9*01	2-7*01
APSQTSVYFCASRPRTGAYEQYFGPGTRLTVTEDLKNVFPP	6-5*01	2-7*01
ASSQTSVYFCASSHRDPGADTQYFGPGTRLTVLEDLKNVFPP	10-1*02	2-3*01
APSQTSVYFCASSYRRTSGGVYEQFFGPGTRLTVLEDLKNVFPP	6-2*01	2-1*01
LQPEDSALYLCASSLGVTEAFFGQGTRLTVVEDLNKVFPP	4-1*01	1-1*01
QRTEQRDSAMYRCASSLARILDNSPLHFGNGTRLTVTEDLNKV	7-7*01	1-6*02
ESATSSQTSVYFCAISQKGDQYFGPGTRLLVLEDLKNVFPP	10-3*02	2-5*01
APSQTSVYFCASSPVSGSGSYGYTFGSGTRLTVVEDLNKVFP	6-1*01	1-2*01
AELEDSGVYFCASSDGSGANVLTFGAGSRLTVLEDLKNVFPP	14*02	2-6*01
AELEDSGVYFCASSQDMPGEQYFGPGTRLTVTEDLKNVFPP	14*01	2-7*01
LELEDSALYLCASSLQGSYEQYFGPGTRLTVTEDLKNVFPP	5-8*02	2-7*01
ELGDSALYFCASSVGAGQGGTYEQYFGPGTRLTVTEDLKNVF	9*02	2-7*01
APSQTSVYFCASRRMGGSYEQYFGPGTRLTVTEDLKNVFPP	6-1*01	2-7*01
KLEDSAMYFCASSEALRATQYFGPGTRLLVLEDLKNVFPP	2*01	2-5*01
NRETRHCISAPAVSLGNEQYFGPGTRLTVTEDLKNVFPP		2-7*01
APSQTSVYFCASSSVIAGAGTGELFFGEGSRLTVLEDLKNVFPP	6-2*01	2-2*01
HPEDSSFYICSAPAPGARSYTDTQYFGPGTRLTVLEDLKNVFPP	20-1*01	2-3*01
SEPRDSAVYFCASSLTGGAYEQYFGPGTRLTVTEDLKNVFPP	12-4*01	2-7*01
PNQTALYFCATSVSGDNEQFFGPGTRLTVLEDLKNVFPP	24-1*01	2-1*01
TSSQTSVYFCATSSGEGTDTQYFGPGTRLTVLEDLKNVFPP	10-3*01	2-3*01
VRGDSAAYFCASSLAPSRATIDNEQFFGPGTRLTVLEDLKNV	18*01	2-1*01
VVRGDSAAYFCASSQFPGQASYNSPLHFGNGTRLTVTEDLNK	18*01	1-6*02
APSQTSVYFCASSPQGGYGYTFGSGTRLTVVEDLNKVFPP	6-5*01	1-2*01
TQQEDSAVYLCASSSTGFKEQYFGPGTRLTVTEDLKNVFPP	7-2*01	2-7*01
APSQTSVYFCASNPDRGIYNEQFFGPGTRLTVLEDLKNVFPP	6-1*01	2-1*01
ASSQTSVYFCASSDKGTSGGTNEQFFGPGTRLTVLEDLKNVF	10-1*01	2-1*01
APSQTSVYFCASSFTYEQYFGPGTRLTVTEDLKNVFPP	6-6*01	2-7*01
APSQTSVYFCASSYSRGNEQFFGPGTRLTVLEDLKNVFPP	6-6*01	2-1*01
APSQTSVYFCASSYRAGVTEAFFGQGTRLTVVEDLNKVFPP	6-5*01	1-1*01
TERGDSAVYLCASSLTSGSYNEQFFGPGTRLTVLEDLKNVFPP	7-3*01	2-1*01
APSQTSVYFCASRYSTDTQYFGPGTRLTVLEDLKNVFPP	6-2*01	2-3*01
APSQTSVYFCASSPYRADQPQHFGDGTRLSILEDLNKVFPP	6-2*01	1-5*01
APSQTSVYFCASRPAGIPNTGELFFGEGSRLTVLEDLKNVFPP	6-6*01	2-2*01
APSQTSVYFCACLGSGRLLEQFFGPGTRLTVLEDLKNVFPP	6-2*01	2-1*01

TRSQTSVYFCASQPDSAYNEQFFGPGTRLTVLEDLKNVFPP	10-2*01	2-1*01
IPNQTALYFCATGQGYNEQFFGPGTRLTVLEDLKNVFPP	24-1*01	2-1*01
APSQTSVYFCASSPPAGTREQFFGPGTRLTVLEDLKNVFPP	6-5*01	2-1*01
APSQTSVYFCASSYSQGGYEQYFGPGTRLTVTEDLKNVFPP	6-5*01	2-7*01
GDSALYFCASSPLQFLAKNIQYFGAGTRLSVLEDLKNVFPP	9*02	2-4*01
APSQTSVYFCASSRLLAGVDEQFFGPGTRLTVLEDLKNVFPP	6-5*01	2-1*01
APSQTSVYFCASRGGTGSTGELFFGEGSRLTVLEDLKNVFPP	6-5*01	2-2*01
TSSQTSVYFCASRDIRTEAFFGQGTRLTVVEDLNKVFPP	10-3*01	1-1*01
PEDSALYLCASSQEGGIAQYFGPGTRLTVLEDLKNVFPP	4-3*01	2-3*01
APSQTSVYFCARHRQGATGELFFGEGSRLTVLEDLKNVFPP	6-6*01	2-2*01
APSQTSVYFCASSQGQWEETQYFGPGTRLLVLEDLKNVFPP	6-6*01	2-5*01
APSPTYVYLCASSYSSRGGSDTEAFFGQGTRFTVVEDLNKVF	6-5*01	1-1*01
APPQTSVYFCASRRNTGELFFGEGSGLTVLEDLKNVFPP	6-2*01	2-2*01
APSQTSVYFCASNVQGSTEAFFGQGTRLTVVEDLNKVFPP	6-3*01	2-2*01
APSQTSVYFCASSYPRGGTMAQYFGPGTRLLVLEDLKNVFPP	6-3*01	2-5*01
APSQTSVYFCASSFYPRSVKNIQYFGAGTRLSVLEDLKNVFPP	6-1*01	2-4*01
TSSQTSVYFCAISDRVSYEQYFGPGTRLTVTEDLKNVFPP	10-3*02	2-7*01
APSQTSVYFCASNVQGSTEAFFGQGTRLTVVEDLNKVFPP	6-5*01	1-1*01
GDSALYFCASSSGQKNTEAFFGQGTRLTVVEDLNKVFPP	9*02	1-1*01
APSQTSVYFCASIRQGATEAFFGQGTRLTVVEDLNKVFPP	6-5*01	1-1*01
TQQEDSAVYLCASSLGLAGGGAETQYFGPGTRLLVLEDLK	7-2*01	2-5*01
PEDSALYFCASRQGETQYFGPGTRLLVLEDLKNVFPP	4-3*01	2-5*01
APSQTSMYLCASSLVPGDFYEQYFGPGTRLTVTEDLKNVFPP	6-6*01	2-7*01
APSQTSVYFCASSRQGRLGYTFGSGTRLTVVEDLNNVFPP	6-5*01	1-2*01
AIPNQTALYFCATSDLTPGANVLTFGAGSRLTVLEDLKNVFPP	24-1*01	2-6*01
LELGDSAVYFCASSRSTGGSYEQYFGPGTRLTVTEDLKNVFPP	3-1*02	2-7*01
APSQTSVYFCASSYVSQNNEQFFGPGTRLTVLEDLKNVFPP	6-6*01	2-1*01
LQPEDSALYLCASSLTQAYEQYFGPGTRLTVTEDLKNVFPP	4-3*01	2-7*01
LGDSALYFCASSVGGSTDTQYFGPGTRLTVLEDLKNVFPP	9*01	2-3*01
TEQGDSAVYLCASSLVAGEQYFGPGTRLTVTEDLKNVFPP	7-4*01	2-7*01

Sequences from data set were compared with known TCR sequences to determine variable region and joining region usage (Imgt.cines.fr - version 3.2.16 (Accessed 24 September 2010) (Brochet, X et al, 2008)).

Appendix – A6

Table of T Cell Receptor CDR3-Derived Peptides Predicted to bind to HLA Alleles from SYFPEITHI and BIMAS

HLA-A1^{+}	HLA-A1^{-}	HLA-A2^{+}	HLA-A2^{-}
YGDGTYEQY	SQDMPGEQY	SLIGGTRFT	TLTVNTGEL
VLEDLKNVF	SSRQGRLGY	SVLEDLKNV	ALYFCASSL
ISEKGFEQY	VTEDLKNVF	GLAGGGAET	GLGGNTGEL
SPDLIYEQY	ILDNSPLHF	SMYLCASSL	GLGARTGEL
SADFRGEQY	VTEDLNKVF	DLTPGANVL	GLAGNTGEL
VTEDLKNVF	ELEDSGVYF	YLCASSPEL	CISAPAVSL
KLEDSAMYF	KLEDSAMYF	YLCASSPDL	SLAPSRATI
ELEDSGVYF		AMYRCASSL	TVVEDLNKV
VTEDLNKVF		YLCASSPGL	ALYFCASSV
		LVLEDLKNV	SILEDLNKV
		YLCAIKPDL	LVLEDLKNV
		ALYFCASSV	YLCASSPGL
		ALYFCASSL	ALYLCASSL
		YLCASSLGV	ALYFCATSV
		ALYLCASSL	TVTEDLKNV
		YLCASSLGL	TVLEDLKNV
		TVTEDLKNV	SVLEDLKNV
		TVVEDLNKV	
		TVLEDLKNV	
		SILEDLNKV	
		LIYEFGPGT	
		YLCASSFIA	
		YLCASSFIT	
		LVYFCAISV	
		YLCASSSGT	
		YLCASSLGI	

HLA-B7^{+}	HLA-B7^{-}	HLA-B8^{+}	HLA-B8^{-}
APSQTSMYL	GPGTRLTVL	GLGARTGEL	GPGTRLTVL
GPGTRLTVL	GPGTRLLVL	GPGTRLTVL	CASSRPGPL
APSPTYVYL	SPGQGRTPL	GPGTRFTVL	GPGTRLLVL
GPGTRLLVL	GPGTRFTVL	GAGSRLTVL	GAGTRLSVL
AVYLCASSL	QPEDSALYL	GPGTRLLVL	CASSRQGRL
QPEDSALYL	SSRGLGSPL	FCAIKADTL	GAGSRLTVL
AVYFCASSL	AVYLCASSL		
AAYFCASSL	SPEDSSIYL		
WPRHPADSA	QAFGPGTRL		
CASSPLQFL	ASRGVGSPL		
ASRRNTGEL	SVYFCASTL		
AVYLCASSL	AVYFCASSL		
	ASRPGLGPL		

Key

Peptides predicted to bind to: SYFPEITHI
Both Algorithms
BIMAS