

**Investigating the role of HLA class I derived peptide in
alloreactivity.**

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Investigating the role of HLA Class I derived peptide in alloreactivity

Richard Battle

A thesis submitted in partial fulfilment of the requirements of
Sheffield Hallam University
for the degree of Doctor of Philosophy

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Abstract

The peptide complexed with human leukocyte antigen (HLA) can be said to have a significant role in the generation of alloresponses, both in terms of cellular and humoral alloresponses. Previous researchers have identified minor histocompatibility antigen (mHAG) derived peptides such as HA-1, and their contribution to alloresponses. However, little research has been performed regarding the contribution of peptides, which are themselves derived from HLA molecules. To redress this, this thesis focused upon identifying and assessing the function of endogenous peptides, in particular peptides which are themselves derived from HLA class I molecules, which may have a role in the alloresponses, within the context of clinical transplantation.

This research describes the creation of a database of HLA class I derived peptides predicted to bind to HLA-A*02 molecules, and then utilises this database to identify HLA class I derived peptides which are bound by the HLA-A*02 molecule, present upon the surface of the monocytic THP-1 cell line. This process identified two peptides, which were derived from one HLA class I molecule and presented by another (HLA-A*02). One of the identified peptides (VMAPRTLIL) belongs to a group of peptides, known as leader peptides, which have functions in both innate and adaptive immune responses. A clinical audit was performed to assess the effect of mismatching the HLA class I derived leader peptides within the context of renal transplantation and identified a correlation with a poorer functioning allograft at 12 months post transplant, when the donor and recipient have 3 leader peptide mismatches ($p < 0.05$).

Further experimental work attempted to determine a functional role of the endogenous peptides. In particular seeking to establish if variation within peptides bound by the same HLA molecule can influence the subsequent binding of HLA specific antibodies, and if so, seek to elucidate the mechanisms involved. Using a T2 cell line peptide binding assay, as a target for a HLA-A*02 specific antibody, a variation between the ability of the antibody to bind when alternative peptides were bound was observed. Through the use of structural modelling it was demonstrated that changes within HLA class I bound peptides can induce conformational changes to epitopes,

which were previously described for HLA class I molecules as being the targets of HLA class I specific antibodies.

These findings suggest an important role for HLA class I derived leader peptides in the outcome of renal transplantation, which requires further study within a validating cohort of patients. While the observation that peptides can induce conformational variation within identified epitopes provides further insight into the complex nature of alloantibody binding, and aids the understanding of this process.

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Publications, posters and prizes

Publications

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Battle R, Turner D, Woodroffe N and Clark B (2014) Identified HLA class I epitopes can undergo conformational induced variation due to changes within HLA bound peptides. *Immunology Letters* 161: 57-58.

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Published abstracts

Battle R, Clench M, Woodroffe MN, Clark B (2012) 131-P Preliminary *in-vitro* data supporting the concept of peptide exchange by cell surface HLA-A*02:01. *Human Immunology* 73:S132.

Battle R, Woodroffe MN, Clench M and Clark B (2013) HLA class I leader peptide sequence mismatches between renal transplant donors and recipients are associated with a reduction in allograft function within the first 12 months post transplant. *International Journal of Immunogenetics* 40: 403.

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List of abbreviations

ACN, Acetonitrile

AI, Artificial intelligence

APC, Antigen presenting cells

AUC ROC, Area under receiver operator curve

β_2 M, β_2 -microglobulin

BSA, Bovine serum albumin

BSHI, British Society of Histocompatibility and Immunogenetics

BTS, British Transplant Society

CDC, complement-dependent cytotoxicity

CDR, Complimentary determining regions

CHCA, α -cyano-4-hydroxycinnaminic acid (CHCA)

CLIP, Class II associated invariant chain peptide

Cn3D, See in 3D

CNX, Calnexin

cPRA, Calculated panel reaction frequency

CRF, Calculated reaction frequency

CRT, Calreticulin

CREGs, Cross reacting epitope groups

cTEC, Cortical thymic epithelial cells

D, Diversity gene

dAPC, Donor antigen presenting cell

DBD, Donation after brain stem death

DCD, Donation after cardiac death

DMSO, Dimethyl sulphoxide

DRiPS, Defective ribosomal products

DSA, Donor specific antibodies

EP, Electrostatic potential

ER, Endoplasmic reticulum

ERAD, ER-associated protein degradation system

ERAP1, ER aminopeptidases

ESI, Electrospray ionization

FCS, Fetal calf serum

GvHD, Graft versus host disease

HC, Heavy chain

HFE, Haemachromatosis

HLA, Human Leukocyte Antigens

HPLC, High performance liquid chromatography

HSCT, Haematopoietic stem cell transplantation

IFN- γ , Interferon- γ

IHWS, International Histocompatibility Workshops

li, Invariant chain

IMGT/HLA, Immunogenotyping and HLA

J, Joining gene

MALDI-TOF, Matrix-assisted laser desorption/ionization time of flight

MFI, Median channel fluorescence

MHC, Major Histocompatibility Complex

mHAGs, Minor histocompatibility antigens

MICA, Major histocompatibility complex related chain A

MICB, Major histocompatibility complex related chain B

MIIC, MHC Class II compartment

mm, Mismatches

MOPS, 3-(*N-morpholino*) propaenesulfonic acid

MS, Mass spectrometry

mTEC, Medulla thymic epithelial cells

M/Z Mass to charge ratio

NHS, NHS Blood and Transplant Organ Donation and Transplantation

ODT, Organ donation and transplantation

PBS, Phosphate buffered saline

PE, Phycoerythrin

pHLA, peptide complexed HLA

PLC, Peptide loading complex

PSSM, Position specific scoring matrices

RAG1 Recombination-activating gene 1

RAG2 Recombination-activating gene 2

rAPC, Recipient antigen presenting cell

RT, Room temperature

SD, Standard deviation

SDS, Sodium dodecyl sulphate

SPI, Solid phase immunoassay

SPR, Surface plasmon resonance

T cells, T lymphocytes

TAP, Transporter associated with antigen processing

TFA, Trifluoroacetic acid

TNF Tumour necrosis factor

TRAV, T cell receptor α -variable genes

TRBV, T cell receptor β -variable genes

TRM, Transplant related mortality

T2 cells, TAP deficient cell line

WHO, World Health Organisation

1.1.1 Organ Transplantation

The use of organ transplantation as a clinical therapy in modern medicine is the result of many successive advances within multiple disciplines including: advances in the available pharmacology, improved surgical and intensive care techniques, greater success in tissue preservation and a vital understanding of the immunology of transplantation. These advances have led to the ability to transplant an ever increasing range of complex organs and tissues into patients for their life saving function (for example in the cases of cardiothoracic, renal and liver transplantation) and the quality of life that is associated with successful transplantation (Neipp *et al.*, 2006).

Perhaps the first documented attempt at organ transplantation is depicted in the works of renaissance painters, who portrayed the patron saints of surgery, Cosmos and Damian, performing a transplant in the 3rd century (Figure 1.1). However without these saintly powers it would be many centuries before transplantation became a reality; indeed many early attempts at organ transplantation resulted in failure either immediately or shortly after transplantation. Consequently the modern era of transplantation can be argued to have begun in the 1950s with the success of renal allografts, which survived for longer than 6 months (Murray *et al.*, 1962). These were quickly followed by successful heart transplantation (Barnard, 1968) and orthotopic liver transplantation (Starzl *et al.*, 1968).

Understanding the immunology of transplantation was crucial to these successes, and improvements to this understanding have helped lead to the remarkable success of modern transplantation. The father of transplant immunology and founder of the British Transplant Society, Sir Peter Medawar, first demonstrated allograft rejection as having an immune cause using skin grafts in animal models (Figure 1.2) (Medawar, 1945) and created the start of a new field of science, which would ultimately become known as histocompatibility and immunogenetics. Medawar's experiments demonstrated that rejection of skin grafts on animal models were T cell mediated, had immunological memory and could be transferred from one animal to another. Medawar was unaware at the time of his experimentation that the phenomenon he was witnessing was a response primarily directed against mismatched Major Histocompatibility Complex (MHC) encoded antigens. Further work into histocompatibility and immunogenetics over the decades following Medawar's work

Figure 1.1 Twins Cosmos and Damian perform a miraculous leg transplantation in the 3rd century.

As depicted by an artist in the 1500s. Image reproduced for research purposes courtesy of the Wellcome Image Library.

- A) Syngeneic skin graft, is tolerated
- B) An allogeneic skin graft is rejected within 10-13 days. Medawar termed this a 1st response. A second allograft using the same murine model as B) results in rejection in 6-8 days, the kinetics referred to as a 2nd set response
- C) The T lymphocytes were removed from the murine model displaying 2nd set kinetics D) and then inoculated into a different mouse (E)
- D) T cells transferred from yellow to green mouse
- E) A skin graft from the 1st mouse (Grey), is now rejected with the second set kinetics (6-8 days) in a naive mouse (Green) after its inoculation with the T cells, i.e . the response kinetics were transferred with the T cells

Figure 1.2, An overview of the experiments performed by Medawar

(Adapted from Janeway, CA. Travers, P. Walport, M. (1999) Immunobiology, 4th Edition, Churchill Livingstone, Edinburgh, p510)

revealed the complex nature of the genes and proteins which govern rejection reactions and have led to a greater understanding of immunology as a whole.

1.1.2 *A basic overview of the immune system*

This increased understanding of immunology has led to the current view of the immune system as a complex network of organs, tissues, cells and subsets of cells, which function and cooperate together in the defence of the organism against foreign invasion, most usually in the form of attack from pathogenic bacteria and viruses. The organs of the immune system, referred to as lymphoid organs as they are host to lymphocytes, include the tonsils, adenoids, lymph nodes, thymus, spleen, Peyer's patches, appendix and the bone marrow. Lymphatic and blood vessels facilitate migration of cells around the body and between these lymphoid organs. Lymphoid organs have specialised roles, the bone marrow for example is the ultimate source of all blood cells, including lymphocytes. B lymphocytes mature within the bone marrow and once activated they develop into plasma cells and secrete antibody which provides an effective response against pathogens, while T lymphocytes migrate from the bone marrow to the thymus for further development and can provide a variety of functions from direct cellular cytotoxicity of infected cells and pathogens to aiding B lymphocytes and their responses (Paul, 2013).

Fluid is exchanged between the blood and lymphatic vessels enabling the lymphocytes to encounter any invading pathogens. In addition the lymph nodes, which are located throughout the body (specifically clusters of lymph nodes are present in the neck, armpits, abdomen and groin), enable the lymphocytes to encounter antigen. Containing specialized anatomical structures, the lymph nodes concentrate T cells in the paracortex to facilitate their encounter with antigen, allow the formation of B cell germinal centres and aid the production of humoral responses by the formation of plasma cells within the medulla. The regions within the body which facilitate the encounter of lymphocytes, and indeed other immune cells with antigen, are too numerous to list as they can be said to consist of areas which act as gateways to the body, such as the linings of the gastrointestinal tract (Paul, 2013). However a crucial element of a competent immune system is the ability to differentiate between self and

non self, in order to generate an appropriate response. It is this ability which is most pertinent to the setting of organ transplantation.

1.1.3 *Histocompatibility and Immunogenetics*

In order to distinguish between self and non-self, immune cells recognise antigens via specialised receptors, such as the pattern recognition receptors of the innate immune system or the B and T cell receptors of the adaptive immune system. In transplantation, in which cells, tissues or organs are transferred from one individual to another, recognition of non-self antigens from the transplanted material can occur, which can lead to damage and/or rejection of the transplant. As Medawar demonstrated this is best avoided by use of a syngeneic transplant (i.e. between a genetically identical donor and recipient), such as in the case of monozygotic twins, thus allowing 'self-recognition' of the transplant due to the expression of the same antigens, and therefore allowing histocompatibility (Afzali *et al.*, 2007). However as most individuals do not have a genetically identical twin this is rarely an option in clinical transplantation, and an allograft is performed (the transplant of genetically different material from an individual of the same species). An immune response against the differing antigens on the allograft can be formed, as a consequence of its lack of histocompatibility (Afzali *et al.*, 2007).

The genes expressing the antigens of the greatest significance in histocompatibility are encoded for on the short arm of chromosome 6, specifically 6p21.3, in a region known as the Major Histocompatibility Complex (MHC). In man, within the MHC it is the genes which encode the Human Leukocyte Antigens (HLA) which have the greatest impact in transplantation (Howell *et al.*, 2010).

1.1.4 *The Major Histocompatibility Complex*

Our current understanding of the MHC has been pieced together from observations spanning several decades (Thorsby, 2009). The Nobel prize for Physiology or Medicine in 1980 being awarded to Benacerraf, Dausset and Snell for discovering the MHC. In the 1940s, Snell first discovered components of the MHC through their role in transplant rejection in murine models. This knowledge was added to in the 1950s as Dausset identified the first HLA molecules after observing immune reactions following blood transfusions between ABO compatible individuals and establishing the cause as

being white blood cell agglutination, this agglutination was a result of HLA specific antibodies, while Benacerraf provided the evidence that immune reactions are controlled by genes. Working alongside these three pioneers, were a host of other investigators who also identified HLA molecules. Through international collaborative efforts, facilitated by a series of International Histocompatibility Workshops (IHWS), these researchers exchanged sera and cells and identified that several locally identified HLA were identical (Thorsby, 2009). The sera used by these researchers to identify HLA molecules was obtained from multiparous women, whose immune system was exposed to the HLA molecules of their partners during their pregnancies, consequently their sera contained HLA antibodies, which were formed against the different HLA molecules of the foetus, which were paternally inherited. Through careful population and familial studies these investigators went on to establish different loci encoding the identified HLA. Leaping forward to present day, modern molecular biology techniques have revealed the exquisite polymorphism of HLA molecules and the complexity of the MHC. At the time of writing there are 8,794 HLA alleles identified on the IMGT/HLA sequence database (Robinson *et al.*, 2013), the nomenclature system for which is governed by the World Health Organisation (WHO) nomenclature committee and the MHC itself contains some 224 genes (Figure 1.3).

1.1.5 *The diversity of the Major Histocompatibility Complex*

The remarkable polymorphism of the MHC has been revealed due to its significance in infection, autoimmunity and transplantation, which has led the MHC to be the most widely studied region of the human genome (Horton *et al.*, 2004). The primary function of the genes within the MHC is to confer protection from pathogens. It incorporates many of the genes which have a role in the processing and presentation of antigen, such as the transporter associated with antigen processing (TAP) genes, which are required for transporting peptides from the cytosol into the endoplasmic reticulum (ER) (Cresswell *et al.*, 1999), as well as the HLA genes.

HLA molecules are membrane bound heterodimer glycoproteins which bind, and then present, peptides to T lymphocytes (T cells). HLA Class I molecules, HLA-A, B and C, are expressed on almost all nucleated cells (as well as platelets) and present intracellular

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|--------------------|----------|-------------|--------------|
| | | HLA-N | |
| | | HCG19P | |
| | | UBQLN1P | |
| | | MICC | |
| | | TMP0P1 | |
| | | SUCLA2P | |
| | | RANPI | |
| | | HLAE | |
| | | GNL1 | |
| | | | HLAF |
| | | | RPL23AP1 |
| | | | |
| | | | HCG9P5 |
| | | PTMAP1 | IFITM4P |
| | | C6orf136 | HCP5P14 |
| | | DHX16 | HCG4 |
| | | KIA1949 | HLA-75 |
| | | NRM | HCP5P12 |
| | | RPL7P4 | HLAD |
| | | | RPL7AP7 |
| HLA-DRA | PPIP9 | MDC1 | |
| HLADRB3 | RPL15P4 | TUBB | |
| HLADRB3 | BAT1 | FLOT1 | HCP5P12 |
| HLADRB3 | ATP6V1G2 | IER3 | HLA-G |
| | NFKBIL1 | HCG29 | |
| HLADRB2 | LTA | C6orf214 | HLA H |
| HLA-DRB1 | TNF | TIGD1L | HLA 16 |
| HLA-DQA1 | LTB1 | DDR1 | HCG4P7 |
| | LST1 | GTF2H4 | HCG4P6 |
| HIADCI.BI | NCR3 | VAR2L | HLA-K |
| MITC03P1 | UQCRHP1 | SFTPG | HLA-21 |
| HLADQB3 | AIF1 | HCG21 | HLA-A |
| | BAT2 | DPRI | HCP5P3 |
| HLA-DQA1 | BAT3 | HCG21 | HLAD |
| | APOM | C6orf205 | MICD |
| HLADQB2 | C6orf47 | C6orf15 | HCG9 |
| | BAT5 | CDSN | HCP5P2 |
| HLADOB | LY6G6D | P50RS1C2 | |
| | LY6G6E | PS0RS1C2 | HCG8 |
| | LY6G6C | POLR2LP | ETF1P1 |
| PSMBS | C6orf25 | C6orf18 | C6orf12 |
| | DDAH2 | TCF19 | ZNRD1 |
| | CLIC1 | P0U5F1 | PPP1R11 |
| PSMBS | M5H5 | PS0RS1C3 | RNF39 |
| PPP1R2P1 | C6orf26 | HCG27 | TRIM31 |
| | C6orf27 | HLA-C | TRIM-10 |
| HLAZ | VAR2 | USPSP | TRIM10 |
| HLA DMB | L5M2 | RPL3P2 | TRIM20 |
| | HSPA1L | WA5F5P | TRIM15 |
| HLA DMA | HSPA1A | HLAB | TR M26 |
| | HSPA1B | DHFRP2 | PAIIP'1 |
| | C6orf46 | FGFR3P | HCG17 |
| HLA-DOA | NEU1 | HLAS | TRIM20P |
| | C6orf15 | MICA | HLAL |
| HLA-DPA1 | BATS | HCP5 | HCGIS |
| HLADPB1 | ZBTB12 | HCG2S | TR M39 |
| | C2 | MICB | RPP21 |

Figure 1.3 An overview of the MHC on chromosome 6, from the centromeric HLA-DPB*01 gene to telomeric HLA-F.

HLA-DRB*3,*4 and *5 chains which are present only on certain haplotypes are also shown. Adapted from Horton *et al.*, (2004) and Defranco *et al.*, (2007).

processed peptides (e.g. of viral origin) to cytotoxic CD8⁺ T cells (Long and Jacobson 1989). HLA class II molecules, HLA-DR, DQ and DP, have a more restricted distribution, being expressed on cells of the monocyte/macrophage lineage, B cells and activated T cells. HLA Class II molecules can also be induced by the cytokine interferon- γ (IFN- γ) on some cell types, such as endothelial cells. HLA Class II molecules present extracellular derived peptides (e.g. bacterial peptides) to T helper CD4⁺ cells (Howell *et al.*, 2010).

The peptides bound by HLA molecules possess a set of anchor residues which fit specifically into pockets within the HLA molecule's binding groove (discussed in further detail later), this confers a specific set of characteristics upon the peptides bound by HLA molecules. These characteristics have been suggested as one of the key driving forces behind the extensive polymorphism of HLA molecules. Specifically referred to as the heterozygote advantage theory, which proposes that individuals who are heterozygous for HLA molecules, are able to respond to a greater number of pathogen derived peptides than homozygotes, due to their ability to present a greater range of peptides, as a consequence of the variation in the peptide binding characteristics of their greater range of HLA molecules (Spurgin and Richardson, 2010). Additionally another advantage can be seen in rare HLA alleles, whereby it is argued that the ongoing immune-evasion strategies of pathogens leads them to overcome the peptide presentation of the most common HLA molecules, therefore new HLA alleles which arise are able to provide a greater protection than common alleles, therefore offering a survival advantage (Spurgin and Richardson, 2010). Another interesting feature regarding the nature of MHC diversity can be observed within these common alleles, as some HLA haplotypes are seen with a far greater frequency than others. Indeed linkage disequilibrium, which refers to the characteristic of certain genes to be inherited together due their proximity to each other upon the chromosome, means that certain common haplotypes are observed in far higher frequencies than they would be if haplotypes were the consequence of a random formation. Furthermore the phenomenon of a founder effect can be observed within some HLA haplotypes. Whereby some genetic variation is lost when a sub population is established from a

larger population by a limited number of individuals, this is seen within patients suffering from haemochromatosis (HFE), an iron overload disorder. The carrier of the original mutation on the HFE gene was believed to be of Celtic or Viking origin, and possessed the HLA haplotype HLA-A3; B7 or A3; B14. Although recombination events have led to new haplotypes which include the HLA-A3 molecule, linkage disequilibrium between HLA-A3 and the mutated HFE gene means a strong association between these two genes is still seen today (Olsson *et al.* 2009).

1.1.6 *The association of transplant outcome and HLA*

The significant effect of HLA upon outcome of transplant can be observed by looking at the survival of allografts in relation to HLA matching between donor and recipient pairs, as shown for kidney transplants (Figure 1.4). Non HLA genes within the MHC have also been linked to transplant outcome, including major histocompatibility complex related chain A (MICA) (Sanchez-Zapardiel *et al.*, 2013) and B (MICB) (Hankey *et al.*, 2002) and tumour necrosis factor (TNF) gene polymorphisms (Sahoo *et al.*, 2000) amongst others.

The significance of HLA matching on transplant outcome is not uniform across all transplanted tissues, the effect of mismatches being profound in haematopoietic stem cell transplantation (HSCT), and less so in liver transplantation. The expression and distribution of the HLA antigens influences this effect, as well as various other factors such as the risk of graft versus host disease (GvHD) in HSCT and type and dose of immunosuppression utilised. To understand the mechanism by which HLA matching influences the outcome of allotransplantation, it is essential to understand how the immune system is able to distinguish between self and non-self and in particular the role which HLA plays within this process.

1.1.7 *The role of HLA in distinguishing between self and non-self*

The interaction of the T cell receptor (TCR) on CD4⁺ and CD8⁺ T cells with peptide complexed HLA (pHLA), is central to the discrimination of self and non-self antigens in triggering adaptive immune responses (Huang *et al.*, 2010; Zingernagal *et al.*, 1974). The ability of the TCR to recognise a pHLA as foreign or self is achieved via a process of positive and negative T cell selection of appropriately armed T cells in the thymus, as described in detail by

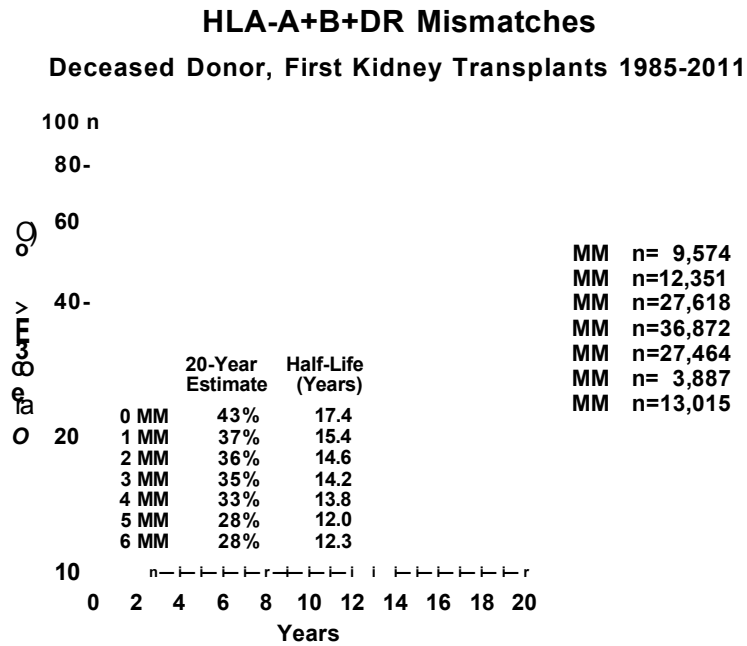


Figure 1.4. The impact of HLA mismatches (mm) upon renal allograft survival over a 20 year period.

Adapted from the Collaborative Transplant Study.

<http://www.ctstransplant.org/servlet/ArchiveServlet?group=K-21101-0214&archivemode=false&ts=1400939174138>

Ziegler *et al.*, (2009). This process, referred to as central tolerance, allows the destruction of self-reactive T cells while retaining those T cells with appropriate peptide complexed HLA (pHLA) discrimination capabilities (Edelmann *et al.*, 2011).

1.1.8 *T cell development within the thymus*

The presence of pHLA is thought to be crucial in the establishment of this central tolerance. Briefly, T cell education occurs within the thymus, pHLA is expressed upon cortical thymic epithelial cells (cTEC), if the TCR of the developing T cell does not recognise a pHLA on cTEC, or if the TCR reacts with high affinity to these pHLA molecules, then the T cells do not survive positive selection. If the TCR interacts with the pHLA with a low affinity then the T cell survives positive selection as self restricted (i.e. it recognises self pHLA with low affinity), but has the potential for self reactivity. These positively selected cells then migrate within the thymus via the cortico-medullary junction and into the medulla, where they interact with the medulla thymic epithelial cells (mTEC), where negative selection occurs. This mechanism involves the deletion of T cells whose TCR reactivity to self antigens is greater than an acceptable threshold, thus removing self reactivity (Ziegler *et al.*, 2009).

The ability for T cells (and B cells) to distinguish between self and non self is vital for an appropriate immune response to develop. The consequence of a transplanted allograft expressing differing HLA to the recipient can be severe, as recognition of the transplanted antigens can occur in a process known as allorecognition and an immune response generated against the allograft, which can be both cellular and/or humoral in nature (Petra *et al.*, 2013).

1.1.9 *The processes of allorecognition in transplant rejection*

In order to understand allorecognition a basic understanding of 'normal' immune recognition processes is required. Immunology text books tell us that for recognition of an antigen and an immune response to occur, the response to foreign antigen is initiated by antigen presenting cells (APC), which take up antigen in the periphery and migrate to the secondary lymphoid organs, the spleen, lymph nodes and the gut and mucosa-associated lymphoid tissue. Naïve T cells, which constantly patrol through secondary lymphoid tissues, engage and sample the foreign antigen, displayed by the

APCs as pHLA complexes. A naïve T cell, which recognises an APC displaying a pHLA for which its TCR is specific, engages the APC in an interaction between the TCR and pHLA (as well as co-receptors and co-stimulatory molecules, which are essential for an effective immune response). This interaction leads to the generation of armed effector T cells, which express the necessary receptors for migration to the site of inflammation (Lakis, 2003).

In transplant immunology this process has the added complexity of donor APC and non-self HLA molecules. These complexities are described in the mechanisms of direct and indirect allorecognition and the concept of peripheral sensitisation (Figure 1.5). Peripheral sensitisation describes the process whereby donor endothelial cells, which line vascularised allografts, are capable of directly activating allospecific naïve T cells (Al-Lamki *et al.*, 2008). Direct recognition can be summarised as T cell recognition of intact donor HLA on donor APC, whereas, indirect recognition is the result of T cell recognition of allopeptides bound to self HLA on recipient (self) APC (Benchou and Thomson, 2009). Both the indirect and direct allorecognition pathways play an important role in the response to an allograft. Direct allorecognition is believed to be the driving force behind early acute allograft rejection episodes, due to the high frequency of T cells capable of recognising intact donor HLA on donor APC (Bolton *et al.*, 2008), whereas indirect recognition is thought to be responsible for later allograft damage when the source of donor APC has been exhausted (Afzali *et al.*, 2008). T cells are the principle effector cells of allorecognition and alloreactive T cells are readily detectable in humans, between 0.1% and 10% of T cells have been shown to react with alloantigens (Colf *et al.*, 2007), which is significantly higher than the less than 1/100,000 (0.001%) of T cells which are reactive towards nominal antigens (Heeger, 2003).

The relative simplicity of the direct and indirect pathways is complicated by the theory of semi-direct allorecognition (Afzali *et al.*, 2008). Whereby instead of the traditional immune response 'cross-talk' between T cells, where CD4⁺ and CD8⁺ T cells are stimulated by the same APC. Semi-direct recognition in transplantation requires cross-talk between direct and indirect recognition. Where CD4⁺ T cells with indirect

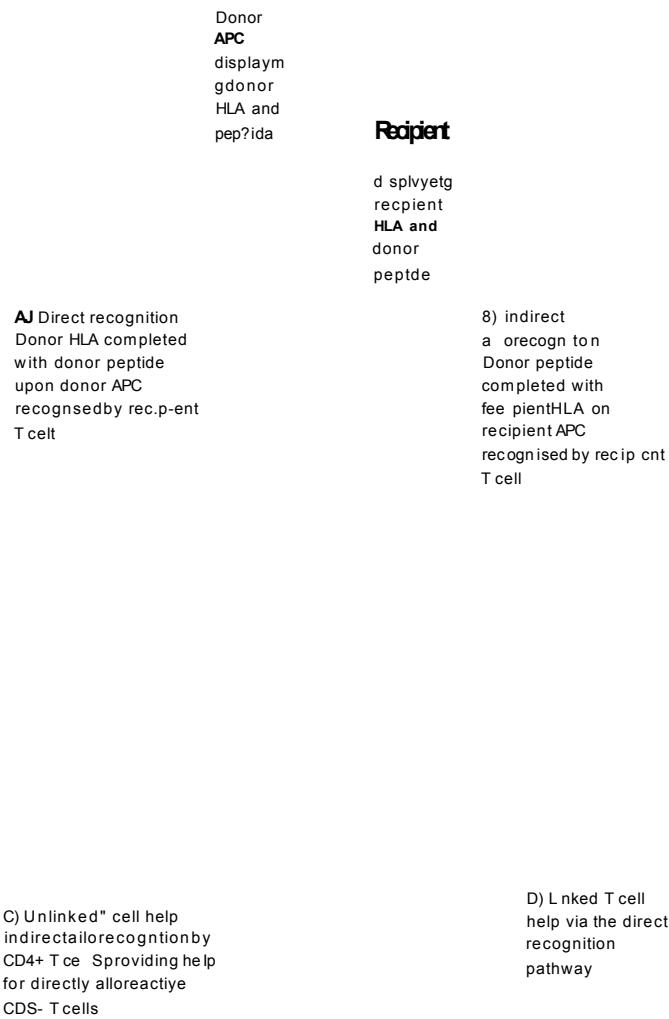


Figure 1.5 The pathways of allorecognition.

Direct allorecognition (A), and indirect allorecognition (B), as well as examples of semi direct allorecognition (C) and linked help via the direct recognition pathway (D).

dAPC - donor antigen presenting cell, rAPC - recipient antigen presenting cell.

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8. The diagram shows the indirect activation of T helper cells by a proposed semi-direct mechanism. TCR - T cell receptor, BM - Bone marrow, adapted from Ali et al., (2013).

responses activated via indirect activation of T helper cells, while the red arrows show cellular responses activated by a proposed semi-direct mechanism. TCR - T cell receptor, BM - Bone marrow, adapted from Ali et al., (2013).

allospecificity can amplify or regulate directly allospecific CD8⁺ T cells. This appears to require a 'four cell' or 'unlinked model' as the CD4⁺ indirectly allospecific T cells would be activated by self APC, whereas, the CD8⁺ directly allospecific T cells would be activated by donor APC. Such 'unlinked' help may not be required however, as dendritic cells have previously been shown as being able to acquire intact MHC peptide complexes from other dendritic cells (Afzali *et al.*, 2008 and Brown *et al.*, 2011). A potential consequence of the semi-direct pathway could be sustained stimulation of directly alloreactive T cells when donor APC has been exhausted.

In terms of which T cell lineages are responsible for specific graft damage induced via indirect and direct mechanisms, Ali and colleagues (2013) argue that indirectly stimulated CD8⁺ T cells are 'largely clinically irrelevant' due to graft parenchyma remaining completely of donor origin in vascularised allografts, and thereby the cytolytic property of CD8⁺ T cells can only be activated through direct recognition of allogeneic class I HLA (the graft having no recipient HLA presenting donor antigen upon it therefore negating indirect recognition). This can be said to be true to some extent for CD4⁺ T cells as well, in that no recipient class II HLA will be present upon the allograft either. However CD4⁺ T cells indirectly activated via recipient APC can, theoretically at least, help mediate a humoral response (and more controversially, a cellular effector response (Ali *et al.*, 2013) if the semi-direct mechanism of allorecognition is assumed to be correct). In particular, evidence seems to show that memory CD4⁺ T cells derived by indirect recognition can provide help to naïve B cells responding to a new, secondary, target alloantigen. This would explain the observation of late developing *de novo* HLA alloantibody (Ali *et al.*, 2013).

1.1.10 *The categories of transplant rejection*

Allorecognition then, can generate both cellular and humoral responses to the allograft, and consequently rejection of an allograft can also be made up of both cellular and humoral responses, or indeed may be comprised of one response predominantly over the other (Petra *et al.*, 2013). Transplant rejection episodes are categorised according to the time frame in which they occur post transplant, and are known as, hyperacute, accelerated acute, acute and chronic rejection episodes.

1.1.10.1 *Hyperacute allograft rejection*

Hyperacute rejection occurs minutes to hours after the vascular clamp to the transplanted organ is removed allowing reperfusion (Nankiville and Alexander, 2010). The cause for hyperacute rejection was established in the 1960s (Patel and Terasaki, 1969) as being due to preformed donor specific antibodies (DSA), specifically towards HLA antibodies (although donor specific ABO group antibodies are also a cause of hyperacute rejection this can be avoided by ensuring ABO compatibility) which are present in the recipient due to previous exposure to HLA molecules, through previous transplant, pregnancy or transfusion, bind to their specific HLA target upon the allograft vascular endothelium, and mediate allograft damage, via activation of the complement cascade, a feature which can be observed in the biopsies of rejected allografts in the form of C4d deposition (Girnita *et al.*, 2007). This process results in vascular thrombosis and ischaemic necrosis to the allograft, the consequence of which is nearly always removal of the allograft (Nankiville and Alexander, 2010).

1.1.10.2 *Accelerated acute allograft rejection*

In a similar fashion to hyperacute rejection, accelerated acute rejection is mediated by HLA antibodies, which were either at a low level and not detected by a pre-transplant crossmatch (XM), or due to an anamnestic response (Nankiville and Alexander, 2010). The rejection event occurs within the first few days post transplant and is caused by HLA antibody as with hyperacute rejection. The sudden increase in DSA is caused by activation of memory B cells post transplant. In other aspects, accelerated acute allograft rejection is comparable to hyperacute rejection.

1.1.10.3 *Acute allograft rejection*

Acute rejection occurs over the first months post transplant and is comprised of both cellular and humoral components (Nankiville and Alexander, 2010). Predominately caused by direct allorecognition (Bolton *et al.*, 2008), acute rejection develops as intra-allograft immature donor dendritic cells carry donor antigens to the draining lymph nodes and spleen, in the process of which they mature into APC (Nankiville and Alexander, 2010) and present their antigen to T cells. Activated T cells then migrate to the allograft and mediate allograft damage. In sites of acute rejection within an allograft, interstitial mononuclear cells including CD4+ and CD8+ T cells can be seen to

accumulate upon histological analysis of biopsies, where direct contact with tubular epithelial cells generates cell mediated cytotoxicity, and CD4+ T cells can promote a humoral response (Nankiville and Alexander, 2010).

1.1.10.4 *Chronic allograft rejection*

Chronic rejection occurs in an ongoing process years post transplant, and is thought to be the result of indirect recognition (Afzali *et al.*, 2008). However the contribution of this ongoing process to eventual allograft loss can be difficult to determine, as allograft damage is also caused by immunosuppressant toxicity as well as the patient's primary disease over the same time period. In the case of kidney transplantation for example, the term chronic rejection is often replaced with chronic allograft nephropathy, to highlight the contribution of these varied factors to damage to the allograft. The features of chronic rejection include: the gradual loss of allograft function over time, with T cell infiltration of the allograft parenchyma and deposition of T cells and macrophages in the interstitium (Nankiville and Alexander, 2010).

1.1.11 *The role of peptides in direct and indirect allorecognition*

The role of the direct allorecognition pathway in acute rejection seems to contradict the classic self restriction property of T cells, i.e. that T cells can only recognise foreign antigen when complexed with self HLA (Ziegler *et al.*, 2008), as direct recognition involves the recognition by self TCRs of intact donor HLA on donor APC. Self restriction is a consequence of the establishment of central tolerance (previously discussed in section 1.1.7 and 1.1.8), and classically two models have previously been proposed to explain the mechanism by which a T cell is able to 'break' self restriction, and recognise donor HLA directly on donor APC, these are known as the high density determinant model and multiple binary complex theory (Afzali *et al.*, 2007). The 'high density determinant' model proposes that directly alloreactive T cells recognise differences in the amino acids of the HLA molecules of the donor cells, and that the nature of the peptide within the groove is unimportant. This means all donor cells expressing HLA can act as a ligand for directly alloreactive T cells, creating a very high density of ligand. As a result of this high density, the affinity of the directly alloreactive T cell receptor is hypothesised to be lower than that of a self-restricted T cell receptor and can thus overcome self restriction (Bharat and Mohanakumar, 2007).

In the multiple binary complex theory however, the peptide has a key role in direct allorecognition. This model proposes that alloreactive T cells recognise specific peptides within the donor HLA grooves. These peptides are derived from homologous intracellular proteins which are also present within the recipient. However differences in the donor HLA groove due to HLA polymorphism, result in a different set of peptides being presented from the same intracellular proteins, when compared to the peptides presented by the recipient's HLA (Bharat and Mohanakumar, 2007). This model fits with the current understanding of TCR/pHLA interaction, which is discussed later, and assumes that a TCR is able to recognise the donor HLA due to the relative structural similarity of the molecule in TCR binding regions in comparison to the peptide binding HLA groove regions.

The indirect allorecognition pathway is perhaps a closer representation of the way a T cell recognises an antigen in a 'normal' immune response. The donor antigens have been internalised, processed and presented in the context of self HLA on recipient APC. The role of the peptides is central to the recognition by TCR (as is the case with normal antigen recognition) and the generation of an alloimmune response in the indirect recognition pathway.

Most recently, the significance of the peptide within both direct and indirect recognition is becoming clear, and many studies have now demonstrated the existence of peptide specific alloreactive T cells, and the ability in some of these cases, for distinct T cells to respond to multiple allopeptides (Felix *et al.*, 2007). Recent reviews have highlighted the fact that peptides are intrinsic to TCR recognition and that there are many similarities between allorecognition and conventional recognition (Felix and Allen, 2007). Indeed the ability of the TCR receptor to recognise multiple peptides complexed within the same HLA molecule (which emphasizes the importance of the peptide within recognition pathways) was recently reported by Wooldridge and colleagues (2012) in an autoimmune context. They described a single CD8⁺ T cell derived from a patient with type I diabetes, which was specific for a preproinsulin derived peptide, and was identified as pathogenic in this context. The autoimmune TCR of this CD8⁺ T cell was identified as being able to recognise more than 10⁶ different peptides. The authors most significant finding is perhaps not the surprising extent of a

single TCR to identify such a large array of peptides, but that the initial peptide antigen can be improved upon in terms of its stimulation of the TCR, the authors reporting an increase in the functional response of greater than 100 fold in a CD8⁺ T cell already established as pathogenic.

1.1.12 *The effect of altering the peptidome through transplantation*

Peptides, according to the literature, play a key role in TCR recognition and, according to the multiple binary complex model, are crucial to direct recognition. Furthermore the peptides available to be bound by HLA, the so called peptidome (Milner *et al.*, 2013), are altered through allotransplantation, due to the presence of non-self antigens. This change within the peptidome can therefore allow an alloreactive T cell's TCR to recognise a target pHLA with a higher affinity than self pHLA, otherwise the T cell bearing the TCR would have been deleted during the establishment of central tolerance (if self reactivity was high, the T cell would have been deleted), and this change in affinity leads to a response from the T cell. The change of the peptidome it can be then argued, reduces in amount, degree and intensity, the effects of negative selection, or in more concise terms abates negative selection. A measure of this response can be observed in the high frequency of alloreactive T cells, although other factors influence this high number of alloreactive T cells, and include the number of pHLA complexes upon the surface of APC and the influence of co-stimulatory signals, as well as the number of TCR upon the surface of the T cell (Smith *et al.*, 2012).

As an example of the influence peptides can have upon binding affinities of the TCR, the minor histocompatibility antigen (mHAG), HA-1, demonstrates how the change of a single amino acid within a peptide bound by HLA can significantly affect TCR recognition and affinity. mHAGs result from the presence of different gene polymorphisms between the donor and recipients, and can be encoded for by autosomal genes, or from proteins encoded upon the Y chromosome in sex mismatched grafts. These different polymorphisms result in the presence of different peptides being bound by HLA molecules which then act as alloantigens and induce an alloresponse (Roopeen *et al.*, 2002). The HA-1 mHAG results from the products of a dimorphic gene, *HMHA-1*. HA-1 is an HLA-A*02 restricted peptide, only one of the two

variants is known to cause alloreactivity, this being VLHDDLLEA, the other variant, VLRDDLLEA, differs at position 3 by a His(H)/Arg(R). Although both peptides bind to HLA-A*02, the allogenic H containing peptide allows greater structural stability when complexed with the HLA-A*02 molecule, and is presented at the cell surface in greater numbers than the R variant (Nicholls *et al.*, 2009). However, the R peptide is thought capable of forming complexes with HLA-A*02 and being expressed in the thymus during central tolerance. Consequently HA-1 can serve to highlight a process reducing the effect of negative selection. Whereby, if the immunogenic HA-1 H peptide was presented by HLA-A*02 upon thymocytes during the establishment of central tolerance and a TCR receptor was specific to this target above an appropriate threshold then it would have been deleted. However if HA-1 H was absent during central tolerance, and later introduced to the TCR (e.g. as a consequence of indirect allorecognition) then a response can be formed. In this concept a portion of the alloreactive T cells could be thought to be quasi autoimmune in nature, due to the 'undoing' of central tolerance, Figure 1.7.

The HA-1 R peptide has not been shown to generate an immune response and appears to form a less stable structure with HLA-A*02. Studies using Surface Plasmon Resonance (SPR) to measure HA-1 specific TCR and HLA-A*02 complexed with H and R peptide show a decreased affinity for the HA-1 R, with extremely rapid off rates (Nicholls *et al.*, 2009), which most likely accounts for this observation. In a direct allorecognition situation, assuming the binary complex theory is correct; where donor and recipient combinations represent an HLA mismatch, the HLA polymorphism results in a different set of peptides being bound, to which an alloreactive TCR can have an inappropriate affinity for, thus also abating negative selection and allowing the generation of a T cell response.

Comparison of the binding affinities of T cell's TCR, between TCRs of T cells which are specific for viral antigens, cancer antigens, autoimmune antigens and allo antigens, demonstrates that there are similar and 'robust' affinities between allo, viral and cancer antigen specific TCR in measurements carried out by SPR, and greater affinities for alloreactive TCRs than the autoimmune specific TCR affinities (Smith *et al.*, 2012).

1.1.13 *Factors that influence alloreactivity*

The overall contribution to alloresponses of the TCR upon alloreactive T cells, which are the result of altering the peptidome is uncertain, and must vary greatly between different donor and recipient combinations. Indeed, alloreactivity is influenced by a number of variables such as the number of mismatched alleles (the alloreactivity of certain mismatches also varies, with some being permissive mismatches and others being strongly immunogenic, which is believed to be a function of structural similarities between certain HLA molecules) and gender; females generally exhibit higher alloresponsive T cell frequency. Additionally, in healthy individuals a proportion of viral specific memory T cells exist, which have been shown to be cross-reactive in an alloresponsive HLA class I specific manner. Considering that Epstein-Barr virus (EBV) and cytomegalovirus (CMV) specific CD8⁺ T cell frequencies have been shown to be between 10%-40%, a significant pool of anti-viral memory T cells are available, some of which are capable of cross-reacting with donor antigens (Smith *et al.*, 2012). Although the mechanism by which this cross-reactivity occurs remains to be elucidated, given the orthogonal binding mechanism of TCR to pHLA, the bound peptide is likely to play an integral role, due to its interaction with the TCR (see section 1.1.8), the complementary-determining regions (CDR)3 somatically rearranged variable- (diversity)-joining regions of the $\alpha\beta$ TCR, which are the most polymorphic regions (see section 1.1.15), being positioned at the binding interface with the HLA bound peptide (Garcia *et al.*, 2009).

1.1.14 *The contribution of peptide to the mechanism of TCR-pHLA interaction*

Garcia and colleagues (2009) outlined the molecular bias of the TCR binding to pHLA, where they describe how this binding interface is composed of four structurally distinct components, which form composite surfaces. The portion of the TCR which binds to the pHLA can be divided into the invariant germline variable (V) gene encoded regions, which comprise the CDR1 and CDR2, and the junctions between the somatically rearranged variable-diversity (J) gene encoded- joining regions, which form the CDR3 region. The TCR binding region upon the pHLA is mostly comprised of conserved regions of scaffold upon the α -helix of the groove, within which the diversity of the peptide binding region of the HLA encloses a peptide. The binding of

the TCR to pHLA allows the interaction of the TCR's most variable region, CDR3, to the centre of the pHLA, allowing interaction with diverse peptides (Colf *et al.*, 2007), while the germ-line encoded CDR1 and CDR2 regions interact with HLA around the peptide binding region (Figure 1.8 and 1.9). Binding in this manner allows for 75-80% of the contact of the TCR and pHLA being achieved by CDR1 and CDR2, and this interaction is postulated as being a facilitator of the rapid TCR 'scanning' of pHLA, allowing identification of peptides within the pHLA which stabilize the half-life of TCR-pHLA interaction via CDR3 for a period long enough for signalling to occur (Garcia *et al.*, 2009).

This description highlights the intrinsic nature of the peptide in the activation of T cells via their TCR. In another paper, Corse and colleagues (2011) conclude that the stronger the interaction between TCR-pHLA, the more efficient the T cell response *in vitro*. While they report that in *in vivo* responses, there are likely to be constraints upon high and low levels of T cell stimulation, for example attenuation of strong signals to prevent detrimental inflammation, or, following a time lag, similar levels of T cell reactivity can be seen for low potency pHLA, whereby interrogation of pHLA by TCR may involve multiple encounters prior to activation of T cells. In addition reports state that variability in TCR-pHLA interactions could lead to expansion and differentiation of different T cell clones (Corse *et al.*, 2011 and Mirshahidi *et al.*, 2004), such as memory cells, or regulatory T cells. Indeed low dose of pHLA has been shown to favour transient FoxP3 positive T regulatory cell formation above FoxP3 negative T effector cells (Long *et al.*, 2010).

1.1.15 *The mechanisms of obtaining diversity within the TCR*

The TCR is unusual in being both highly diverse, consisting of an estimated 10^{15} potential sequences, and yet being restricted to recognising its antigen in the context of HLA (Birnbaum *et al.*, 2012). The mechanism of binding to the pHLA, as described in section 1.1.8, reveals a binding interface which consists of both highly diverse (CDR3) and more conserved (CDR1 and 2) regions, which facilitate these properties. The development of diversity within the TCR is achieved by recombination of the Variable (V), Diversity (D) and Joining (J) genes, so called V(D)J recombination, which is achieved

Figure 1: Binding groove. Image generated in Deepview/swissPDBviewer from PDB ID: 103J (Guesx & Peitsch 1997)

through a single recombinase enzyme, encoded for by the lymphoid-specific recombination-activating genes 1 and 2 (RAG1 and RAG2) (Hewitt *et al.*, 2010). Specifically the CDR1 and CDR2 regions are encoded for by the germline T cell receptor α -variable (TRAV) and β -variable (TRBV) genes, while the highly diverse CDR3 is encoded for by V and J gene segments in the α -chain of the TCR, and the V(D)J segments in the β -chain (Gras *et al.*, 2012). Furthermore additional variation can be generated within the CDR3 regions by the addition/deletion of non-nucleotide (N) templates at the V-(N)-J, V-(N)-D and D-(N)-J junctions (Gras *et al.*, 2012).

1.1.16 *The influence of HLA bound peptide on TCR mediated alloreactivity*

In support of the importance of the peptide in the binding mechanism and activation of alloreactive T cells, Morris and colleagues (2010) demonstrated that alloreactive and non-alloreactive T cells differed specifically at the CDR3 region of the TCR, supporting the hypothesis that CDR1 and CDR2 regions provide a germ line affinity for HLA, and offering an explanation for the ability for alloreactive T cells to recognise non-self HLA. In addition, there are at least two cases reported within the literature of the CDR3 region of the TCR providing the majority of the binding energy due to TCR recognition of bulged peptides (Liu *et al.*, 2012; Speir *et al.*, 2001).

The orientation of the TCR in relation to pHLA also appears to have an influence upon the ability of a TCR to recognise pHLA, with the orientation of the most variable region of the TCR positioned such that it engages with the peptide, highlighting the significance of the peptide in its ability to generate TCR mediated immune responses. There is some variation described within the literature between the precise mechanisms, but generally an orthogonal docking angle is seen in reported TCR/pHLA crystal structures (Garcia *et al.*, 1996; Garcia *et al.*, 2009). The α -chain of the TCR alignment falling over the NH₂-terminal end of the peptide, with the β -chain over the COOH-terminal. In the reported structures, the variation that has been observed is seen in the degree of flexibility within the TCR binding of pHLA. Whereby the angle to which TCR engages pHLA can vary from between 22 to 84 degrees. It appears the TCR approaches the binding of HLA with a single binding conformation, it then flexes and readjusts the interaction with pHLA to allow the formation of the single binding conformation (Felix and Allen, 2007).

Considering these findings the nature of the peptide presented by HLA in an alloimmune setting can induce a variety of effects upon the T cells which recognise them. This ranges from the generation of a response targeted to the allograft, resulting in allograft damage and/or rejection, to inducing allograft protection via the formation of FoxP3+ T regulatory cells (Sakaguchi *et al.*, 2001). The significance of the peptide bound by HLA to the triggering of immune responses has led many investigators to study the processes of peptide processing and presentation by HLA, as well as attempting to characterise the properties of peptides bound by specific HLA alleles.

1.1.17 Peptide processing and presentation

Given the extensive impact that HLA bound peptide has been demonstrated to have upon TCR recognition, whether in the context of alloimmune, autoimmune or immune responses against pathogens, the nature of this peptide binding and its orientation within the HLA molecule is likely to be of consequence. As such, the structure of HLA molecules and their mechanism of peptide presentation have been extensively studied. The nature of peptide processing and presentation is dependent upon the peptide source, and the class of HLA molecule to which they are bound for presentation.

1.1.17.1 The source of peptides in the HLA class I processing pathway

Class I HLA molecules present peptides derived from endogenous proteins to cytotoxic (CD8⁺) T lymphocytes. These peptides are generated via proteasome mediated degradation of proteins at the end of their functional lives, as well as newly synthesised proteins in the form of defective ribosomal products (DRiPs) (Schubert *et al.*, 2000), the source of these proteins can be derived from self proteins, or from pathogens, such as viral proteins due to a viral infection. The functional life of different proteins varies considerably and can range from minutes to hours, however a significant proportion of proteins are degraded immediately post synthesis, prior to the formation of functional proteins as a consequence of DRiPs. DRiPs being the result of defective transcription or translation, alternative reading frame usage and failed assembly into functional proteins via other mechanisms (Neefjes *et al.*, 2011). The DRiPs are degraded immediately to prevent aggregation of protein, this process therefore allows peptides to be presented by HLA class I extremely rapidly. Indeed the

influenza virus can be recognised by T cells, 1.5 hours post infection due to DRiPs, instead of 8 hours later when the first stable viral proteins are degraded by the proteasome of infected cells (Neefjes *et al.*, 2011). In addition to peptides being generated from proteins at the end of their functional life and from DRiPs, peptides which have been generated from non-continuous sequences which are a consequence of ligation have been observed (Neefjes *et al.*, 2011). Dalet and colleagues (2011) report the splicing of two protein fragments in the proteasome to form a peptide which is presented to CD8+ T cells by Class I HLA. In the transplant setting, this raises the intriguing theoretical possibility that allo and self protein fragments could be spliced to form a novel peptide.

The repertoire of peptides which can be bound by HLA class I molecules, known as the HLA class I peptidome (Milner *et al.*, 2013), can therefore include peptides from proteins which are non functional, peptides which are not encoded for by the genome due to ligation, and peptides from proteins which have reached the end of their functional life, all of which have been shown to be capable of eliciting an immune response. In addition to this variety of peptide origin, a different degradation pattern has been observed between the constitutive and the immuno proteasomes (Eleftheriadis, 2012), which can also produce variety in the peptides generated. Regardless of this wide source of peptides, loading onto the HLA class I molecule for presentation is essential for effective immunity. In order to facilitate this, a peptide loading complex is formed.

1.1.17.2 *The HLA class I antigen processing pathway*

As the peptides for class I HLA molecules are generated within the cytosol their transport into the ER, where HLA molecules are assembled, is required. This access to the ER is granted by the transporter associated with antigen processing (TAP), which also forms a part of the peptide loading complex (PLC). TAP is formed from two gene products, TAP1 and TAP2, and transports peptides of 8-16 amino acids in length into the ER. Once inside the ER, the peptides are processed to ensure their suitability for loading onto HLA molecules by the action of ER aminopeptidases, specifically ERAP1 and ERAP2, which trim the N-terminal ends of peptides (Wearsch and Cresswell, 2008),

These ER aminopeptidases are not however part of the PLC, and do not otherwise facilitate loading of peptides and assembly of HLA molecules.

The assembly of HLA and loading of peptides is achieved via a two stage process (Figure 1.10). Initially calnexin (CNX) and ERp57, which is a protein disulfide isomerase (promotes disulphide bond formation), facilitate early folding of the heavy chain (HC) and association with β_2 -microglobulin (β_2 M). After which, a second stage occurs whereby the HC and β_2 M are recruited into the PLC (Figure 1.11). The PLC is comprised of tapasin, which acts as a bridge between TAP and the HC, and is covalently associated with ERp57. ERp57 itself is also bound to calreticulin (CRT) and CRT is bound to a monoglucosylated *N*-linked glycan attached to the HC at Asn86 (Wearsch and Cresswell, 2008). In terms of PLC function, tapasin stabilizes the HLA molecule while it is empty (Elliot and Williams, 2005) and promotes high affinity peptide loading, in a process known as peptide editing. Interestingly the influence of tapasin upon peptide binding appears to be HLA allele specific, with peptide loading being heavily reliant upon tapasin in some HLA alleles, such as HLA-B*44:02, while others appear to be able to load peptide without the function of tapasin, for example HLA-B*27:05 (Elliot and Williams, 2005). HLA molecules and peptides, which do not form a complex with each other, are transported back into the cytosol for further trimming or destruction via peptidases and the proteasome. This transport out of the ER is achieved via the ER-associated protein degradation (ERAD) system (Neefjes *et al.*, 2011). Successfully complexed HLA class I and peptide is transported to the Golgi body and then transported from the Golgi to the cell surface with the aid of CD99, a leukocyte adhesion molecule. The absence of CD99 has been shown to result in a build up of MHC Class I within the Golgi (Bremond *et al.*, 2008).

1.1.17.3 The HLA Class II antigen processing pathway

HLA class II molecules are also assembled in the ER, where an α and β chain associate with an invariant chain (Ii), the resulting heterodimer-Ii complex is then transported to the late endosomal compartment known as the MHC Class II compartment (MIIC), again via the Golgi. The Ii blocks the peptide binding groove thus preventing endogenous peptide binding (Wolters and Chapman, 2000), the class II associated invariant chain peptide (CLIP) region of the Ii chain occupies the binding groove

(Neefjes *et al.*, 2011) and the Ii undergoes stepwise proteolytic degradation so that only the CLIP region remains. This CLIP/HLA class II complex can now load peptide for presentation at the cell surface, as CLIP, unlike the larger Ii, rapidly dissociates from the HLA class II molecule and is replaced by an exogenous peptide from a protein degraded in the endosomal pathway (potentially an exogenous pathogen, or donor protein). This replacement of CLIP with exogenous peptide requires the presence of another HLA class II molecule, the HLA-DM protein (Neefjes *et al.*, 2011). The successfully loaded class II HLA molecule is then displayed upon the cell surface. Crucially the polymorphism of both class I and class II HLA molecules provides a range of peptide binding characteristics upon different HLA molecules. To some degree this allows then, an ability to determine the likelihood of a specific peptide to be bound by particular HLA molecules using bioinformatic approaches (Helmberg, 2012), discussed in section 1.9.

1.1.18 The characteristics of peptide binding by HLA molecules

The genes within the MHC are highly polymorphic; indeed the HLA-B gene is believed to be the most polymorphic gene within the human genome (Yanover and Bradley, 2011). The polymorphism within HLA molecules is at its greatest in the sequences lining the peptide binding grooves, specifically the greatest polymorphism is encoded for by exons 2 and 3 of the Class I α -chain (HLA-A, B and C) and exon 2 for the class II β -chain of HLA-DR, DQ and DP molecules, in addition polymorphism is also seen within the alpha chain of DQ and DP molecules. This polymorphism produces a selective peptide binding motif for each HLA molecule, which results in the ability to bind a specific set of peptides, referred to as the peptide repertoire or peptidome (Rao *et al.*, 2011). The peptide repertoire of different HLA molecules can overlap, as a consequence the same peptide can be presented by numerous HLA molecules. Indeed this promiscuous binding of peptide may be as high as 60% (Rao *et al.*, 2011). This promiscuity of binding may be a disadvantage in terms of the immune response, due to the principles of the heterozygous advantage theory. Whereby, individuals who are heterozygote are able to present a wider range of peptides than homozygotes, due to the potential ability to display a wider peptide repertoire. The promiscuous binding of peptide by some HLA molecules will obviously reduce this effect for those peptides.

Indeed, individuals with HLA molecules which have unique peptide binding motifs have a lower chance of transmission of a pre-adapted virus (Rao *et al.*, 2011).

In the transplant setting these properties could have various effects, which are dependent upon the uniqueness of the peptide binding motifs of HLA mismatches, and the promiscuity of donor and recipient peptides. A promiscuous peptide could be bound by a donor or recipient HLA molecule and may or may not initiate an alloimmune response, depending upon the state of tolerance to the epitopes generated. Use of structural simulations to investigate peptide binding landscapes for HLA-A* and B* alleles have revealed that the HLA-B locus encoded antigens are, on average, able to bind a greater range of peptides, although this is not uniform for all alleles, some HLA-A* alleles being able to present a greater range of peptides than some HLA-B* alleles (Yanover and Bradley, 2011).

HLA alleles which share similar peptide binding characteristics are grouped into HLA supertypes, which has allowed the development of 'immunological hotspots' whereby regions of some antigens (such as the SARS coronavirus nucleocapsid) have regions of promiscuous T cell epitopes (Zhang *et al.*, 2011) i.e. they contain peptides which bind to multiple HLA molecules, these have therefore been used as potential targets for vaccine development. For HLA-Class I, nine supertypes have been identified, based upon overlapping peptide binding repertoires and groove structures, these being HLA class I supertype A1, A2, A3, A24, B7, B27, B44, B58 and B62 (Zhang *et al.*, 2011).

1.1.19 Predicting peptide binding to HLA

In order to allow a greater understanding of the relationship between peptide and HLA encoded molecules, several bioinformatic tools have been generated to provide an *in silico* assessment of peptide binding, such tools include the SYFPEITHI and the immune epitope databases (Helmberg, 2012). These tools utilise the described properties of peptide binding to the groove of HLA encoded molecules, and include bioinformatic systems for prediction of peptide binding to the HLA class I and class II molecules.

In the peptide binding groove of the HLA class I molecules, six binding pockets have been identified, A-F, which have allele specific characteristics, towards specific peptide

side chains, known as anchor residues (Fagerberg *et al.*, 2006). The six binding pockets allow amino acids, which have similar physicochemical properties, to be bound in a specific manner. The contribution to peptide binding is not uniform across all binding pockets, and peptide residues can be bound in pockets which are highly specific for that residue, these residues are termed primary or main anchor residues, the less specific residues are termed secondary anchor residues. The greatest contribution to peptide binding within HLA class I molecules comes from the N and C terminals of the peptide which are usually bound firmly by the ends of the binding groove, by pockets A and F respectively (Fagerberg *et al.*, 2006). Further binding energy is produced by hydrogen bonds between the HLA and backbone of the peptide. This concept of binding pockets has been broadened more recently, with some evidence suggesting the ability to predict peptides, which will bind to less well structurally documented alleles, such as the HLA-B*41 variants, requires a fine understanding of the amino acids present within the groove and each pocket (Bade-Doeding *et al.*, 2007).

Assessing peptide binding within HLA class II is more complex than that of HLA class I. Indeed state-of-the-art performance in peptide prediction reaches an AUC ROC (area under receiver operator curve) of 0.85-0.95 for HLA class I but only 0.70 to 0.85 for HLA class II (Zhang *et al.*, 2012), indicating a higher accuracy for HLA class I binding prediction than class II. The increased complexity of predicting HLA class II peptide binding is due to various differences to the class I binding. The binding groove in HLA class II molecules which accommodates peptide is open at the terminal ends, allowing longer peptides to bind, and also allowing peptide to bind at multiple sites. In addition, peptide residues which fall outside the peptide binding groove can interact with the HLA molecule and enhance its structural stability (Liao and Arthur, 2011).

An interesting feature of peptides eluted from HLA class II molecules and subsequently sequenced, is that a significant fraction of them are derived from intracellularly synthesised proteins, between 70-90%, depending upon the HLA allele from which the peptides were eluted (Leddon and Sant, 2010). This observation demonstrates that in the absence of a pathogenic challenge, HLA class II molecules present peptides which are most likely sourced from APC derived intracellular proteins, and not, in the main, extracellular derived proteins. Furthermore, it has been reported that 10-60% of these

peptides are themselves derived from HLA molecules themselves (Leddon and Sant, 2010). This is an interesting finding, considering the higher risk of mismatching HLA-DR over HLA-A and B in renal transplantation (Opelz 1997), and the role of endogenous peptides in allorecognition, described in section 1.1.20.

1.1.20 The role of endogenous derived peptides in allorecognition

The evidence for peptide dependent alloreactivity is increasing within both solid organ transplantation and HSCT. Within the limited number of structural models of this phenomena it is interesting to note that, where the peptide has been identified, the evidence suggests that alloreactive T cells recognise endogenous peptide (D'Orsogna *et al.*, 2013). Indeed in an investigation into 50 different alloreactive T cell clones, which were generated in a GvHD response *in vivo*, they were not only peptide specific, but led the authors to conclude, 'that the alloreactive T cells exert a high-avidity recognition for a single endogenously processed and presented peptide' (Amir *et al.*, 2011).

1.1.21 The importance of peptides in the alloresponse

The evidence then that peptides bound within HLA molecules have a significant influence upon allorecognition is incontrovertible. These peptides can be of MHC origin, or minor histocompatibility antigen (mHAG) derived, and can be from both the donor and the recipient depending upon the context in which they are presented. The alloresponse can be generated by a change in the peptidome, or the HLA molecules expressed, to which the recipient has not previously been exposed to during the establishment of central tolerance, which results in a change in the affinity to which T cell's TCR recognise pHLA, and results in a response from the T cell.

Given the effects of pHLA upon T cell clone formation by differential engagement of the TCR, it is not surprising that carefully selected peptides have been investigated as potential therapies for use in vaccination (Lovgren *et al.*, 2012), cancer therapy (Goulmy, 2004), preventing autoimmunity and tolerance induction (Mirshahidi *et al.*, 2004). In one such paper which examines the impact of peptide dose upon CD8⁺ T cell responses (Lövgren *et al.*, 2012), the authors conclude that low dose peptide vaccination generated stronger CD8⁺ T cell responses than high dose peptide, and

showed a lower dependence upon CD8 co-receptor. This is an intriguing finding considering the observation that alloreactive T cells have shown TCR cross-reactivity for MHC class I and II molecules (Smith *et al.*, 2012), and offers the potential explanation that this cross-reactivity is as a result of low frequency peptides. In the transplant setting, intrathymic injection of allopeptide has been shown to induce acquired systemic tolerance (Oluwole *et al.*, 2001), a process which re-establishes the central tolerance lost due to transplantation.

Recently the generation of a chimeric state in renal transplant recipients by simultaneous HSCT has been shown to be capable of providing an environment suitable for renal allograft survival, without maintenance immunosuppression (Kawai *et al.*, 2008). This work was carried out following the hypothesis of the interrelationship of chimerism and transplant tolerance outlined by Starzl and Demetris (1998). The proposed mechanism of achieving tolerance following chimerism has been postulated to be the elimination of 'reactive cells within the thymus which are reactive to donor antigen' (Kawai *et al.*, 2008), this would again restore central tolerance. Intrathymic deletion was found to be the 'dominant tolerance induction mechanism' through mixed chimerism, reviewed by Pilat and Wekerle (2010).

However central tolerance is certainly not the only player in such events (Pilat and Wekerle, 2010). Indeed administration of allopeptides, or cells which express single donor class I HLA, has been reported to result in down regulation of alloantigen specific cell-mediated responses by Tregs (Wood and Sakaguchi, 2003), the authors called for further characterisation of the allopeptides involved. The phenomena of Treg cells being critical for the maintenance of central tolerance is well reported, and can be seen in the development of severe autoimmune disease in regulatory T cell transcription factor Foxp3 deficient individuals (Kim *et al.*, 2011). Given the ability of changes within pHLA to alter the affinity of TCR-pHLA interactions, and that the affinity of the TCR for pHLA can influence T cell clone formation, the pHLA environment will have an impact upon this event. The differentiation of specific T cell clones will also be dependent upon the environment within which the pHLA is displayed, with a significant role for costimulation (Kim *et al.*, 2011). Therefore the pHLA can be said to have a significant role in both the generation of the alloresponse, due to the

phenomena of reducing or abating negative selection, and to achieve transplant tolerance through re-establishing central tolerance, via appropriate introduction of donor peptide antigens. Of particular interest is the role of endogenously sourced peptides within these phenomena.

1.2 Thesis aims

This thesis will seek to increase the understanding of the role of the peptide bound by HLA molecules in the context of alloreactivity in transplant recipients.

This will be achieved by identifying and assessing the function of endogenous peptides, which may have a role in the alloresponse within the context of clinical transplantation.

The key research approaches include:

In chapter 2 the research attempts to identify potential endogenously derived peptides, which may provide targets for allorecognition. Given that HLA class II molecules have been shown to present peptides derived from HLA class II molecules. A method for assessing this phenomena in HLA class I molecules will be investigated, specifically:

- A bioinformatic database of potential HLA class I derived peptides, which may bind to HLA-A*02, will be generated using peptide binding software
- Peptides bound by HLA-A*02 will be isolated from the HLA-A*02 molecule and characterised by mass spectrometry
- The characterised peptides will be searched within the bioinformatic database to determine whether they are derived from HLA class I molecules.

Research described in chapter 3 will seek to assess the role of peptides, identified from the research undertaken within chapter 2, in the context of clinical transplantation in relation to outcomes, specifically:

- HLA class I derived peptides, which are of particular interest in the alloresponse setting, will be identified and a 'key' showing the HLA alleles which encode these peptides will be generated

- This key will be utilised in a cohort of renal transplant recipients, which will be assessed for the effect of matching/mismatching these peptides, in relation to the performance of the allograft within the first 12 months post transplant.

In chapter 4, the experimental work will attempt to determine a functional role of the endogenous peptides identified in chapter 2, and seek to establish if variation within peptides bound by the same HLA molecule can influence the subsequent binding of HLA specific antibodies, and if so, seek to elucidate the mechanisms involved.

- A cell line which expresses HLA-A*02 will be loaded with peptides identified in chapter 2
- An HLA-A*02 specific antibody will be used to assess the influence of these peptides on the ability of an antibody to bind to its target
- Structural modelling will be utilized to examine the influence of peptide upon antibody binding, using the current understanding of electrostatic potential energy upon an antibody target and the antibody binding characteristics.

Chapter 2 - Identifying HLA Class I derived peptides as potential ligands in inducing alloreactivity

2.1.1 Introduction

This chapter will seek to create a database of peptides which can theoretically be derived from HLA-Class I molecules and then presented by another HLA-class I molecule. The database will then be utilised to identify any HLA derived peptides eluted from an HLA molecule.

2.1.2 Bioinformatics within transplant immunology and HLA peptide binding predictions

Within the field of transplant immunology there are a wide range of frequently used bioinformatic tools (Helmberg, 2012), which provide both a collection of experimental data, such as the HLA/IGMT database (Robinson *et al.*, 2013) or allele frequency database (Gonzalez-Galarza *et al.*, 2010). These allow access to information contributed to by the entire transplant immunology community. There are also bioinformatic tools which allow analysis of an investigator's own data, using the described properties of the bioinformatics tool in question, such as the use of HLAmatchmaker (Duquesnoy *et al.*, 2011), to ascertain permissible epitope mismatches between donor and recipient pairs.

The use of bioinformatics to predict peptide binding to HLA molecules dates back to the late 1980s, and essentially three approaches to determining the probability of binding are used, these being: (1) position specific scoring matrices (PSSM), (2) artificial intelligence (AI) systems and (3) structural based methods (Liao and Arthur, 2011). Some systems utilise a combination of these techniques. Both PSSM and AI use the sequences of previously eluted peptides from specific HLA molecules to predict the ability of peptides to bind to a given HLA molecule. As the particular binding pockets of an HLA allele will favour certain amino acid characteristics (acidic, basic, polar, non-polar, etc.) a likelihood of favourable binding can be established. The AI component allows for subtle relationships between amino acids within the peptide to be considered, most commonly PSSM and AI systems are used in parallel (Liao and Arthur, 2011). Structural based systems use the ever increasing number of crystal structures (Bjorkman *et al.*, 1987) to estimate the stability and strength of binding of a peptide to a particular HLA molecule, determining the free energy. A review of the peptide predicting bioinformatics tools available conclude that PSSM and AI combined systems

currently provide the best predictions (Liao and Arthur, 2011). Of these systems, perhaps the most widely reported within the literature is that of the SYFPEITHI database, first reported by Rammensee and colleagues (1999). The SYFPEITHI database was named after the sequence of the first peptide eluted from an MHC molecule, and contains a repository of such sequences combined with PSSM and AI peptide prediction. SYFPEITHI peptide binding prediction is based upon published peptide sequences, and only includes sequence prediction for HLA molecules for which a large amount of data is available. In line with all such bioinformatics tools, careful judgement regarding appropriate use of the data generated is required and, in the case of peptide prediction, certainty regarding actual binding of a peptide requires confirmation within the laboratory, either by eluting peptides from selected HLA molecules, or by assessing the binding of HLA molecules, by particular peptides, experimentally.

2.1.3 Confirmation of HLA peptide binding and sequencing of isolated peptides.

Various approaches within the literature are reported for isolating peptides from HLA molecules and their subsequent characterisation (Castellanos *et al.*, 2001; den Haan *et al.*, 1998; Bade-Döding *et al.*, 2011; Heinhold *et al.*, 2010; Harndhal *et al.*, 2009). Typically peptides are eluted from HLA by acid elution following the isolation of the HLA molecule itself (if not produced in a pure soluble form). Often High Performance Liquid Chromatography (HPLC) is used to separate the peptides prior to their sequence characterisation. Recently, sequence characterisation by mass spectrometry (MS) has been favoured within the literature, whereas previously Edman end terminal sequencing was the methodology of choice (Hoppes *et al.*, 2010). The combination of separation of HLA from a target cell (if not in a soluble form), isolation of peptide from the HLA itself, and then sequencing of the peptide makes this a time consuming process. However new approaches such as those reported by Heinhold and colleagues (2010), have demonstrated the utility of a two-step sequential immunoprecipitation and Matrix-Assisted laser desorption/ionization Time of Flight (MALDI-TOF) method to identify HLA-B bound peptides. The increasing use of MS for peptide characterisation is largely due to the increased sensitivity of mass spectrometers, and has led to the identity of the characteristics of large pools of peptides derived from different HLA grooves to be determined, this has aided the development of the previously described

PSSM (Falk *et al.*, 1991). However, it is often difficult to determine between self-peptides and non-self-peptides (either of pathogen or donor origin) when eluted from HLA, and consequently further characterisation of the identified peptides are often performed by measuring functional responses, meaning the overall process of identifying allogenic peptides can be time consuming (Hoppes *et al.*, 2010).

2.1.4 Mass spectrometry sequencing of HLA eluted peptides

Mass spectrometers ionise compounds (i.e. generate a positive (cation) or negative (anion) electrical charge), this generally causes fragmentation of the compound, some of these fragments can in-turn fragment further (Hoffmann and Stroobant, 2007). The ions, which are thus generated, are separated within a mass spectrometer according to their mass-to-charge ratio and detected in accordance with their relative abundances.

The visual representation of the data generated is a plot of the mass-to-charge ratios of the detected fragments and their abundances, and can be displayed as a table showing mass-to-charge ratio (m/z) vs relative abundance (%) or most commonly the data is plotted as a mass spectrum, with m/z plotted upon the x axis and relative abundance upon the y axis. The ions generated and displayed within the mass spectrum provide information regarding the nature and structure of the precursor compound. Where a pure compound has been ionised and is still present in an unfragmented form, it is referred to as the molecular ion. The molecular ion is present at the highest m/z value, giving the molecular mass of the compound, while fragments from the molecular ion will be of a lower m/z ratio. For example, if methanol is analysed the molecular ion appears at m/z 32 of the spectrum (methanol's molecular mass is 32), while there is also a peak on the spectrum at 15 m/z . Therefore there has been a loss from the methanol molecular ion of 17 ($32-15=17$). This equates to the loss of a hydroxyl group and is the m/z of methanol minus a hydroxyl group (Hoffmann and Stroobant, 2007). These principles are also used to study proteins and peptides, not only with the capability to detect complexes of linked amino acids, but also post-translational modifications (Hoffmann and Stroobant, 2007). In terms of peptide sequencing, as is appropriate here, the peptide must undergo fragmentation in the same manner as a chemical compound. In simplistic terms, fragmentation causes the bonds between adjoining amino acids to break, this allows the amino acids, and

fragments of amino acids which remain joined together, to have their molecular mass determined by the m/z ratios produced. In cases where a residual amount of the unfragmented peptide still remains, the molecular mass of the peptide itself can be determined. Two mass spectrometry techniques which are most often used in the study of peptides and proteins are electrospray ionization (ESI) and the previously mentioned MALDI. These two techniques are particularly suitable to the study of peptides and proteins as they do not require the analyte to be in a volatile state, a requirement which limited the study of non-volatile biological molecules prior to the introduction of ESI and MALDI (Hoffmann and Stroobant, 2007).

In previous reports of HLA derived peptide sequencing, MALDI has been used to great effect (Heinhold *et al.*, 2010). MALDI tandem mass spectrometry (MS/MS) peptide sequencing relies upon the peptide being dissolved in a matrix of small organic molecules, which is then dried and placed under vacuum conditions within the MALDI MS and pulsed with a laser. The organic compounds within the matrix have a strong absorbance at the wavelength of the laser, causing ablation of the matrix and also the peptide, generating an ionised gas phase (Hoffmann and Stroobant, 2007) which can then be separated within the MS, based upon the m/z of the peptide. In addition, through the use of tandem mass spectrometry (MS/MS) it is possible to isolate a particular peptide by identifying it through its m/z ratio in the first MS, then the peptide either undergoes spontaneous or induced fragmentation to yield peptide fragments for analysis within the second MS. In this way it is possible to select a peptide of a particular molecular weight from a mixture of peptides and then fragment and sequence that peptide (Hoffmann and Stroobant, 2007). MS/MS has been used in this way specifically for the purpose of characterising MHC class I peptides (Escobar *et al.*, 2011), the data produced however, is complex. Fortunately various 'free to use' bioinformatic data analysis tools are available, which can provide likely peptide fragmentation products given a known sequence (Steen and Mann, 2004), or allow comparison to previously characterised sequences.

2.1.5 Roepstorff-Fholmman-Biemann nomenclature and describing peptide fragments

When fragmentation of a peptide is induced, the fragments generated are comprised of different components of the peptide. In order to allow an accurate description of

these peptide fragments, a system known as Roepstorff-Fohlmann-Biemann nomenclature (Figure 2.1) is used to describe the fragmentation products (Steen and Mann, 2004). Essentially the fragmented peptide ions are referred to as a_n , b_n and c_n ions, when the amino terminus group remains, and z_n , y_n and x_n ions, when the carboxyl group remains (n equals the number of amino acid residues). The assigned a , b or c (or z , y and x) is dependent upon the position within the peptide to which the peptide is fragmented. As the 20 common amino acid residues have known masses, the mass difference between consecutive ions produced allows deduction of the peptide sequence (Table 2.1). The fragmentation of the peptide can produce a complex array of ions derived from the peptide, as stated earlier, various bioinformatic tools are available to help decipher this data and allow the sequence to be derived. Those bioinformatic tools most pertinent to this thesis, calculate the likely fragment ions of a given peptide, allowing the interrogation of a mass spectrum for those fragments. Such bioinformatic tools include the MS Product resource from the University of California mass spectrometry bioinformatics suite (www.prospector.ucsf.edu).

2.1.6 Assays to assess the binding of peptide by HLA

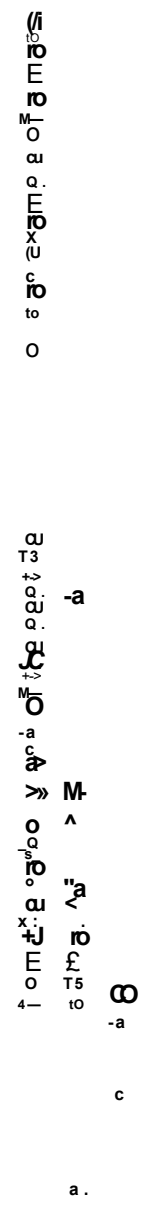
The ability of HLA to bind particular peptides can be investigated experimentally. Investigations of peptide binding are often used in conjunction with the bioinformatic tools discussed in section 2.1.1, or as a measure of the strength of binding peptides identified through those techniques discussed in section 2.1.3. Techniques assessing the binding of peptides to HLA and currently reported within the literature include: binding measurements assessed by the ability to inhibit binding of a fluorescently labelled reference peptide to HLA (Baxter *et al.*, 2004; Haan *et al.*, 1998), refolding of HLA molecules with test peptides (Harndahl *et al.*, 2009), surface plasmon resonance (Chen *et al.*, 1994) and TAP deficient cell line binding of specific peptides (Gatfield *et al.*, 1998; Wang *et al.*, 1998; Gricks *et al.*, 2001; Stuber *et al.*, 1995; Castellanos *et al.*, 2001). One advantage of the TAP deficient cell line approach to assessing peptide binding is that subsequent to successful binding, the cell line loaded with the bound peptide can be utilised in various functional assays (Castellanos *et al.*, 2001) or as targets for HLA antibody binding.

Figure 21 Røepstorff-Fohlmann-Biemann nomenclature used to describe the fragmentation

Z

x

x



in

b.g

| Residue | Three-letter code | One-letter code | Average Mass | Properties | Definition of Fragments |
|---------|-------------------|-----------------|--------------|------------|-------------------------|
|---------|-------------------|-----------------|--------------|------------|-------------------------|

here for completeness. Adapted from Hoffmann & Stroobant (2007).

The T2 cell line is one such cell line. T2 is a hybrid of T and B lymphoblasts and was first described by Salter and colleagues in 1985. As described in chapter 1 (section 1.1.17.2), TAP forms an essential component of the antigen processing and peptide loading pathway. Consequently cell lines deficient in TAP have reduced (Salter *et al.*, 1985) or absent (Gatfield *et al.*, 1998) expression of HLA at the cell surface. It should be noted that where encoded, HLA synthesis continues within TAP deficient cell lines, but when presented at the cell surface, without the stabilizing effect of bound peptide, the HLA dissociates rapidly. This has led to the discovery that incubating these cell lines with a peptide fitting the binding characteristics of the HLA molecule, allows stabilization of the HLA molecule at the cell surface (i.e. peptide binding is not achieved via traditional routes and is taken up at the surface). This can be measured as an increase in surface expression levels of HLA by the use of an HLA specific antibody, commonly the antibody used is specific to a conformational dependent epitope, to ensure correct structural orientation of the HLA detected (Gatfield *et al.*, 1998).

2.1.7 Aims of chapter 2

The work undertaken within chapter 2 will seek to establish a database of peptides derived from HLA-A*, B* and C* encoded alleles, which are theoretically capable of binding to HLA-A*02 encoded molecules. This database will then be assessed by obtaining peptides from an HLA-A*02 molecule and searching for matching peptide ligands within the database. This will provide information which will give an insight into the nature of HLA molecules binding HLA derived peptides, and allow the identification of specific combinations of HLA molecules and HLA derived peptide complexes. This insight will further the understanding of the allorecognition process.

2.2 Materials and Methods

A flowchart overview of all the methods described here is given at the end of this section on page 56, to provide an overview of the work flow.

2.2.1 Creating a bioinformatic database of peptides derived from HLA-A*, B* and C* encoded alleles with theoretical HLA-A*02 binding potential.

The SYFPEITHI database was used to generate peptides which have a high probability of binding to HLA-A*02 (Rammensee *et al.*, 1999; Liao and Arthur, 2011). Amino acid sequences of HLA Class I encoded alleles were obtained from the HLA/IGMT database (Robinson *et al.*, 2013) and entered into the SYFPEITHI database, to generate potential peptides theoretically capable of binding to HLA-A*02, which were derived from HLA-Class I encoded alleles. SYFPEITHI has a reported minimum reliability of retrieving the most 'apt' peptide in 80% of all predictions, identifying a naturally presented peptide within the top 2% of all peptides predicted in 80% of cases (Rammensee *et al.*, 1999). Thus for a protein sequence of 400 amino acids in length, the correct peptide will be in the top 8 scoring peptides in 80% of cases. The HLA-Class I α -chain is less than 400 amino acids in length, but to ensure the utility of the bioinformatic database generated, the top 10 scoring peptides were recorded in the order of their binding score. Briefly the SYFPEITHI binding score system examines every amino acid within a peptide, giving a value of 1 for an amino acid only slightly preferred at its respective position, while amino acids which are optimal anchor residues are given the value of 15. Amino acids are then scored depending upon their frequency within natural peptides previously eluted and characterised from the HLA molecule of interest. The scoring ranges from 1-15 based upon these frequencies, while negative values are also assigned when an amino acid has been shown as being disadvantageous in a particular position (Rammensee *et al.*, 1999).

The molecular weight was calculated for each peptide generated, and the position of the peptide within the HLA class I α -chain was also recorded. This process generated a database containing peptides derived from the amino acid sequences of all HLA-A*, B* and C* encoded alleles (>6000) listed upon the HLA/IGMT database, which were predicted to match the binding characteristics of HLA-A*02.

*2.2.2 Identification of HLA-A*02 expressing THP-1 cells and recovery of their membrane fragment*

The monocytic cell line THP-1 (ATCC TIB-202) was used as a source of HLA-A*02, as the previously reported HLA expression included HLA-A*02 (Tsuchiya *et al.*, 1980). THP-1 cell line authentication was performed by HLA typing according to the PCR sequence-specific primers (SSP) phototyping method (Bunce *et al.*, 1995), and through commercially available short tandem repeat (STR) cell line authentication (LGC standards, UK). THP-1 cells were cultured in RPMI 1640 medium (5% Fetal Calf Serum (FCS), 200mM L-Glutamine, 100 µg/ml penicillin/streptomycin (Life Technologies, Fulton, UK), until confluent. Cells were washed twice in 0.85 % phosphate buffered saline (PBS) (Life Technologies, Fulton, UK) (pH 6.8-7.2,) with 5% FCS and then resuspended in PBS. A confluent flask of THP-1 cells provided approximately 1g of cells post washing. A 3ml glass homogeniser was used to lyse the resuspended THP-1 cells, (5 plunges on ice), the resultant lysed cells were centrifuged at 5000g at 4°C for 10 minutes, to remove the nuclear and mitochondrial components from the lysed cells. The pellet containing the nuclear and mitochondrial components was discarded, while the supernatant was retained and centrifuged at 100,000g at 4°C for 1 hour to isolate the membrane fraction. The supernatant was discarded and the pelleted membrane fraction was resuspended in 0.5M TRIS-HCl (pH 7.2).

*2.2.3 Dynabead isolation of HLA-A*02 encoded molecules from the isolated membrane fraction*

HLA-A*02 molecules were isolated from the membrane fraction using a Dynabeads co-immunoprecipitation kit (Life Technologies, Fulton, UK), this kit allows a pure protein isolate, without contamination from the isolating antibody. To obtain HLA-A*02 molecules from the cell membrane fraction, monoclonal mouse anti-HLA-A*02, BB7.2 (Abcam, Cambridge, UK), was coupled to M-270 magnetic dynabeads according to the kit protocol. Briefly, 10mg of M-270 magnetic dynabeads were washed in 1ml of kit C1 wash buffer by pipetting, the magnetic beads were placed upon a Dynamag (Life Technologies, Fulton, UK) for 1 minute. This allowed attraction of magnetic beads to the Dynamag and therefore to move to the side of the separation tube, the C1 wash was then aspirated and discarded. 14µg of BB7.2 antibody was transferred to the separation tube with the addition of C1 wash buffer to give a total volume of 500µl. An

equal volume of C2 kit coupling reagent was added to this solution and gently mixed by pipetting, and placed on a rotating mixer for 24hrs at room temperature (RT). Post incubation, the separation tube was placed upon the Dynamag for 1 minute and the supernatant was aspirated. 800µl of HB kit wash reagent was added, and the beads were mixed by gentle vortexing and placed upon the Dynamag for 1 minute, the supernatant was then removed. Subsequent washes using LB and then SB kit reagents repeated this process, the final SB wash buffer included a 15 minute incubation period on a rotating mixer at RT, prior to placing on the Dynamag and subsequent removal of the supernatant. The BB7.2 coupled M-270 dynabeads were then resuspended in 1ml of kit SB wash, giving a final concentration of 10mg/ml BB7.2 conjugated M-270 dynabeads.

1ml of BB7.2 coupled dynabeads at 10mg/ml concentration was added to a tube containing the separated cell membrane fraction and placed upon a rotating mixer, ensuring the dynabeads remained in suspension for 40 minutes at 4°C. After this time the tube was placed upon the Dynamag, drawing the dynabeads to the side of the tube and the remaining liquid removed. The dynabeads were then washed using 200µl of kit extraction buffer by gentle pipetting, before placing upon the Dynamag and aspirating the supernatant as before, this was repeated three times. A final wash using kit LWB buffer consisted of the addition of 200µl of LWB buffer and a 5 minute incubation on a rotating mixer at RT, before aspiration post Dynamag incubation as before. After these washing stages, the BB7.2 captured protein was eluted by means of a glycine elution buffer, via a 5 minute incubation at RT on a rotating mixer and then placing on the Dynamag for 1 minute, the aspirated supernatant, containing the isolated HLA-A2, was transferred to a clean 1.5ml Eppendorf tube. Protein concentration of the eluted product was measured using the NanoDrop (Thermo Scientific, Wilmington, USA) spectrophotometer by measuring absorbance at 280nm, with the elution buffer being used to correct for background.

2.2.4 Confirmation of protein capture via Dynabead isolation

Following precipitation of HLA by BB7.2 anti-HLA coupled Dynabeads as described above, the HLA protein was obtained while the capture antibody remained bound to the Dynabeads. To confirm the presence of the protein purified by BB7.2 anti-HLA

antibody, the elutant containing the protein was separated on a 12% Bis-Tris 1.6mm gel (Life Technologies, Fulton UK), in conjunction with a Novex sharp protein standard (Life Technologies, Fulton UK) protein size marker, via MOPS SDS (3-(N-morpholino)propanesulfonic acid, sodium dodecyl sulfate) (Life Technologies, Fulton UK) electrophoresis and then stained using silver staining (ProteoSilver Plus Pro Silver stain, Sigma-Aldrich, Dorset, UK) according to the manufacturer's instructions, with the single exception being within the stage of the silver staining assay termed sensitisation (whereby pores within the gel are generated to allow silver staining of the protein within the gel to occur). Briefly, the protocol fixes the gel, post electrophoresis, using a 50% ethanol, 40% acetic acid with 10% H₂O solution, followed by washing, firstly with a 30% ethanol solution and then with distilled H₂O. The sensitisation stage was then performed using the kit sensitisation solution. As the 12% Bis-Tris gels used were of a greater thickness (1.6mm) than those recommended by the manufacturer (1.0mm) of the silver staining kit, optimum staining was obtained when the incubation time for the sensitising stage was increased to 20 minutes as opposed to the manufacturers' recommended 10 minutes. Further washing was performed using distilled H₂O, post sensitisation, before the kit silver stain solution was added for 10 minutes, and then decanted. The gel was then washed briefly (90 seconds) with distilled H₂O, the H₂O was decanted and a kit developer solution was added. A constant visual inspection was maintained during gel development, when bands became visible upon the gel, a kit stop solution was added for 5 minutes to stop the reaction, before a final wash and storage in H₂O. The stained bands on the gel were excised using a scalpel and destained according to the silver staining kit protocol, by addition of gel destaining solution until the band disappeared and then washing with H₂O. An 'in-gel' digest was performed upon the excised destained fragments to release the peptides bound by HLA captured by BB7.2 for analysis via MALDI MS/MS.

2.2.5 'In-gel' digestion of excised gel fragments

Each excised and destained gel fragment was placed in a 1.5ml Eppendorf tube with 75µl of 100mM ammonium bicarbonate : acetonitrile (ACN) (1:1), vortexed and incubated for 15 minutes at room temperature, the solution was then aspirated and the process repeated once. 75µl of 100% ACN was then added for 30-60 seconds until the gel fragments had shrunk and turned white, the ACN was then aspirated and

discarded. The gel fragments were then rehydrated in digestion buffer, containing 50mM NH_4HCO_3 , 5mM CaCl_2 , 12.5ng/ml proteomic grade trypsin (Sigma-Aldrich, Dorset, UK), ensuring the gel fragments were covered and then incubated on ice for 15 minutes. The supernatant was then removed and replaced with 70 μl 50mM NH_4HCO_3 , 5mM CaCl_2 solution and incubated at 37°C overnight. The gel fragments were briefly centrifuged for 60 seconds at 500g to pellet the fragment and allow aspiration and transfer of the supernatant into a new 1.5ml Eppendorf tube. The resultant supernatant was concentrated and desalted using an Amicon Pro 3kDa filtration device (Millipore, Maine, USA) and then analysed via MALDI MS immediately.

2.2.6 Alternative method of peptide isolation using Superdex 75 column

An alternative protocol to obtain peptides from the HLA-A2 molecules isolated in section 2.2.3 utilized an Akta purifier (Amersham Biosciences, UK) coupled with a Superdex 75 column (GE Healthcare Lifesciences, Buckinghamshire, UK), to achieve liquid chromatographic separation based upon size in a 0.05M PBS (Life Technologies, Fulton, UK) 0.15M NaCl pH7.0 buffer run at 0.5ml/min. Protein isolated with an approximate size of 500-1500 Da was predicted to contain peptide which was derived from the protein bound by the BB7.2 isolated HLA and was utilized for MALDI MS/MS. The protein separation characteristics of the column are determined for known RMM standards separated upon the column. Bovine Serum Albumin (BSA) (67,000 kDa) 8mg/ml, Ovalbumin (43,000 kDa) 25mg/ml, Ribonuclease (13,700 kDa) 5.0mg/ml, Aprotinin (6,512 kDa) 0.5mg/ml and Vitamin B12 (1,355 kDa) 0.1 mg/ml were used for this purpose, protein standards were filtered through a 0.45 μm filter to remove aggregates. As fractions were eluted from the column, protein was detected by absorbance at 280nm. All fractions from the column were collected in 1ml aliquots directly from the column and were retained. Fractions predicted to contain the isolated peptide based on size were concentrated and desalted using an Amicon Pro 3kDa filtration device (Millipore, Maine, USA) before MALDI MS analysis to remove any aggregates.

2.2.7 MALDI MS/MS and bioinformatic database assessment of isolated fragments

As recommended for protein and peptide analysis of targets <10kDa, a MALDI matrix constituting 10mg/ml α -cyano-4-hydroxycinnaminic acid (CHCA) in 50% ACN, 50% H_2O

and 0.1% trifluoroacetic acid (TFA) (Hoffmann and Stroobant, 2007) was pipetted (0.5µl) onto a MALDI OPTI TOF 192 target plate (Applied Biosystems, CA, USA) and left to dry for 20 minutes. The proteins isolated as described in 2.2.5 and 2.2.6 were pipetted (0.5µl) directly onto the dried MALDI matrix and left to dry for 10 minutes. After drying, the MALDI plate was loaded onto a QSTAR mass spectrometer for protein analysis. Any resultant peaks upon the mass spectra were searched within the bioinformatic database generated within 2.2.1 of this section for corresponding molecular weights of the predicted peptide ligands. For example if an m/z ratio of 1115 was observed within the mass spectra, then a search for a peptide within the database corresponding to this molecular weight was performed. If any hits within the database were observed, MALDI MS/MS was performed upon the specific identified peptide to derive its sequence. The sequence described within the database was entered into the MS product peptide fragment calculator (www.prospector.ucsf.edu) to aid this process. In addition, the potential sequence as described within the database were commercially synthesized (ThinkPeptides, Oxford, UK and Pepceuticals, Enderby, UK) and reconstituted from lyophilised powder via a step wise addition of dimethyl sulphoxide (DMSO) (Sigma-Aldrich, Dorset, UK). These commercially synthesized peptides were analyzed by MALDI MS/MS therefore allowing direct comparison with the peptides generated from the HLA-A*02 molecules isolated from THP-1 cells. Thus, if a peptide was identified within the isolated HLA-A*02 elutant, which corresponded to the molecular weight and sequence of a peptide within the database then it could confidently be stated to be identified as an HLA-class I derived peptide, which is presented by HLA-A*02. In a final step however a binding assay was performed to assess the ability of the identified peptide to be bound by HLA-A*02.

2.2.8 HLA-A*02 peptide binding assay using the T2 cell line.

Previously described minor histocompatibility antigens (mHAG), shown to bind to HLA-A*02 (Spencer *et al.*, 2010) were used as positive controls. These were HA-1, HA-2 and SMCY mHAG, with the following sequences VLHDDLLEA, YIGSVLISV and FIDSYICQV respectively. The positive controls were synthesized as outlined for the test peptides in section 2.2.7. Optimal concentration of peptide (25µg/ml) for the binding assay was established via titration with the HA-1, HA-2 and SMCY peptides at 5µg/ml – 200µg/ml concentrations.

The binding assay was performed as previously described (Pinilla-Ibraz *et al.*, 2006). Briefly 1×10^6 T2 cells were washed in serum free RPMI 1640, and incubated overnight at 37°C 5% CO₂/95% air with 10µg/ml β₂-microglobulin (Sigma-Aldrich, Dorset, UK) and optimal test peptide concentration (25µg/ml). T2 cells were then washed to remove peptide not bound to HLA-A*02, in serum free RPMI 1640 (Life Technologies, Fulton, UK) media and stained with mouse monoclonal anti-w6/32 (w6/32 is the name of the cell line first used to produce the antibody) IgG2a conjugated to Phycoerythrin (PE) (Abcam, Cambridge, UK) for 30 minutes, followed by washing (x3 in RPMI 1640 at 500g) to remove unbound antibody and fixing with 0.5% paraformaldehyde (Sigma-Aldrich, Dorset, UK)/0.05M PBS (Life Technologies, Fulton, UK). Analysis was performed using a FACSCalibur (BD biosciences, Oxford, UK) flow cytometer and fluorescence measured upon the FL-2 channel. Negative controls of DMSO alone and Class II derived peptides in DMSO, previously described as being able to be bound by HLA class II molecules (Costantino *et al.*, 2012) which were a kind gift from Dr Aravind Chekuri (St James University Hospital, Leeds, UK) were also used. Gates were set using a monoclonal isotype IgG2a PE conjugate control (Abcam, Cambridge, UK). Positivity was calculated by means of a fluorescence Index (FI), this being:

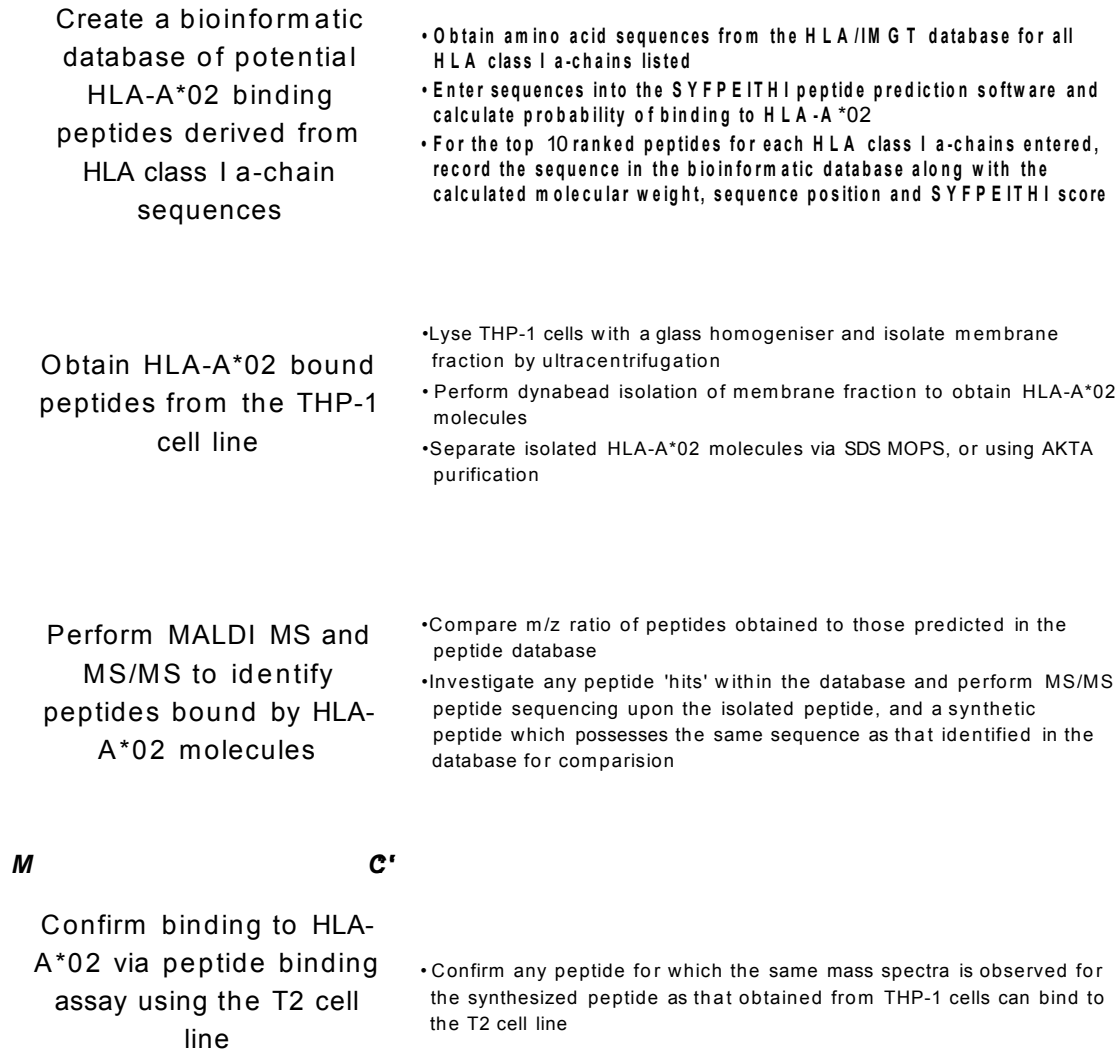
FI test peptide – FI negative control

FI negative control

A FI >0.5 was considered positive, which corresponds with the previously reported methods of determining positive reactions within this assay (Zhu *et al.*, 2003; Stuber *et al.*, 1994)

2.2.9 Reproducibility of assays described in section 2

All experiments described within this chapter were set up in triplicate, and repeated on at least three occasions unless otherwise stated.



v; .

Figure 2.2 An overview of the work flow for experimental methods utilised within Chapter 2.

2.3 Results

2.3.1 Generation of the HLA-A*02 specific HLA class I derived peptide database

The peptide database currently contains nearly 3000 peptides, with sequences derived from HLA class I molecules, which are predicted to bind to HLA-A*02 encoded antigens with a high affinity, according to the algorithm utilized within the SYFPEITHI program. A screenshot of the database is shown in Figure 2.3. The database groups peptides into the 10 top scoring peptides for each HLA allele, these alleles in turn are grouped into P groups (i.e. they contain the same protein sequence within their peptide binding region). The database was structured in this way to keep the data as concise as possible to aide in searching. For each peptide sequence recorded, the HLA alleles encoding the peptide are shown, along with the position of the peptide sequence within the HLA allele encoding it, followed by the score assigned by the SYFPEITHI program and then the calculated molecular weight. This allows the database to be searched for peptide sequences which match a particular molecular weight, or to search for peptides derived from particular regions within the amino acid sequences of the HLA-class I α -chain. This is achieved by using the 'find' function within the Microsoft excel program. For searching for a peptide of a molecular weight of 999 Da, for example the user would enter 999 in the 'find' function, and any peptide with this molecular weight can be viewed and searched through, recording the alleles encoding the peptide, the peptide sequence and position of the peptide as the user progresses through the search.

2.3.3 Obtaining peptides from HLA-A*02 molecules

In order to validate the database, peptides were obtained from HLA-A*02 molecules expressed by the THP-1 cell line. Figure 2.4 shows the eluted products of the Dynabead isolation of HLA-A*02 by the HLA-A*02 specific BB7.2 monoclonal antibody, from the membrane fraction of THP-1 cells. The gel was stained via the sensitive silver staining method, and the images reveal three distinct bands obtained by the immunoprecipitation, this corresponds to previously described properties of BB7.2 (Peace-Brewer *et al.*, 1996; Brady *et al.*, 2000). The three bands visible have been described as a result of different sialic acid / glycosylation states by these authors. 'In gel' digests were performed upon all three excised bands and multiple common

peptides were identified within the bands as would be expected from a single HLA-A*02 encoded antigen.

Separation of the BB7.2 purified product from the Superdex 75 column reveals several protein peaks, as determined by measurement of absorbance at 280nm, as fractions were eluted from the column (Figure 2.5). The reported optimum separation capacity of the Superdex 75 column is between 70,000-3,000Da, as a consequence the fractions most likely to contain the peptide, which were obtained from bound HLA-A*02 (approximately 500-1,500Da), would be expected to be eluted off within the last fractions from the column. The last fractions collected showed a corresponding estimated size of approximately 1000Da, according to the characteristics of the column in terms of the elution time for the protein standards.

2.3.4 MALDI MS for preliminary identification of peptides

The MALDI MS of the peptides produced by both methods, produced MS spectra containing peptides, which were within the size range of peptides which expected to be bound by HLA class I molecules (approximately 500-1500Da) (Figures 2.6, 2.7 and 2.8). MALDI MS analysis of the matrix only, aided determination of the m/z peaks which were associated with the matrix, allowing analysis of true peptide m/z data only. The m/z ratios of the peptides were entered into the peptide database, as described in section 2.2.7. Hits within the database are marked on the MS spectra. Figure 2.6 shows 8 peptides with m/z ratios ranging from 1013.66 to 1098.01. When these were entered into the peptide database, 1013.66, 1050.12, 1066.08 and 1098.01 produced a hit with potential sequences of VMAPRTLIL and/or VMAPRTLLL (1013.66), QLRALEGT (1050.12), YLENGKETL (1066.08) and YWDGETRKV (1098.01) being given, these were shown to be encoded for by various HLA molecules by the peptide database.

In terms of the size of the peptides within the final fractions eluted from the Superdex 75 column, the MALDI MS analysis determined their size to be within 900 and 1200Da as expected by the determined characteristics of the column (Figure 2.5[B]).

Furthermore, MALDI MS/MS established the sequences corresponded with those peptides obtained from the excised gel fragments and 'in gel' digests, and those from the column separation.

2.3.5 Determination of HLA type for THP-1 cells and characterisation of HLA derived peptides bound to HLA-A*02

The cell line validation of THP-1 included the determination of the HLA type, this identified a discrepancy with the previously reported HLA type for THP-1 (Tsuchiya *et al.*, 1980), allowing a higher resolution revised THP-1 HLA type, HLA-A*02; B*15; C*03; DRB1*01, DRB1*15; DRB5*01/02; DQB1*05, DQB1*06, to be determined (Battle *et al.*, 2012). The previous type being reported as HLA-A2, A9, B5, DRw1 and DRw2 (Tsuchiya *et al.*, 1980) (the nomenclature used here corresponds to the contemporary system at the time of THP-1 cell line establishment), specifically the reported higher resolution type now includes alleles at the additional HLA-C*, DRB5*, and DQB1* loci, and identifies HLA-A as being homozygote for HLA-A*02 rather than co-expressing HLA-A9. The HLA-B* alleles were shown to be B*15 rather than those encoding for the B5 antigen, and finally the reported DRw2 serological antigen is split into the DRB1*15 alleles which encode for the DRw2 antigen.

The HLA type of THP-1 included those antigens which encoded the peptides identified as hits within the database from the MALDI MS data in Figure 2.6 VMAPRTLIL (1013.66) being encoded for by HLA-C*03, QLRALEGT (1050.12) by HLA-A*02, YLENGKETL (1066.08) by HLA-A*02 and B*15, and YWDGETRKV (1098.01) encoded for by HLA-A*02.

VMAPRTLLL (1013.66) was not encoded for by HLA molecules expressed upon THP-1. The peptide database identified VMAPRTLLL as being encoded for by HLA-A*01, A*03, A*11, A*29, A*30, A*31, A*32, A*33, A*36, A*74; C*02, C*15 molecules.

All the HLA alleles which are capable of encoding the peptides identified in Figure 2.6 and producing hits within the peptide database are shown in table 2.2. In addition the table catalogues all peptides identified as hits within the database within figures 2.7 and 2.8. Showing a total of thirteen peptides, seven of which produced hits within the database. The MALDI MS spectrum in Figure 2.7 reveals a single peptide hit, ALGFYPAEI (MW 980 Da) which is encoded by HLA molecules which include HLA-B*15 antigen on THP-1. Two other peptides did not produce a hit within the database. In Figure 2.8 two further hits were observed, with peptides SMRYFTTSV (MW 1137 Da) and SMRYFYTSV (MW 1153 Da), both of which are encoded by various HLA molecules (see table 2.2) including HLA-A*02, which is expressed by THP-1 cells.

Those peptides which did not produce a hit within the peptide database are also shown within table 2.2 which include masses of 1002, 1018, 1029, 1031, 1060 and 1082 Da. Other peaks within the MS spectra shown within figures 2.6, 2.7 and 2.8 represent those associated with the MALDI matrix used within the method to aid the ionisation of the proteins. The m/z ratio of 1013.66 was the only result which provided two hits within the database, all other peaks resulted in a single, or no hit within the database. In addition the 1013.66 m/z ratio was the only peak which resulted from a HLA molecule which was not encoded for by THP-1 cells. Albeit this was in conjunction with a peptide hit which did have a HLA molecule encoding it, which was demonstrated as being expressed upon THP-1 cells, VMAPRTLIL (1013.66), being encoded for by HLA-C*03 on THP-1 cells. The identification of peptides in this manner produced a tentative identification, which required further analysis to confirm that the sequences of the peptides identified by their MW, have the same amino acid sequences as those within the peptide database.

2.3.6 MALDI MS/MS sequencing of peptides identified in 2.3.4

In order to confirm the identity of the peptide sequences MALDI MS/MS was performed. Table 2.3 and 2.4 shows the predicted fragmentation products of the peptides identified with a MW of 1013Da. These being VMAPRTLIL and VMAPRTLLL, which were obtained using the MS product peptide fragment calculator (www.prospector.ucsf.edu). Tables 2.3 provides the MW of the b, c, y and z ions (as per Roepstorff-Fohlmann-Biemann nomenclature described in section 2.1.5) which would be expected to be produced if the peptide was in fact VMAPRTLIL, while table 2.4 contains a more comprehensive list of potential peptide fragmentation products, which includes fragmentation products which have lost H₂O, NH₂ and COOH groups (amongst others) during the MS/MS analysis. Unfortunately, as VMAPRTLIL and VMAPRTLLL differ only by the presence of amino acids leucine and isoleucine, at the same positions within the peptide, and both of these amino acids have the same molecular weight (131.18 Da), their fragmentation patterns are identical. This confounds the ability to determine between them in this manner and only the presence of both of these peptides should be considered. The fragmentation pattern observed during MALDI MS/MS does however match the expected patterns of these two peptides (Figure 2.9).

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Table 2

Mass Spectrometry Data

The predicted m/z ratios are shown in bold. Calculated using MS Product (prospector.ucsf.edu) and checked using Hoffmann and Stroobant (2007).

In an additional confirmation step, a synthetic peptide commercially synthesized to have the same sequence as VMAPRTLIL was also subjected to MALDI MS/MS. This also shows the same peptide fragmentation products induced through collision induced dissociation (CID) during MS/MS (Figure 2.10). This reveals a MS/MS spectrum that matches the predicted peptide fragmentation patterns of VMAPRTLIL given in tables 2.2 and 2.3, and the peptide fragmentation pattern seen within the MS/MS spectrum of the peptide identified by the MW of 1013 Da. Confirming that this is most likely either peptide VMAPRTLIL or VMAPRTLLL. As the MALDI MS/MS was able to confirm the presence of either VMAPRTLIL or VMAPRTLLL, but not determine between them, the flow cytometry T2 peptide binding assay was used to establish if both of these peptides could in fact be bound by HLA-A*02, the source of the initial bound peptides. Figure 2.11 presents this data as overlaid flow cytometry histograms, demonstrating assay validity via the positive and negative controls and then showing the overlaid VMAPRTLIL peptide result over the negative control. This demonstrates this peptide binds to HLA-A*02 on the T2 cells. However, the VMAPRTLLL peptide does not appear to stabilize the HLA-A*02 molecules present on T2 cells. This can be seen in the histogram showing the VMAPRTLLL peptide laid over the negative control. Comparison of the histograms shows VMAPRTLIL strongly stabilizes the HLA-A*02 molecules, while VMAPRTLLL closely resembles the negative control.

Interestingly the peptide database reveals that both the VMAPRTLLL and VMAPRTLIL peptides are found at a common site of their respective HLA antigens, both being present at position 3 of the HLA-class I α -chain. This region contains the leader peptide sequences of HLA class I molecules, and was investigated further in chapter 3.

However, since the leader peptides appear to have common characteristics, an analysis was performed to determine if any other leader sequences could be bound by HLA-A*02. Commercially synthesized leader peptides were subjected to the same T2 binding assay as the VMAPRTLLL and VMAPRTLIL peptides.

This reveals that VMAPRTLIL (identified as leader peptide 7 and highlighted yellow) is the only leader peptide which is capable of binding to HLA-A*02 as this is the only peptide stabilizing HLA-A*02 at the surface, all the other leader peptides, shown in Figure 2.12 as leaders 1, 2, 3, 4, 5, 6, 8, 9 and 10 (with their sequences also given), have a FI of less than 0.5.

Over the course of the study, the authors found that the binding of the peptide to the class II HLA-A*02

was significantly reduced in the presence of the peptide. This suggests that the peptide may be acting as a competitive inhibitor of the binding process.

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negative control was an HLA class II binding peptide previously shown to bind to the class I HLA-A*02

This includes the VMAPRTLLL peptide (also highlighted yellow to enable easier comparison with VMAPRTLIL), this again confirms that VMAPRTLIL is able to be bound by HLA-A*02 and VMAPRTLLL is not.

Considering that THP-1 cells do not express the HLA molecules which include a VMAPRTLLL peptide, and that the VMAPRTLLL peptide cannot be bound by HLA-A*02, the molecule used as a source of these peptides, then the VMAPRTLLL peptide is extremely unlikely to be the potential 1013 MW peptide identified in the peptide database. The VMAPRTLIL peptide however, which was also identified within the database, can be bound to HLA-A*02 and is also present in one of the HLA molecules which is expressed upon THP-1, specifically the HLA-C*03 molecule.

MALDI MS/MS of the other peptides identified as hits within the peptide database did not suffer the potentially confounding problems of two identical peptide masses being identified within the peptide database. Of those other peptides tentatively identified by the peptide database and MALDI MS, the peptide with the ALGFYPAEI (MW 980) sequence was the only other peptide which was present within the HLA molecules present upon THP-1 cells, which was also not present in the HLA-A*02 molecule (table 2.2), ALGFYPAEI being present in the HLA-B*15 molecule on THP-1 cells. Again to confirm its identity MALDI MS/MS sequencing was performed.

The fragmentation products which would be expected from the ALGFYPAEI (MW 980) peptide are shown in table 2.5 for the b, c, y and x ions and table 2.6 for other potential fragmentation products.

The MALDI MS/MS spectrum for ALGFYPAEI (Figure 2.13) reveals a fragmentation pattern which matches that predicted for ALGFYPAEI, confirming the presence of this peptide. Therefore both ALGFYPAEI and VMAPRTLIL are confirmed as being bound by HLA-A*02. Neither of these peptides sequences are present in HLA-A*02 molecules, indicating that they are a result of peptides being bound by HLA-A*02. The ALGFYPAEI peptide is found within the HLA-B*15 molecule (table 2.2) present upon THP-1 cells, and this peptide is then presented by HLA-A*02. Interestingly ALGFYPAEI is present in several other HLA alleles according to the peptide database, these being HLA-A*01, A*03, A*11, A*23, A*24, A*30; B*15, B*18, B*27, B*35, B*38, B*39, B*40, B*41, B*42, B*44, B*45, B*46, B*47, B*48, B*49,

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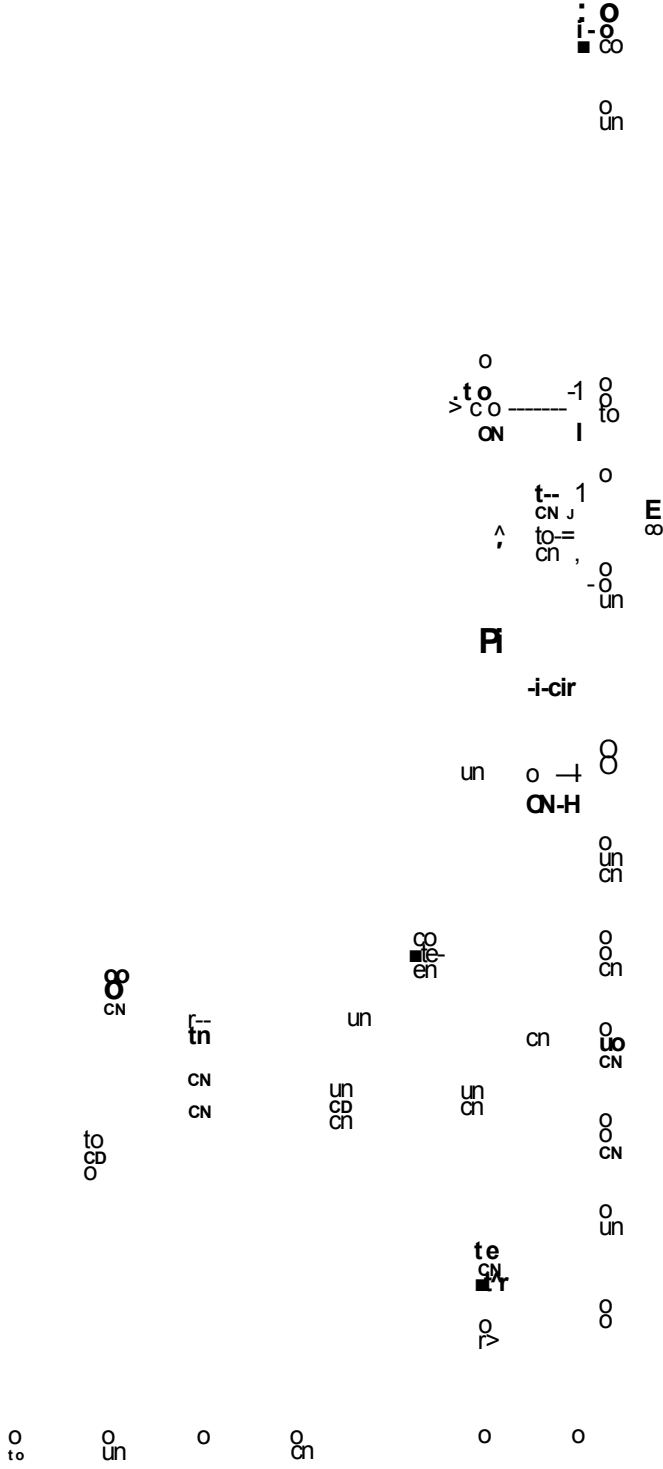
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B*50, B*51, B*52, B*53, B*54, B*55, B*56, B*57 (table 2.2), incorporating both HLA-A and B alleles. The VMAPRTLIL peptide in comparison is present in several HLA-C molecules, specifically HLA-C*01, C*03, C*04, C*05, C*06, C*08, C*12, C*14, C*16, C*17:02 (table 2.2).

2.3.7 Assessing peptide binding to HLA-A*02 via the T2 peptide binding assay

As with peptide VMAPRTLIL, to ensure peptide ALGFYPAEI was capable of being bound to HLA-A*02, the T2 binding assay was performed for ALGFYPAEI. The results of this experiment are shown in Figure 2.14 and reveal ALGFYPAEI has a FI of 1.5, demonstrating binding, highlighted in yellow and identified as peptide 19. In addition to this data, Figure 2.14 also contains the T2 binding assay data of 8 peptides commercially synthesized from sequences contained within the peptide database. 8 peptides within the database were selected at random. The T2 binding assay of these peptides, reveals two peptides had a FI of greater than 0.5 demonstrating they are correctly predicted as being able to bind to HLA-A*02. The remaining six did not bind with FI of less than 0.5. A final peptide with the sequence of SMRYFYTSV, identified as peptide 20 in Figure 2.14, is also shown as not binding to HLA-A*02 (FI<0.5). This peptide is present in one of the HLA antigens expressed by THP-1 cells, HLA-A*02 and also by six other HLA-A allele groups which are not present on THP-1 cells, HLA-A*11, A*25, A*26, A*34, A*66 and A*68. The SMRYFYTSV peptide was tentatively identified within the peptide database after a providing a hit from the MALDI MS (Figure 2.8) with a MW of 1153 Da, and was speculatively synthesized for the T2 binding assay. The lack of binding within this assay however means it is unlikely to be the correct sequence. In order to confirm this, MALDI MS/MS analysis was performed upon the identified MW 1153 Da peptide. The fragmentation products for SMRYFYTSV predicted to be present are given in table 2.7 and 2.8, while the MS/MS spectrum for the MW 1153 Da peptide is shown in Figure 2.15. The MS/MS spectrum does not correspond to the predicted fragmentation products, with the only fragmentation product observed being that of arginine, as indicated on the spectrum. Demonstrating that the peptide with the MW of 1153 Da, is not the SMRYFYTSV peptide which produced a hit within the database, although it does contain arginine. A summary of these experiments is shown in table 2.9, which provides the sequences of all the potential peptides tentatively identified as hits within the peptide database, followed by the results of the MALDI MS/MS analysis and the T2 peptide

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Products include those which have lost components, such as H₂O, and internal peptide fragment ions are shown. Calculated using MS Product (prospector.ucsf.edu) and checked using Hoffmann and Stroobant (2007).

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binding assay. This summary table includes three peptides which, as with the SMRYFYTSV peptide, were demonstrated as not corresponding to those providing a hit in the peptide database when analysed by MALDI MS/MS. These peptides have the following sequences and MW, SMRYFTTSV (1137 Da), TLLLLLLGA (926 Da) and YLENGKETL (1066 Da). Because the peptides were not confirmed as present by MALDI MS/MS they were not commercially synthesised to perform the T2 binding assay in order to reduce costs, with the exception of the TLLLLLLGA (926 Da) peptide, which was included in the random selection of peptides analysed in Figure 2.14, for their ability to bind to HLA-A*02 in the T2 assay, this produced a negative result.

In total, two peptides were confirmed as being bound by an HLA-A*02 molecule, these being ALGFYPAEI and VMAPRTLIL (highlighted in yellow in table 2.9), which were present in the HLA-B*15 and HLA-C*03 molecules respectively, expressed on the THP-1 cell line.

2.4 Discussion

The creation of a peptide database, which contains those sequences which are predicted as likely to be bound by HLA-A*02, and are themselves derived from HLA class I molecules, has provided a mechanism for identifying two peptides, which are encoded for by one HLA class I molecule and bound and presented by another (HLA-A*02). Specifically the ALGFYPAEI and VMAPRTLIL peptides, which are encoded for by HLA-B*15 and HLA-C*03 alleles within the THP-1 HLA type (Battle *et al.*, 2013) respectively. In addition to these alleles, several other HLA alleles encode these peptides, ALGFYPAEI is encoded for by HLA-A*01, A*03, A*11, A*23, A*24, A*30; B*15, B*18, B*27, B*35, B*38, B*39, B*40, B*41, B*42, B*44, B*45, B*46, B*47, B*48, B*49, B*50, B*51, B*52, B*53, B*54, B*55, B*56, B*57 and VMAPRTLIL by HLA-C*01, C*03, C*04, C*05, C*06, C*08, C*12, C*14, C*16 and C*17:02.

In terms of allorecognition this finding is of some significance. If an allograft recipient has an HLA type which includes the HLA-A*02 molecule, but does not include any of the other HLA molecules containing the ALGFYPAEI and VMAPRTLIL sequences, then the recipient will have had no experience of these peptides when central tolerance was established, although they will have been selected to recognise HLA-A*02 molecules.

As a consequence if the organ donor's HLA type included both the HLA-A*02 molecule as well as one (or more) of the HLA alleles encoding the ALGFYPAEI and VMAPRTLIL peptides, then the allograft recipient will be able to recognise the donor's HLA-A*02 and then form a response to the ALGFYPAEI and/or VMAPRTLIL peptide which is bound within it. This is a similar mechanism to that of mHAGs allorecognition, with the exception that instead of a non-HLA polymorphism encoding the protein being presented, it is another HLA antigen.

Extending this concept further, the HLA type of donor and recipient pair could be considered not only as being matched or mismatched in terms of their shared HLA molecules, but when they are mismatched, the extent to which their mismatches are able to generate peptides capable of being bound by the matched antigens could also be considered. In some situations permissible mismatches may be possible in this context. Whereby the HLA antigens which are mismatched contain either peptides

which cannot bind to the matched antigens, or have peptide sequences which are shared with other HLA antigens which the donor does possess.

The concept of matching of peptides in this manner would require a significant amount of time and effort to become fully realised. The work described here provides a proof of principle and includes only 3000 peptides within the peptide database, and only in the context of binding to the HLA-A*02 molecule. Both of these limitations would need to be expanded further to generate any realistic utility. However the proof that alloresponses are derived from mismatching of peptides can be seen in the role of mHAG in HSCT cases, and via the concepts such as the binary complex theory.

In terms of the location of the peptides which were identified within chapter two, it is interesting to note that two peptides which were predicted as likely to bind to HLA-A*02 were leader peptides, and present at position 3 of the α -chain. Analysis of the leader peptides is performed in greater detail within chapter three, which discusses the role which these peptides have within the immune system. However in terms of being able to be bound by HLA-A*02 only one of the leader peptides tested produced a positive HLA-A*02 T2 peptide binding assay result, the VMAPRTLIL peptide.

Chapter 2 provides a proof of principle of this approach to identifying the different peptides which are derived from one HLA molecule and presented by another.

In summary the work described includes:

- The creation of a peptide database, which contains peptides derived from HLA molecules predicted to be bound by HLA-A*02
- The utility of this peptide database in characterising peptides derived from one HLA molecule and presented by another
- The identification of peptides within this context, ALGFYPAEI and VMAPRTLIL, and demonstration of which HLA molecules these peptides can be derived from
- Evidence that only one HLA class I leader sequence peptide can be bound to HLA-A*02.

**Chapter 3 – Endogenous derived class I leader
peptides and renal transplant outcome**

3.1.1 Introduction

In chapter 2 an endogenous HLA class I leader peptide was identified which bound to HLA-A*02 molecules. Specifically the leader peptide from HLA-C*03 molecule was detected on HLA-A*02 molecules expressed on THP-1 cells, which was used for the BB7.2 (anti-HLA-A*02) antibody targeted magnetic bead purification. Although there is variable interpretation of the use of the term leader peptide (Molhoj and Degan, 2004), in the cases of HLA class I derived leader peptides, which are reported within the literature (Lee *et al.*, 1998; Braud *et al.*, 1997), the definition of a peptide which 'directs the ribosome to the ER, and initiates the growing protein across it before being cleaved and secreted into the external environment', is appropriate here. These proteins are of potential significance within alloresponses, as they can clearly be presented at the cell surface by HLA, therefore they could be potential targets for recognition by CD8⁺ T cells if bound by HLA-Class I. The leader sequences would be present at high levels due to their secretion into the cytosol whenever HLA is synthesized, thus potentially providing an abundant target for alloreactive T cells in accordance with the principles discussed in chapter 1. Furthermore HLA class I leader sequences have been shown to have novel roles regarding non-classical HLA-E expression and function, which may have an influence upon the alloresponse (Petrie *et al.*, 2008).

3.1.2 HLA class I leader peptides and HLA-E

Expression of HLA-E has been shown to rely significantly upon the binding of HLA class I leader sequences in a TAP dependent manner (Lee *et al.*, 1998). This expression is crucial for HLA-E to act as a ligand for the CD94/NKG2 family of receptors, which are expressed upon Natural Killer (NK) cells (Petrie *et al.*, 2008). Furthermore HLA-E has been shown to act as a presenter of antigen to the α - β TCR on T cells, via the presentation of cytomegalovirus (CMV) derived peptides in a HLA-E restricted manner to the α - β TCR of CD8⁺ T cells (Tamouza *et al.*, 2006; Allard *et al.*, 2012). Thus HLA-E mediated immunity spans both innate and adaptive immune responses. Currently the HLA/IMGT database lists 13 HLA-E alleles which encode for 5 proteins, however the reports within the literature regarding HLA-E focuses upon alleles HLA-E*01:03 and 01:01, as these are the only two widely represented alleles (Hosseini *et al.*, 2013).

The importance of HLA-E within the transplantation setting has received a relatively small amount of interest in comparison to classical HLA molecules. However several studies now suggest that HLA-E polymorphisms are associated with a lower incidence of GvHD and transplant related mortality (TRM) following HSCT (Hosseini *et al.*, 2013; Tamouza *et al.*, 2006; Danzer *et al.*, 2009). In particular a better overall survival was seen in patients homozygous for HLA-E*01:03, post HLA matched HSCT (Tamouza *et al.*, 2006; Danzer *et al.*, 2009). The influence of HLA-E within solid organ transplantation is relatively unreported; however HLA-E expression has been shown to be significantly increased within renal biopsy specimens undergoing a rejection event (Crispim *et al.*, 2008).

A comparison of the two most common alleles, HLA-E*01:01 and 01:03, reveals that they differ by a single amino acid at position 107, which is found within the $\alpha 2$ domain of HLA-E (Danzer *et al.*, 2009). This variation allows HLA-E*01:03 to bind the HLA class I derived leader peptides with a greater affinity than HLA-E*01:01, and consequently HLA-E*01:03 molecules are expressed at significantly higher numbers at the cell surface (Danzer *et al.*, 2009). Once at the cell surface, HLA-E interacts with the CD94/NKG2 family of receptors, which comprise both inhibiting (NKG2A and B) and activating receptors (NKG2C, E and F) on NK cells (Heatley *et al.*, 2013). Interaction of HLA-E with these activating and inhibitory receptors results in an activating or inhibitory signal to the NK cell, dependent upon the balance of these stimuli (Vales-Gomez *et al.*, 1999).

Interestingly the NKG2 inhibitory receptor, NKG2A, which prevents NK cell dependent cell lysis after activation, has been exploited by certain pathogenic viruses in an immunoevasive mechanism (Heatley *et al.*, 2013). As variation in the leader sequences bound by HLA-E affects the ability of CD94/NKG2 receptors to recognise their HLA-E target (Hoare *et al.*, 2008), certain CMV strains have evolved to take advantage of this, by mimicking HLA-class I leader sequences themselves. The human CMV strain AD169 for example, contains a nonamer sequence exactly homologous to a leader peptide sequence from HLA-C, which has been shown to upregulate HLA-E surface expression and inhibit NK mediated cell lysis (Tomasec *et al.*, 2000). This mimicry presumably evolved due to evolutionary pressure induced by the viral strategy of down regulating

classical HLA class I surface expression to avoid CD8+ cytotoxic T cell immunity (Mazzarino *et al.*, 2005). Due to this down regulation of HLA class I, the virus becomes susceptible to NK mediated lysis mechanisms, which result from the detection of a reduction in surface expression in HLA. One such mechanism being a lack of HLA-E expression as a result of reduced leader peptide availability (a consequence of down regulating the classical HLA expression); by providing a homologous HLA-E leader sequence, AD169 maintains HLA-E surface expression (Mazzarino *et al.*, 2005) and thus avoids the NK cell mediated lysis. Demonstrating the specificity of this mechanism, CMV specific effector memory cells, which target infected cells in an HLA-E restricted manner, are detectable only within those patients who do not have the leader peptide sequence mimicked by CMV within their HLA type (Mazzarino *et al.*, 2005), i.e. patients who possess a different leader sequence to the one CMV is mimicking can effectively target and kill the viral infection. This finding has potentially profound implications in the setting of organ transplantation. Transplant recipients who possess CMV-specific effector memory cells, which are HLA-E restricted, and receive an allograft which expresses HLA class I molecules encoding the leader peptide mimicked by CMV, will form an alloresponse via the CMV specific memory effector cell recognition of leader peptide (mimicked by the CMV strain) and bound by HLA-E expressed on the allograft.

3.1.3 Allorecognition and HLA class I derived leader sequences

It is interesting to note that of the reports on HLA-E polymorphism and transplantation outcome, it is the HLA-E*01:03 allele, which encodes a surface antigen expressed at higher levels than its equally common counterpart HLA-E*01:01, that is associated with reduced TRM post HSCT, the explanation for this being the increased availability of HLA-E*01:03 to bind to inactivating CD94/NKG2 receptors (Hosseini *et al.*, 2013). Given this observation, the finding that changes within the leader peptide bound to HLA-E, can profoundly affect recognition by CD94/NKG2 receptors on NK cells in a discriminatory manner (Hoare *et al.*, 2008), and that they can be peptide specific (Lampen *et al.*, 2013) leads to the question of whether differences between donor and recipient leader sequences, due to classical HLA mismatches, would influence the post transplant outcome. This would result in alternative HLA-E/leader peptide combinations, which may prevent interaction with inactivating CD94/NKG2 receptors and lead to a cytolytic response.

Additional evidence for a potential influence of HLA class I derived leader sequences was provided by Allard and colleagues (2012), who showed that HLA-E restricted CD8+ T cells, responding to CMV infection in a renal transplant recipient, can be alloreactive when the CMV and mismatched donor class I antigens contain the same sequence, i.e. the HLA class I derived leader sequence and the CMV homologue for this sequence. Therefore evidence exists that HLA Class I derived leader sequences can influence both innate alloresponses through the change of recognition by CD94/NKG2 receptors, and adaptive alloresponses through HLA-E restricted CD8+ T cells.

3.1.4 Assessing the effect of endogenous HLA-Class I derived leader peptides upon transplant outcome

Given that it is clear that HLA-class I derived leader sequences can affect allorecognition, and that currently there is a lack of clinical data regarding any influence of differences, between the HLA class I derived leader peptides expressed between transplant donors and recipients on transplant outcome, this chapter will specifically assess matching levels of HLA class I derived leader sequences, between renal transplant donors and recipients, and relate the level of matching to transplant function, during the first 12 months post transplant.

3.2 Materials and Methods

A flowchart overview of all the methods described here is given at the end of the section on page 97, to provide an overview of the work flow.

3.2.1 Renal transplant recipients

A retrospective study of consecutive renal transplants carried out at St James University Hospital, Leeds, was undertaken in the form of a clinical audit. Ethical approval for the study approval was granted by Leeds central ethics committee and the local NHS research ethics committee. Approval letters are provided in the appendix.

3.2.2 Data collection

The data for over 300 renal transplant recipients was retrospectively analyzed for patient demographics, recipient sex and age, and transplant related variables: donor type (donation following brain stem death (DBD), donation following cardiac death (DCD) or live donation), presence of anti HLA IgG and whether it was donor specific (DSA), recipient HLA type, donor HLA type and recipient creatinine levels at 3, 9 and 12 months post transplant. When the laboratory data was incomplete e.g. no 12 month serum creatinine levels recorded, then the transplants were not included in the audit. This resulted in a total cohort of 139 renal transplant patients with complete datasets for analysis. Data was collected from the laboratory database at the Transplant Immunology Department, at St James University Hospital in Leeds. Where data was incomplete within these records the raw data was obtained from the patient's renal file, also held within the Transplant Immunology Department. With the exception of creatinine levels, which were obtained from the Proton database within the Renal unit at St James University Hospital.

3.2.3 Analysis of HLA class I leader sequence matching

To ensure a complete record of the currently defined HLA class I derived leader sequences, the HLA/IMGT database was searched for all recorded HLA-A*alleles (n=2365 alleles encoding n=1695 proteins), B* alleles (n=3005 alleles encoding n=2277 proteins) and C* alleles (n=1848 alleles encoding n=1321 proteins), and the amino acid sequence of the leader peptide encoded by each of these alleles was recorded.

Specifically, the leader sequence is located within the HLA-Class I α -chain at amino acid residues at position 3-11 (Braud *et al.*, 1997), this sequence was recorded along with the HLA type encoding it in a spreadsheet. This allowed for a comparison of the leader peptides between the recipient and donor pairs, by using their HLA typing data to establish the leader peptides encoded within their HLA types and then assessing the level of leader peptide matching between them.

3.2.4 Post transplant renal function analysis

Although serum creatinine level measurements taken at a single time point post transplant have previously been utilized, and validated, as an approach to assess post transplant outcome (Hariharan *et al.*, 2003), it can be influenced by a variety of factors, including sex, age and patient size. Therefore to accurately utilize the serum creatinine level data for renal function in this retrospective analysis, the change of serum creatinine level between 3 and 12 months post transplant was calculated for each recipient, by using data obtained from serum samples collected from the recipient post transplant. This approach has previously been reported, and has been shown to have utility as a predictor of long-term renal allograft survival (First, 2003; Hariharan *et al.*, 2003). A decrease in serum creatinine levels between 3 and 12 months indicates an improvement in renal allograft function, while an increase indicates deterioration, this change in serum creatinine is referred to as Δ creatinine within this thesis from this point forward. To allow confirmation of any trends observed within the data set, serum creatinine was recorded at 3, 9 and 12 months.

3.2.5 HLA matching

In order to assess the impact of HLA matching within the cohort of patients, the HLA matching grade was calculated for each donor and recipient pair. HLA matching is recorded by the number of mismatches at the HLA-A locus, followed by the HLA-B locus, and then the HLA-DR locus, giving a three number matching level, 000 indicating no mismatches at HLA-A, B or DR, while 222 indicates the maximum 2 mismatches at HLA-A, B and DR. A single mismatch of HLA-A, two mismatches at HLA-B and no mismatches at HLA-DR would be represented as 120.

This information was used to determine which category of NHS Blood and Transplant Organ Donation and Transplantation (NHS BT ODT) renal allocation matching group the

pairs would fall into, thus allowing patients to be grouped according to similar levels of HLA match and mismatch. In the interest of clarity the NHS BT ODT HLA matching level groups are given in Table 3.1.

3.2.6 Summary of data collected

In summary the following data was collected in the audit.

- Recipient age and sex
- Presence of anti-HLA-IgG
 - Whether this was DSA
- Source of donor kidney
 - Donation following brain stem death
 - Donation following cardiac death
 - Live donation
- Post transplant serum creatinine
 - 3 months post transplant
 - 9 months post transplant
 - 12 months post transplant
 - 3 and 12 month levels were used to generate Δ creatinine
- HLA type of recipients and donors
 - Used to determine HLA matching
 - Used to determine NHS BT ODT matching groups for transplant pairs
 - Used to determine HLA class I leader sequence
 - And therefore HLA class I leader sequence match status

3.2.7 Statistics

The Welch *t*-test, also known as the unequal variance *t*-test, a variation on the student's *t*-test, was used for statistical analysis. The Welch *t*-test was selected due to its appropriate use in analysis of groups which have underlying variance. The Welch *t*-test does not assume equal variances between groups (Ruxton, 2006).

An analysis of variance was performed using the SPSS (IBM, Armonk, NY) statistical software package, in order to assess the variation within groups. Specifically analysis of Δ creatinine within sex v leader peptide mm groups; donor source v leader peptide groups and NHSBT ODT match level and leader peptide mm groups were analysed.

| | |
|---|--|
| 000-No HLA mismatches at HLA-A,B or DR | 000 (The level of HLA A mm is given in the first number, HLA-B in the second number and HLA-DR in the third) |
| 0 HLA-DR and 0/or 1 HLA-B | 100, 010, 110, 200, 210 |
| (0 HLA-DR and 2 HLA-B) or (1 HLA-DR and 0/or 1 HLA-B) | 020, 120, 220, 001, 101, 201, 011, 111, 211 |
| (1 HLA-DR and 2 HLA-B) or (2 HLA-DR) | 021, 121, 221, 002, 102, 202, 012, 112, 212, 022, 122 |

Table 3.1 The NHSBT ODT matching levels used for grouping renal patients according to HLA type in the retrospective clinical audit.

These matching levels are used by NHSBT ODT for categorising patients into HLA matching levels for the national allocation of deceased donor kidneys. The matching levels comprise part of an algorithm, which incorporates many variables not pertinent to this study (such as wait time upon the list for kidney offers). Additionally, as part of the NHSBT ODT allocation scheme a system of default HLA antigens are currently used. For clarity this system refers to the practice of ensuring potential renal recipients who possess rare HLA antigens, are not disadvantaged by defaulting to a common equivalent which is structurally similar to the rare antigen. No default antigens were used in assigning HLA matching levels for this study. Where appropriate a split resolution of HLA type was also used to determine matching level.

The HLA/IMGT database was searched to identify all recorded HLA class I leader sequences



Leader sequences were recorded, assigned a number and grouped with the HLA alleles which encode them



Renal transplant donors and recipients were assigned the leader sequence numbers encoded within their HLA types

Mismatch between leader sequences for renal transplant donors and recipients was calculated

The leader sequence numbers mismatches were calculated in the graft to host direction - including total number of mismatches and HLA loci encoding the mismatch



Relationship of leader sequence matching to A creatinine level was analysed

Figure 3.1 A flow diagram overview of the collection of HLA class I leader sequence data in a cohort of kidney transplant patients and relationship to A creatinine

3.3 Results

3.3.1 Cohort demographics and variance within groups

Complete data was collected on 139 renal transplant patients, with a mean age of 41.8 years. The cohort comprised slightly more males than females (58.3% (n81) v 41.7% (n58)). The number of previous transplants, if any, was not recorded. However 35.97% (n50) had anti-HLA-IgG detected at the time of transplant, 2% (n3) of which was DSA. The donor organs were predominantly from DBDs (55% (n77)), live donation accounted for 30% (n42) of the donor organs, while 14% (n20) of donor organs were from DCDs. A range of NHS BT ODT matching levels were observed, with 11% (n16) level 1, 28% (n40) level 2, 40% (n57) level 3 and 19%(n26) level 4 matches. The mean serum creatinine level post transplant was comparable between 3 and 12 months, being 135 (+/- 60.9 SD) mmol/L and 131 (+/- 54.4 SD) mmol/L respectively. The patient demographics and variance within groups are summarised in Table 3.2. Analysis of variance demonstrated no statistical significant difference between groups. However it should be noted that a reduction of statistical power in groups containing low numbers may contribute to this finding.

3.3.2 Identification of HLA class I derived leader peptides and their frequencies

The search of the HLA/IGMT database identified 11 HLA class I derived leader peptide sequences, and their corresponding encoding HLA allele groups, identified in table 3.3. Each leader peptide is identified with a number (leader 1, leader 2,..etc) along with the leader peptide sequence and the HLA allele groups which encode the leader peptides. The amino acid sequences of the identified HLA class I leader peptides corresponded to those previously reported (Romagnani *et al.*, 2004), although the alleles which encode the leader peptides reported here is more comprehensive, incorporating HLA-A*68; B*48, B*55 and B*46 allele groups, which have not been previously reported, making this the most comprehensive list of documented HLA class I derived leader peptides, and the alleles which encode them currently reported. The frequencies with which the leader peptides were observed within the cohort of renal patients and their donors (table 3.4), shows leader peptide 3 to have the lowest observed frequency (0.06%), while leader peptides identified as leader peptides 4 and 11, were not observed within this cohort of patients at all. Leader peptide 10 was also seen with a

low frequency (0.45%) within this cohort. All the other identified leader peptides were observed with a frequency of between 10-20%

3.3.3 Number of HLA class I derived leader peptide mm observed

In analysis of the level of HLA class I leader peptide mismatches between donors and recipients (table 3.5) 32.4% of renal transplants were shown to have no leader peptide mismatches, while the most common number of leader peptide mismatches observed was a single leader peptide mismatch, observed within 38.8% of the transplants, two leader peptide mismatches and three leader peptide mismatches were observed in 23.7% and 5% of transplants respectively.

3.3.4 HLA matching levels observed within the cohort

In terms of the HLA matching levels observed, those patients which received an 000 mismatched renal allograft and are therefore grouped into NHSBT ODT matching level 1, comprised 11.5% of patients (table 3.6), while NHSBT ODT matching levels 2, 3 and 4, were seen in 28.8%, 40.3% and 19.4% of patients respectively. Demonstrating a range of HLA matching within these patients.

3.3.5 Relationship between leader peptide mm and creatinine level

The comparison of the number of HLA class I leader peptide mismatches and their relationship to Δ creatinine levels (figure 3.2), reveals a striking relationship between the number of leader peptide mismatches to Δ creatinine. A reduction in Δ creatinine approaching 20 mmol/L was found where no leader peptide mismatches were observed, this reduction in Δ creatinine indicates the allograft performance has improved from 3 to 12 months post transplant. Where three leader peptide mismatches were identified, an increase in Δ creatinine was observed, with levels approaching 20mmol/L, indicating the allograft performance had decreased from 3 to 12 months post transplant. The change in Δ creatinine was a statistically significant association, $p < 0.05$ (table 3.7), for the three leader peptide mismatches group. A stepwise increase in Δ creatinine was seen depending upon the number of leader peptide mismatches, zero leader mismatches showing a decrease in Δ creatinine, one leader mismatch a more modest decrease, two leader peptides mismatches producing little change in Δ creatinine, and three leader peptide mismatches an increase. This

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increase in Δ creatinine, associated with an increased number of leader peptide mismatches, demonstrates a relationship with an R^2 value of 0.9999 (figure 3.2). The association between leader peptide mismatch and creatinine levels was also performed so that it included creatinine levels at 9 months post transplant (figure 3.3). With zero leader peptide mismatches showing a stepwise decrease in creatinine levels between 3, 9 and 12 months, the single leader peptide mismatches showing a consistent decrease between 3, 9 and 12 months, while the data for three leader peptide mismatches showing a stepwise increase in creatinine levels between 3, 9 and 12 months. The two leader peptide mismatches however showed an initial decrease in creatinine levels from 3 to 9 months, and then a marked increase from 9 to 12 months. This increase from 9 to 12 months for two leader peptide mismatches was not as steep as that seen for three leader peptide mismatches. The two leader peptide mismatches creatinine level decrease from 3 to 9 months then recovered with an increase from 9 months to 12 months, so that the creatinine levels returned to their 3 month level. Interestingly the three leader peptide mismatch groups creatinine levels, rose modestly between 3 and 9 months and then more sharply between 9 and 12 months. Furthermore this group had the lowest 3 month creatinine levels, which may be explained by the higher number of live donors within this group (Table 3.2).

3.3.6 NHSBT ODT matching levels and relationship to creatinine

The data representing the effect of NHSBT ODT matching levels and relationship to Δ creatinine, figure 3.4, shows the effect of the increasing levels of mismatch and Δ creatinine. With the level 1 group (the best matched group), associated with a decrease in Δ creatinine of 20 mmol/L, while the level 4 group (those with the worst matches), being associated with a more modest decrease in Δ creatinine, approaching 5 mmol/L. All the NHSBT ODT matching levels were associated with a decrease in Δ creatinine, which is in contrast to the leader peptide mismatch analysis described earlier, whereby the three mismatched (mm) group was shown to produce a statistically significant change from a negative to positive Δ creatinine. Unlike the leader peptide analysis, none of the NHSBT ODT groups were shown to be statistically significant, table 3.8, nor was a positive Δ creatinine observed. The relationship between NHSBT ODT levels and Δ creatinine was also shown, however the R^2 value equalled 0.9233. Indicating that the relationship between NHSBT ODT

Leader peptide mismatch (mm) and relationship to mean creatinine levels over a 12 month period



0 10 20 30 40 50 60 70 80 90 100 mm

0 20 40 60 80 100 mg/dL

Effect of ODT matching levels and A creatinine level (3 to 12 month)

Creatinine level mmol/L

A creatinine level (3 to 12 month) and relationship to ODT matching levels

Creatinine level mmol/L

levels and creatinine did not fit the polynomial regression model as well as that of the leader peptide mismatches, where the R^2 value was closer to 1, being 0.9999.

3.3.7 Effect of leader peptide mm within NHSBT ODT matched groups

In an attempt to determine between the effect of leader peptide mm upon Δ creatinine levels and the effect of NHSBT ODT matching levels, an analysis was performed on the effect of leader peptide mm within NHSBT ODT matching levels was made. This analysis revealed the association between an increasing number of leader peptide mm and an increase in Δ creatinine was occurring within NHSBT ODT matching levels, for ODT levels 1 and 2 (figure 3.5) and ODT level 4 (figure 3.6). In the NHSBT ODT level 1 matched group only matched (zero leader peptide mm) and single leader peptide mm were observed. With the matched group showing a reduction in creatinine, with a Δ creatinine of 20mmol/L, while the single leader peptide mm group showing an increase, with Δ creatinine 20mmol/L. The NHSBT ODT level 2 group was again associated with an increase in Δ creatinine associated with the number of leader peptide mm, however unlike level 1, all leader peptide mm levels were associated with a reduced Δ creatinine. Neither NHSBT ODT level 1 or 2 leader peptide mm analysis produced statistically significant observations (table 3.9). The NHSBT ODT level 4 group demonstrated the stepwise increase in Δ creatinine as the number of leader peptide mm increased (figure 3.6), it should be noted the 3 leader mm group only had 2 patients within it.

In the NHSBT ODT level 3 matched group, the data appeared to deviate from the stepwise increase in Δ creatinine level observed within the other NHSBT ODT groups. Whereby the 0 leader mm group appeared to show the poorest performance in terms of reduction of Δ creatinine, with a level of -6mmol/L, while one and two leader peptide mm had levels of -15mmol/L and -10mmol/L respectively. Analysis of the raw data revealed a possible explanation for this observation, in that the zero leader peptide mm group, within the NHSBT ODT level 3 matched analysis, contained data from two allografts which exhibited a significant deterioration in Δ creatinine which may skew the data, these two allografts went on to require allograft nephrectomy

ODT matching level 1

ODT matching level 2

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l/O LUOI aum j|eao v

within 24 months post transplant. However it was important to include all the data collected in this study to prevent any bias.

Attention should also be drawn to the reduction in statistical power when analysing the individual NHSBT ODT groups in this way, with the numbers of patients within the four NHSBT ODT matching levels being low, ranging from 16-54 patients.

3.3.8 The effect of specific HLA class I derived leader peptide mm

To assess if there was any difference when the leader peptide from different HLA class I encoded molecules was mismatched, a comparison of leader peptide encoded just by HLA-A, HLA-B and HLA-C alleles was made (figure 3.7).

This demonstrates that HLA-B encoded leader peptides, identified in table 3.3 as leader peptides 5, 6 and 7, were associated with an increase in Δ creatinine in a stepwise fashion, dependent upon the number of leader mm observed (2 leader peptide mm were only observed twice so a statistical analysis was not performed due to their low frequency) (table 3.10). HLA-C encoded leader peptides, incorporating leader peptides identified as leader peptides 8, 9, 10 and 11 (table 3.3), also demonstrated the association of an increase in Δ creatinine with an increase in leader peptide mm, only zero leader peptide mm and single leader peptide mm were observed within this group however, and this change was not statistically significant. In contrast to the HLA-B and C encoded leader peptides HLA-A encoded leader peptides did not demonstrate an association with a decrease in Δ creatinine, instead there was an increase for zero and one leader peptide mm.

In an attempt to clarify this further, an analysis of the effect of mm of specific leader peptides was performed. This demonstrated that mismatching leader peptides 1 and 2 (HLA-A encoded peptides) resulted in similar Δ creatinine levels as no leader mm at all (figure 3.8), confirming that HLA-A leader peptides do not influence Δ creatinine levels (leader peptide 3, which is also encoded for by HLA-A alleles was only observed once, and is shown in figure 3.9 for clarity). Mismatching of leader peptides 5, 6 and 7 (HLA-B encoded peptides), demonstrated a modest decrease in Δ creatinine levels for peptide 6, a modest increase in Δ creatinine for peptide 7 and more marked increase in

NHSBT ODT

ODT level 1

ODT level 2

ODT level 3

ODT level 4

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Δ creatinine peptide 5. Comparison to the zero leader peptide mismatches confirmed an influence of HLA-B encoded peptides, with the association for peptide 5 approaching statistical significance ($p=0.08$) (table 3.11). Mismatches for the HLA-C encoded leader peptides, peptides 8 and 9, also showed an influence of mismatches and relationship to Δ creatinine levels when compared to the zero mismatched group, however this was not statistically significant (table 3.11), which may be due to the low numbers in the cohort with peptide 8 ($n=8$) and 9 ($n=5$).

3.4 Discussion

The 11 leader peptides identified within this data set, using the HLA/IMGT database, contains the most complete list of HLA class I alleles and their encoded HLA alleles currently available, according to a search within the literature (as of May 2014). The accurate record of the sequences of HLA class I derived leader peptides, and their encoding HLA alleles has been demonstrated of utility within this work, and would also aid those researchers whose work includes the role of HLA class I derived peptides in HLA-E induced responses, such as NK cell mediated cell lysis and HLA-E restricted CD8+ T cell activation. Indeed, the data previously published in the context of CMV infection, NK and CD8+ T cell responses and HLA class I derived leader peptides does not report the leader peptide sequences of HLA-A*68; B*48, B*55 and B*46 alleles (Romagnani *et al*, 2004), making the data reported here more complete. Furthermore, Romagnani and colleagues (2004) assign a leader sequence of VTAPRTLLL for the HLA molecules encoded for by HLA-B*58 alleles, while the data here identifies the sequence as VTAPRTVLL. Cross checking upon the IMGT/HLA database (Robinson *et al*, 2013) confirms the data reported here as accurate.

The frequencies of the 11 identified leader peptides, within the cohort of 139 renal transplant patient and donors, was representative of the frequencies of the HLA class I alleles which encoded them. Leader peptides 4 and 11 were not observed at all and were encoded for by HLA alleles which have a low frequency within the UK population. Leader peptide 4 being encoded for by HLA-A*80, which is seen in less than 1% of 10,000 UK deceased donors according to NHSBT ODT data (<http://www.odt.nhs.uk/transplantation/histocompatibility-and-immunogenetics/handi-information/>), while leader peptide 11 is encoded for by HLA-C*08:09, a rarely seen allele listed as unconfirmed upon the HLA/IMGT database (Robinson *et al*, 2013), which has only been observed within a specific ethnic population, a group of Kolia Amerindians (Little *et al*, 2001). Hence these two leader peptides were unlikely to be observed within the cohort of renal patients reported here. The low frequencies of the observed leader peptides 3 (0.06%) and 10 (0.45%), was also due to correspondingly low HLA encoding allele frequency, being encoded for

by HLA-A*34:01 seen in individuals of Asian pacific islander origin (Robinson *et al.*, 2013; Gonzalez-Galarza *et al.*, 2010), and HLA-C*17:01/02 respectively.

The analysis of the number of mismatches between the identified HLA class I derived leader peptides and Δ creatinine demonstrated a stepwise relationship, with an increase in Δ creatinine associated with an increase in the number of leader peptide mismatches. This indicates that a poorer performance is seen in those allografts which have a greater number of leader peptide mismatches. This relationship was shown to be more pronounced than the relationship observed between Δ creatinine and NHSBT ODT matching levels (R^2 value of 0.999 vs 0.9233). Furthermore the Δ creatinine levels in those patients who received an allograft with 3 leader peptide mismatches was shown to have statistically significant ($p=0.05$) poorer performance than the matched counterparts in terms of Δ creatinine levels. However a larger cohort of patients should be assessed to validate this data, particularly since no statistical significant association was seen within the analysis of variance within the assessed groups.

Analysis of the effect of leader peptide mismatches within NHSBT ODT HLA matching groups shows that this effect appears independent of HLA matching grade. The influence of HLA class I derived peptide mismatch and Δ creatinine being observed within NHSBT ODT level 1, 2 and 4 matched groups. NHSBT ODT level 3 matched patients however did not concur with this finding, specifically the zero leader mismatched group appeared to perform the worst, as determined by Δ creatinine. This may be explained by the confounding influence of two poorly performing allografts within the zero leader peptide mismatched group within the NHSBT ODT level 3 matches (as described within section 3.3.8), this hypothesis would be supported by the observation that the stepwise increase in Δ creatinine associated with an increase in the number of leader peptide mismatches, returned for the single and two leader peptide mismatches within the level 3 group. This data then shows a relationship between the number of leader peptide mismatches and the post transplant outcome of renal allografts as assessed by Δ creatinine (Battle *et al.*, 2013). However the exact mechanism of this influence cannot be determined from this data.

Previously researchers have examined the role of HLA class I derived leader sequences and their ability to bind to HLA-E, showing that not all peptides bind with equal affinity,

and that a consequence of this is the alteration of HLA-E recognition by the CD94/NKG2 receptors upon NK cells (Hoare *et al.*, 2008). A comparison between the reported affinities of the leader peptides and HLA-E (Vales-Gomez *et al.*, 1999), and the data presented here, in terms of the effect of mismatches of individual peptide, reveals some interesting observations. Reports within the literature upon the binding affinities of those leader peptides encoded for by HLA-B alleles demonstrate that the leader peptide, identified here as leader peptide 5, containing methionine at position 2 of the peptide sequence binds with the highest affinity to HLA-E, while those peptides with threonine at position 2 (identified here as leader peptides 6 and 7) bind less well (Hoare *et al.*, 2008; Lee *et al.*, 1998; Lemberg *et al.*, 2001; Vales-Gomez *et al.*, 1999).

The ability to be bound by HLA-E has a direct impact upon the expression level of HLA-E (Vales-Gomez *et al.*, 1999), and therefore changes within peptides which have different binding affinities would be expected to alter surface expression of HLA-E, and alter the ability of HLA-E to interact with the CD94/NKG2 activating/inhibiting receptors. It is interesting to note that mismatches at the HLA-B encoded leader peptides 5, 6 and 7 (figure 3.7) are associated with a poorer Δ creatinine level post transplant (indeed leader peptide 5 mismatches are approaching statistical significance $p=0.08$), while HLA-A encoded leader peptide mismatches appear to exert no influence upon Δ creatinine levels post transplant. This is particularly interesting when considering the reported observation that all HLA-A encoded leader peptides bind to HLA-E with the same affinity (Hoare *et al.*, 2008; Lee *et al.*, 1998; Lemberg *et al.*, 2001; Vales-Gomez *et al.*, 1999).

In terms of the leader peptides encoded for by HLA-C, the majority of which observed here comprised leader peptides 8 and 9 (due to the frequencies of the respective encoding HLA-C alleles), variation is also seen between the binding affinities reported. Peptide 8 showed extremely good binding kinetics (Hoare *et al.*, 2008; Lee *et al.*, 1998; Lemberg *et al.*, 2001; Vales-Gomez *et al.*, 1999) while peptide 9 extremely poor binding kinetics (Vales-Gomez *et al.*, 1999). The Δ creatinine level for these peptides also revealed an increase in comparison to the zero mismatched group, however this was not statistically significant.

These findings, in conjunction with the reported binding affinity for HLA-E, lead to the hypothesis that a change in the peptides available to bind to HLA-E, results in a differential expression of HLA-E at the cell surface, as a consequence of individual peptide binding affinities. Changes in the levels of HLA-E expression could then lead to NK responses which are detrimental to the allograft, this would then be anticipated to be observed in the Δ creatinine levels as seen here. Clearly, functional data is required to support this hypothesis, however it is interesting to note that a reported immunoevasion strategy of CMV is to maintain HLA-E expression at the cell surface by mimicking HLA class I leader peptide sequences (Mazzarino *et al.*, 2005) and those utilized by CMV have a high affinity for HLA-E (Hoare *et al.*, 2008; Lee *et al.*, 1998; Lemberg *et al.*, 2001; Vales-Gomez *et al.*, 1999), which would increase the level of expression of HLA-E. In addition, in the reports on HLA-E polymorphism and HSCT outcome, it is the HLA-E*01:03 allele, which encodes a surface antigen expressed at higher levels than its equally common counterpart HLA-E*01:01, that is associated with reduced TRM post HSCT, the explanation for this being the increased availability of HLA-E*01:03 to bind to inactivating CD94/NKG2 receptors (Hosseini *et al.*, 2013). An analysis of CMV was not undertaken here, but would be an interesting area for further work, which is discussed in further detail in chapter 5. This work strongly suggests that mismatches between the HLA class I derived leader peptides influence Δ creatinine, a marker for renal allograft outcome. However, the exact mechanism requires further elucidation, with changes within the leader peptides which result in changes with affinity for binding to HLA-E, and therefore HLA-E expression a potential mechanism. In addition this work was hampered by the relatively small cohort of patients examined, and a larger validating cohort of patients is required to confirm these findings.

In summary, chapter 3 demonstrates that endogenously derived HLA class I peptides, in this case specifically their leader peptides, can influence the post transplant function of renal allografts (Battle *et al.*, 2013). However a larger validating cohort of patients should now be assessed to validate this data and confirm the findings within chapter 3.

Chapter 4 - Functional analysis of HLA-class I derived peptide influence upon the alloresponse

4.1.1 Introduction

In chapters 2 and 3, peptides were identified which were derived from one HLA class I molecule and presented by another. Subsequently clinical data was analysed to measure the influence of mismatching some of these peptides, within the context of renal transplantation. In this chapter, an investigation to assess a mechanism by which peptides may exert their influence upon humoral alloresponses was undertaken. The thesis introduction outlined the mechanisms by which peptides can generate alloresponses, describing the processes of direct and indirect allorecognition and the peptide involvement within them. In addition to the mechanisms described within the introduction, peptides may also influence the ability of preformed donor HLA specific antibodies to bind to their target epitope.

4.1.2 HLA antibodies and transplant outcome

HLA antibodies are formed in response to exposure to HLA antigens, a phenomena known as sensitisation, this exposure can be generated as a result of transfusion, pregnancy or transplantation (Resse *et al.*, 2013). The detection of donor specific HLA antibodies (DSA) present within the serum of a renal transplant recipient prior to transplantation by complement-dependent cytotoxicity (CDC) crossmatch (XM), has, for many years been an absolute contraindication to transplantation, due to the association of a positive CDC XM with hyperacute and accelerated acute rejection (Girnita *et al.*, 2007). While a positive T cell CDC XM remains a contraindication to transplantation, technical developments within various methodologies used in the detection and identification of HLA antibodies, such as the introduction of solid phase assays, have led to highly sensitive approaches to HLA antibody detection, and consequently the clinical impact of detected antibodies is not completely understood in some circumstances (El-Awar *et al.*, 2009). Current approaches to understand the clinical impact of detected HLA antibodies aim at providing a stratification of risk, based upon the results of XM and antibody identification assays, while considering the type of organ to be transplanted, available immunosuppression strategies, clinical urgency of transplant and various donor factors (Opelz *et al.*, 2013). Currently H&I laboratories utilize HLA antibody screening technologies in those patients who are awaiting transplantation, to determine unacceptable HLA antigen mismatches due to HLA sensitisation (Howell *et al.*, 2010). By using this information, it is possible to

predict the likelihood of a XM to return a negative result for a particular patient and donor combination, when the donor's HLA type is known. This process can prevent the shipment of an organ to a potential transplant recipient, who would produce a positive XM result. In addition, the resultant HLA antibody data allows for an indication of the likelihood of a patient to be offered a kidney to which they would be expected to produce a negative XM result. This is achieved by calculating the patient's HLA antibody reactivity to a panel of HLA antigens, whereby the HLA antibodies detected within a potential transplant recipient are assessed for matches to corresponding HLA types of the previous organ donors offered via NHS BT ODT, giving a percentage of reactivity to these donors. This process is sometimes referred to as calculated reaction frequency (CRF) or calculated panel reactive antibody (cPRA), and aids understanding of how long a patient is likely to wait for a transplant, given the percentage of donors that they would have been expected to have antibody reactivity to, and consequently would not have been offered, those with a cPRA >85% are classified as highly sensitised and are likely to have to wait significantly longer than unsensitised patients (Chang and Kobashigawa, 2012).

Recently, the ability to remove DSA through antibody desensitisation protocols to achieve a negative CDC XM result prior to transplantation, has led to an opportunity to transplant patients previously denied access to a transplant due to DSA . The reported success of desensitisation protocols varies, depending upon a number of factors such as: titre and specificity of DSA and the desensitisation protocol utilised (Marfo *et al.*, 2011). The graft survival in HLA antibody desensitisation protocols remains impaired in comparison to control groups (Becker *et al.*, 2013), while still providing an important clinical option for transplantation for some patients. Those patients who have the best long-term survival, post DSA desensitisation transplant, are those who lose their DSA after their successful desensitisation and transplantation, whereas DSA persistence, recurrence or *de-novo* formation, all affect long term allograft survival negatively (Becker *et al.*, 2013). The development of *de-novo* DSA is also associated with reduced graft survival in non desensitisation cases (Fehr and Gaspert, 2012), even in cases where no antibody mediated rejection is identified (Roelen *et al.*, 2012).

A detailed understanding of the HLA antibodies present in a potential transplant recipient is vital, not only to prevent an unexpected positive XM result, but also to aid post transplant patient management, due to the *de-novo* formation of HLA antibodies against mismatched HLA antigens on the transplanted organ.

Such *de-novo* antibodies do not always occur when an HLA antigen is mismatched however, as the distinct sites which HLA antibodies bind to on HLA antigens, termed epitopes, are shared between some HLA antigens (Duquesnoy, 2011b). Consequently an HLA antigen could be mismatched, but the epitopes upon the mismatched HLA antigen may be present upon the mismatched HLA antigen, or upon other HLA antigens present within the transplant recipient's HLA type. Therefore the epitopes are matched and consequently HLA antibodies are not formed. In order to understand this process better, various mechanisms of characterising the epitopes have been proposed, the two most successful systems are the epitopes identified as 'eplets' by the HLA Matchmaker bioinformatic software (Duquesnoy, 2011a) and the TerEps identified by Paul Terasaki (El-Awar *et al.*, 2007).

4.1.3 HLA antibodies and epitopes

It was not long after the discovery of HLA specificities that serological crossreactivity between certain HLA antigens was observed. To define this crossreactivity, HLA antibodies were utilized to assess their binding to different HLA antigens, using this process it was possible to identify groups of cross reacting epitopes, so called CREGs (table 4.1) (cross reacting epitope groups) (Rodney *et al.*, 1994; Fuller *et al.*, 1990). These groups helped categorise epitopes, which are shared between HLA antigens and, as structural understanding of HLA molecules became defined, it could be demonstrated that the crossreactivity of specific antibodies was likely to be the result of common molecular structures between different HLA molecules (Duquesnoy, 2011b). It is these common molecular structures, which are referred to as epitopes when antibody is capable of binding to them, and which are identified by various systems, such as eplets or TerEps. It was Duquesnoy *et al.*, (2011a/b) who initially suggested that the epitopes themselves consisted of three consecutive amino acid residues, and later revised this to identify epitopes via his eplet system, which used stereochemical modelling of protein antigen-antibody complexes, to identify polymorphic amino acid configurations on HLA antigens,

| | |
|-------------|--|
| A1C | A1, A3, A11, A19(A29, A30,A31), A36, A80 |
| A2C | A2, A9 (A23, A24), A28 (A68, A69), B17(B57, B58) |
| A10C | A10 (A25, A26, A34, A66), A32, A33, A43, A74 |
| Bw4 | A9 (A23, A24), A25, A32, B13, B27, B37, B38, B44, B47, B49, B51, B52, B53, B57, B58, B59, B63, B77 |
| B5C | B5 (B51, B52) B18, B35, B53 |
| B5C2 | B5 (B51, B52) B15 (B62, B63, B71, B75, B76, B77), B17 (B57, B58), B21 (B49, B50), B35, B53, B73, B78 |
| Bw6 | B7, B8, B18, B35, B39, B40 (B60, B61), B41, B42, B45, B46, B48, B50, B54, B55, B56, B62, B64, B65, B67, B71, B72, B73, B75, B76 |
| B7C | B7, B8, B13, B27, B41, B42, B47, B48, B54, B55, B56, B60, B61, B81 |
| B8C | B8, B18, B38, B39, B64, B65 |
| B12C | B12 (B44, B45), B13, B37, B41, B47, B21 (B49, B50), B40 (B60, B61) |

Table 4.1 Cross-reactive epitope groups (CREG) (Reproduced from Laux and Pelz, 2004; Mckenna and Takemoto 2000). HLA antigens enclosed within parentheses identify 'splits' of broad HLA specificities although this may not include all splits within the broad antigen, due to the possession of different epitopes within certain splits.

which were within a 3.5Å radius, in positions within regions accessible to antibodies (Marrari *et al.*, 2010).

4.1.4 The use of HLAMatchmaker to define epitopes

The most frequently used mechanism of defining epitopes upon HLA antigens is the HLAMatchmaker bioinformatic system, which defines the epitopes on an HLA antigen as a series of eplets. This then allows for a comparison of eplets present on different HLA antigens, to see if any or all eplets are shared. This approach has led to a greater understanding of how antibodies are generated to HLA antigens, to which an individual does not appear to have been sensitised to, for example the observation of an HLA-DR1 antibody in response to an HLA-DRw51 antigen was explained by a shared eplet, identified as 96EV (Marrari *et al.*, 2011). Traditionally, HLA antibody identification has been achieved by describing a specific HLA antigen to which the antibody is detected as being capable of binding to, such anti-HLA-A2 or anti-HLA-B7. The HLAMatchmaker system has led to a greater understanding of the actual epitope targets of such antibodies, and can reveal them to be specific to single eplets or eplet pairs (discussed below).

The HLAMatchmaker derived eplets are identified via stereochemical modelling, which assesses each HLA antigen as a string of amino acid configurations in antibody-accessible positions (Duquesnoy and Marrari, 2009). Antigenic proteins have structural epitopes of 15-22 amino acid residues within an antibody binding surface of 700-900Å, within these structural epitopes are 'hot spots' of 2-5 highly energetic residues, which contribute to the strength and specificity of antibody binding. Within HLAMatchmaker, eplets are derived from a 3Å radius of a polymorphic residue upon the surface of a HLA antigen, which define these 'hot spots'. These eplets bind antibody as an individual eplet or as an eplet pair, with eplets in a pair being approximately 6-15Å apart, and at least one of the pair being non-self (Duquesnoy and Marrari, 2009). The amino acid residues outside the hot spots contribute augmenting interactions, which contribute interactions which increase antibody-antigen stability.

The nomenclature of these eplets is derived from the polymorphic residues within the eplet, and the position of the polymorphic amino acid residue within its chain of amino

acid residues. The standard letter code is used to identify the amino acids. Using this system Duquesnoy has defined 199 eplets from 75 polymorphic positions within Class I HLA-A, B and C antigens, of which 110 were positioned on the α -helices, 60 on the side of the surface and 29 in the regions which are less accessible to antibodies such as underneath the molecule and under the peptide binding groove (Duquesnoy, 2011a). While the number of polymorphic regions within HLA Class II molecules consist of 44 DRB, 33 DQB, 29 DQA, 20 DPB and 9 DPA chain polymorphic sites, which were shown to deliver 146 DRB, 74 DQB, 58 DQA, 45 DPB and 19 DPA individual eplets (Duquesnoy, 2011a). As previously mentioned, the HLAMatchmaker program views an HLA antigen as a group of the identified eplets present upon an HLA molecule, these eplets are capable of inducing HLA specific antibodies, but an individual cannot generate HLA antibodies against eplets which are also present in their own HLA type, so called self eplets.

Using these principles, HLAMatchmaker can be used to assess the degree of mismatch in terms of eplets. In some cases, a transplant donor and recipient may be mismatched in terms of the HLA antigens they express, but matched in terms of the eplets present in their HLA antigens. This gives an indication of the likelihood of generating a humoral response to HLA mismatches as a consequence of transplantation. Furthermore the eplet system allows for the analysis of sensitisation pattern induced by an HLA mismatch, accounting for unexpected reactivity patterns such as the HLA-DR1 antibody induced by sensitisation to DRw51 (Marrari *et al.*, 2011). (figure 4.1).

The use of HLAMatchmaker has greatly improved the understanding of the HLA antibody sensitisation status of patients who are awaiting transplantation, as well as helping to minimise sensitisation, by allowing eplet matching in certain situations, when a HLA mismatch is unavoidable. Currently use of the HLAMatchmaker program has been described in various forms of transplantation, including, kidney, cornea, haematopoietic stem cell transplantation (Duquesnoy, 2011b) and cardiac transplantation (Resse *et al.*, 2013), as well as in the allocation of platelets. Despite the use of HLAMatchmaker in these clinical settings, no assessment of the generation of eplets caused by different peptides bound within the HLA groove has been made.

Theoretically at least, changes within HLA bound peptide may alter the epitopes generated, both by generating new epitopes, and by altering the affinity of an antibody to an existing epitope. For example, a change within the peptide could alter the affinity of an antibody to an epitope present upon regions proximal to the peptide binding groove, such as the α -helices; altering the peptide bound within the groove may cause variation between the interaction of the amino acid residues of the α -helices and the bound peptide. As a consequence of this change, the ability of an epitope to be bound by an antibody maybe reduced or increased. Various modelling software is available to assess stereochemical structures in order to investigate the effect of peptide bound upon epitopes (Guex and Peitsch, 1997; Kosmoliaptsis *et al.*, 2011).

4.1.5 *The evidence for the peptide influence on HLA antibodies binding to their epitopes*

In their assessment of a recombinant human Fab fragment for the investigation of tumour associated HLA-A1 melanoma associated antigen (MAGE-1)-A1 complex, Hülsmeier and colleagues (2004) crystalized the MAGE-1 peptide (EADPTGHSY) bound by HLA-A1 and complexed with the Fab fragment. This work demonstrated that several regions of the CDR of the Fab fragments were in direct contact with the MAGE-1 peptide. Indeed the authors concluded that, in this case, the Fab fragment binds the HLA-A1 in a similar manner to a TCR. Indeed after comparing the Fab fragment's binding to HLA-A1 complexed to another MAGE peptide, MAGE-3, the authors stated that the Fab fragment 'does not bind', demonstrating clear evidence that changes within the peptide bound influences the ability of some antibodies to bind.

Furthermore, altering the amino acids at specific regions within the peptide was determined to alter antibody binding ability, from 'weakly' binding to 'strongly' binding to its target.

Although this complex work clearly showed the ability of changes within the peptide bound by HLA to alter the ability of antibody to bind, it should be pointed out that the antibody fab fragment was a recombinant antibody, specifically selected for the purpose of investigating MAGE-1. It does however serve to demonstrate that antibodies can be HLA peptide complex specific, and can have their affinity altered by changing the peptide which is bound by HLA. An image of HLA-A1 complexed with MAGE and interacting with the Fab fragment is shown in figure 4.2.

PdbViewer (Guex & Peitsch 1997).

4.1.6 Structural modelling software

The original article describing the creation of the HLAMatchmaker bioinformatic tool (Duquesnoy, 2006), identifies the use of Cn3D (see in 3D) structure and sequence alignment software program, to identify the location of specific residues in crystallized HLA molecular models, which were obtained from the NCBI website (www.ncbi.nlm.nih.gov/protein). The Cn3D program allows a 'select by distance' function, which Duquesnoy applied around polymorphic residues to the distance of 3.0Å and 3.5Å in order to identify eplets. However Cn3D is by no means the only structural modelling program available, with different modelling programs offering variation within their functionality. One such popular modelling program is Swiss-PdbViewer, which offers many of the functions within Cn3D, including the ability to measure the distance between residues, but with the addition of a mutation function (Guex and Peitsch, 1997). This mutation function allows the user to alter an amino acid residue, for example within a peptide, such as is seen within several minor histocompatibility antigens, such as HA-1 as previously described (section 1.1.12). The mutation function allows for an assessment of how a change of an amino acid would alter the possible structures of the molecule, including the formation of new hydrogen bonds. While the use of Ramachandran plots, which describe the torsion angles of amino acids within proteins (Hollingsworth and Karplus, 2010), incorporated within the Swiss-PdbViewer software, allows for the mutation to be assessed in terms of its correct orientation within a molecule (Guex and Peitsch, 1997). The Swiss-PdbViewer is an ideal tool for the assessment of a change in an amino acid of a peptide bound within an HLA molecule, and how this change may impact upon epitopes which are proximal to the peptide binding groove.

The ability of a peptide to alter the epitopes to which an HLA antibody binds, has several implications within the transplant setting (Mulder *et al.*, 2005). Broadly speaking, this phenomena could affect the ability to detect clinically relevant alloantibodies, if different peptides are bound by HLA molecules used within detection assays to those bound to HLA antigens upon the allograft. While changes within the energy of an epitope as a consequence of peptide variation could potentially alter the function of an antibody which does bind (Kosmoliaptsis *et al.*, 2011).

4.2 Materials and Methods

4.2.1 Purification of an HLA antibody to assess the influence of peptide upon antibody binding

A human antibody specific to HLA-A2, obtained from a patient undergoing a desensitisation protocol was a kind gift from Dr David Lowe (Royal Liverpool and Broadgreen University Hospital, Liverpool, UK), who provided the HLA-A2 antibody in an unpurified form, containing other human plasma proteins. The antibody was purified to remove plasma proteins using Montage antibody PROSEP-G purification kits (Millipore, Maine, USA) according to the manufacturer's instructions. All processes were carried out at 4°C. Briefly the HLA-A2 antibody (300ml) was filtered through a 0.22µm filter (Millipore, Maine, USA) to remove any aggregates and debris. The resultant sample was diluted in a kit binding buffer (1:1) and loaded onto a spin column (10ml per column) containing a protein G plug. Protein-G was selected due to its binding affinity for human IgG_{1,2,3}, and 4, while having no affinity for human IgA, IgD, IgE and IgM, unlike the alternative, protein-A, which does not bind human IgG₃ and does bind human IgA, IgD, IgE and IgM (Akerstrom *et al.*, 1985). The spin column was centrifuged at 150g for 20 minutes to allow the HLA-A2 antibody to bind to the protein-G column. The column was then washed by loading the column with kit binding buffer (20ml) and by centrifuging the column for 5 minutes at 500g, to remove any unbound protein. The bound HLA-A2 antibody was eluted from the protein-G column via pH elution, by addition of a kit elution buffer (10ml) to the column and placing a collection tube containing a kit neutralization buffer (1.3ml), beneath the protein-G column to capture the eluted protein, prior to the centrifugation of the column for 5 minutes at 500g. The neutralisation buffer adjusted the pH of the eluted protein from the column to pH 7.2-7.6, to avoid denaturation of the captured antibody upon storage in elution buffer.

The resultant sample was desalted and concentrated using a centrifugal 30,000 MW Amicon Ultra-15 filtration device (Millipore, Maine, USA) by loading 15ml of the eluted protein and centrifugation at 4000g for 15 minutes, giving a final concentration of 0.30 mg/ml of purified protein. (As determined by NanoDrop (Thermo Scientific, Wilmington, USA) spectrophotometer, by measuring absorbance at 280nm, with the elution buffer being used to correct for background).

4.2.2 *Assessment of the epitope specificity of the HLA-A2 antibody*

The specificity of the isolated HLA-A2 antibody was assessed by the Luminex platform (Luminex, Austin, USA) and Labscreen single antigen HLA Class I HLA beads (referred herein as SAB, single antigen beads) (OneLambda, California, USA). The use of the Luminex platform and SAB for the detection of HLA antibodies, and their subsequent analysis for epitope specificity is highlighted within the 2014 British Society of Histocompatibility and Immunogenetics (BSHI) and British Transplant Society (BTS) joint guidelines for the detection and characterisation of clinically relevant antibodies in allotransplantation

(http://www.bshi.org.uk/BSHI_BTS_Ab_Guidelines_Revision_June_2014.pdf)

4.2.2.1 *Luminex solid phase immunoassay basic principles*

The Luminex is a solid phase immunoassay (SPI), which utilizes polystyrene beads which contain two fluorescent dyes, which emit light at different wavelengths, and are present within the beads at different ratios, the use of the dye ratio allows for up to 100 individual distinguishable bead populations. The surface of the polystyrene beads is coated with different recombinant HLA molecules, which act as a target for any HLA antibodies in a test serum. Antibody bound to the HLA molecules upon the surface of the bead can then be detected by an anti-human IgG antibody conjugated with a third fluorescent dye, phycoerythrin (known as a reporter dye). Analysis of the beads upon the Luminex platform simultaneously assesses the bead in terms of its internal dye ratio and the presence (or otherwise) of the reporter dye, thus allowing the bead identity, which was coated with a known HLA antigen/s, and the reactive HLA antibody detected by the reporter dye, to be determined. The SAB used within this chapter were coated with HLA class I antigens, covering reactivity against HLA-A, B and C antigens.

4.2.2.2 *Luminex solid phase immunoassay protocol*

Briefly the LABScreen beads (OneLambda, California, USA) were resuspended by repeat pipetting, then 5µl of LABScreen beads were incubated with 20µl of the purified HLA-A2 antibody in a 96 well filter plate (N.B. the filter plates were pre-wet with kit wash buffer (OneLambda, California, USA) prior to use) for 30 minutes in the dark (with gentle shaking on a plate shaker). Post incubation, the 96 well tray was washed by adding kit wash buffer (150µl per well), sealing with a tray seal and centrifuging at 1300g for 5

minutes. Wash buffer was removed by vacuum aspiration. This process was repeated 3 times with 200µl per well of wash buffer for all subsequent washes. 100µl of PE conjugated anti-human IgG was added to each well, which was then covered with a tray seal, gently vortexed and incubated in the dark for 30 minutes at room temperature with gentle shaking as above. Post-incubation, the 96 well tray was centrifuged at 1300g for 5 minutes and the supernatant removed by vacuum aspiration. 200µl of wash buffer was added and the tray was sealed and vortexed prior to centrifuging at 1300g for 5 minutes, the wash buffer was removed by vacuum aspiration as before. This wash step was repeated twice. 80µl PBS (Life Technologies, Fulton, UK) (6.8-7.2pH) was added to each well ready for acquisition and analysis upon the Luminex platform. Analysis was performed on the Luminex LABScan 100 instrument with the reactivity of the purified HLA-A2 antibody calculated from raw fluorescence values within the output csv file using HLA fusion (OneLambda, California, USA). The reactivity was normalised for background binding levels by correcting for bead reactivity to LABScreen negative control serum (OneLambda, California, USA), by dividing the reactivity level of a bead by the value of the negative control bead, as per the manufacturers' guidelines.

4.2.3 Determination of the eplet specificity of the HLA-A2 antibody using HLAMatchmaker.

The O1PAIRABSCREEN version of HLAMatchmaker was utilized for the analysis of data generated in 4.2.2 The identity of the HLA antigens coated onto the SAB beads, referred to as the panel of HLA antigens, was entered into the O1PAIRABSCREEN program, this data was derived from the SAB beads specific lot information, which is supplied with the beads. The MFI values of the SAB bead reactivity were transferred from the output.csv file generated in 4.2.2 by copying and pasting. A positive cut-off of 1000 MFI was used according to the criteria for the detection of clinically relevant HLA antibodies in renal transplant patients at St James University Hospital, Leeds. No HLA type of the immuniser or self HLA type was included in the analysis as this was unknown. The reactive eplets were displayed in the resultant HLAMatchmaker eplet results output sheet. The results were manually checked, highlighted and sorted to reveal the reactive eplets which were shared by any reactive SAB.

4.2.4 *Structural analysis of eplets and modelling the effect of changing the peptide sequence upon eplet structure*

The Swiss-PdbViewer (http://spdbv.vital-it.ch/download/binaries/SPDBV_4.10_PC.zip) (Guex and Peitsch, 1997) was used to visualise the structure of HLA-A2 with the peptide bound within the HLA groove, using HLA-A2 structures obtained from the protein database (www.ncbi.nlm.nih.gov/protein). The eplets identified in 4.2.3 via HLAMatchmaker were visualised using Swiss-PdbViewer, to determine their proximity to the peptide and the peptide binding groove. When an eplet was identified as proximal to the peptide, the mutation function of the Swiss-PdbViewer program was used to mutate the amino acid within the peptide. Ramachandran plots were used to visually assess the orientation of the change, in terms of the formation of appropriate torsion angles. Any change leading to improbable torsion angles was not analysed further and deleted (Hollingsworth and Karplus, 2010). The impact of altering an amino acid via the 'mutation' function upon identified eplets was assessed topographically and in terms of electrostatic potential and free energy, as previously described for comparing epitopes (Kosmoliaptsis *et al.*, 2011; Duquesnoy *et al.*, 2013). Electrostatic energy and free energy was calculated using the Groningen molecular simulation computer (GROMOS596) software which is integrated within the Swiss-PdbViewer program using Coulomb calculations (Guex and Peitsch, 1997). Solvent accessibility was also calculated using the Swiss-PdbViewer program and mapped directly to the surface, for rapid assessment of change of amino acid on solvent accessibility, which is used to determine antibody accessibility as described within the HLAMatchmaker system for determining epitopes (Duquesnoy, 2011a).

4.2.5 *Peptide influence on the ability of an eplet to bind its cognate antibody.*

To determine if peptides can influence the ability of antibody to bind to its target, the peptides, VMAPRTLIL, LLLSGALAL and ALGFYPAEI identified in chapter 2 as being capable of binding to HLA-A2, and utilised in the T2 binding assay (figure 2.12 and 2.14 peptides 7, 13 and 19 respectively) were used to determine if any of these peptides could affect antibody binding to those eplets close to the binding groove (as identified in 4.2.4).

The HLA-A*02 antibody described in 4.2.2 and 4.2.3 was used in conjunction with a w6/32 mouse monoclonal antibody (Abcam, Cambridge, UK). The w6/32 antibody is specific to an epitope located away from the peptide binding groove, upon residue 121 of the α -chain, proximal to the β_2 -microglobulin, which is also argued to form a functional portion of the epitope (Trayssac *et al.*, 2012), and therefore will be unaffected by changes within the peptide (figure 4.3). Titrations of the purified HLA-A*02 antibody were performed to determine antibody saturation levels, the w6/32 antibody was used as per the manufacturers' instructions (volumes and concentrations are described below).

The ratio of the fluorescence intensity of w6/32 (FL-2) to HLA-A2 (FL-1) test antibody was calculated and any change in the ratio, when altering the peptide sequence, was taken to indicate peptide influence over binding by HLA-A2, this was calculated as FL-1 / FL-2, such that an increase in ratio indicated stronger binding, while a reduced ratio a decrease in binding. Direct measurement of FL-1 as an indication of HLA-A2 binding was not possible, as individual peptides stabilise HLA-A2 at different rates (see fluorescence intensity values in figures 2.13 and 2.14), which would skew the data if a measurement of the FL-1 fluorescence alone was used.

Peptides ALGFYPAEI, VLLLLSAAL, VMAPRTLIL, and the HA-1 control peptide (VLHDDLLEA) from the T2 binding assay (section 2.2.8), were used to stabilise the T2 cell line. Negative controls of DMSO treated and unstabilised T2 cells were also used (as described in section 2.2.8). The only alteration to the assay described within chapter 2 was the addition of the HLA-A2 antibody purified in 4.2.1 and a subsequent detection phase. The HLA-A2 antibody was incubated with the T2 cell line together with the mouse anti-w6/32 conjugated to phycoerythrin (PE), at saturation conditions (20 μ l of HLA-A2 antibody at 20 μ g/ml and 5 μ l of w6/32 (the antibody was supplied pre-diluted) per 1×10^6 T2 cells), and incubated for 30 minutes in the dark, before washing twice with serum free RPMI 1640 media (Life Technologies, Fulton, UK). To detect the bound HLA-A2 IgG antibody, a mouse anti-human IgG FITC conjugated.

An Unpaired *t*-test was used to assess the influence upon antibody binding using GraphPad analysis software (GraphPad software, CA, USA).

antibody (BD Pharmingen, Oxford, UK) was added at saturation conditions (determined by titration to be 25µl per 1×10^6 cells) and incubated for 30 minutes in the dark, before washing twice with serum free RPMI 1640 media as before. The washed cells were then fixed with 0.5% paraformaldehyde ready for analysis. Analysis was performed on a FACSCalibur (BD biosciences) with fluorescence measured on the FL-1 and FL-2 channels.

4.2.6 Reproducibility of the assays described in chapter 4

All assays were performed a minimum of 3 times. T2 binding assays and flow cytometric assays were performed in triplicate and repeated at least 3 times.

| | |
|---|---|
| Purification of HLA-A2 antibody by protein G | <ul style="list-style-type: none"> •Assessment of reactivity to HLA-A2 by Luminex single antigen beads |
| Determination of purified HLA-A2 antibody epitope reactivity | <ul style="list-style-type: none"> •.CSV file from Luminex single antigen beads entered into HLA matchmaker via import •Determination of epitope reactivity using HLA Matchmaker eplet system |
| Structural analysis of identified eplets to determine the location of the epitopes on the HLA-A2 molecule | <ul style="list-style-type: none"> •Swiss-PdbViewer utilized to assess location of identified eplets upon HLA-A2 molecules obtained from the protein database |
| Structural modelling of the effect of changing an amino acid within the HLA bound peptide upon eplets identified on HLA-A2 which engage/are proximal to the peptide | <ul style="list-style-type: none"> •Swiss-PdbViewer utilized to change appropriate amino acids and measure the effect upon eplets generated |
| Functional analysis of the effect of changing the peptide bound by HLA-A2 | <ul style="list-style-type: none"> •T2 cells loaded with different peptides and the ability of antibody to bind when the different peptides are in place measured |

Figure 4.4 Flow diagram of the work performed in chapter 4

4.3 Results

4.3.1 Determining antibody specificity towards HLA-A*02

The reactivity of the antibody donated by Dr David Lowe, and then purified, towards HLA-A2 was confirmed by analysis upon SAB, using the Luminex platform. A screen shot of the Manhattan graph from the HLA fusion software used is shown in figure 4.5. Each column in the graph represents a bead within the assay, and the antigens coated onto each bead are listed beneath each column, in rows representing HLA-A, B and Cw antigens. Visual inspection of the graph reveals the antibody has reacted with beads coated with HLA-A2, 69, and B57, 58 antigens, while the rest of the beads coated with antigen remain negative, this data was transferred into the HLA matchmaker software.

4.3.2 Identifying epitope reactivity of HLA-A*02 specific antibody

The measure of reactivity against the beads can be seen more clearly in figure 4.6, which shows a condensed version of the output file from the HLAMatchmaker software. The positive and negative controls produce a reactivity value of 11,908 and 7 MFI respectively, demonstrating the validity of the assay. Seven beads show positive reactions with the purified HLA-A2 antibody, with highest reactivity being observed on the three beads present in the assay which are coated with HLA-A2 antigens, specifically HLA-A*02:03, 02:01 and 02:06, which had MFI values of 14,743, 14,378.5 and 13,797 respectively. The HLAMatchmaker software revealed that two reactive eplets were present upon these antigens, both the 62GE and 107W eplet. Reactivity was also observed for the purified HLA-A2 antibody on the HLA-A*69:01 coated bead, which possesses a single eplet shared with the HLA-A2 reactive beads, this being the 107W eplet, the reactivity was lower than for the HLA-A2 beads being 12,198 MFI. The final three reactive beads also shared a single reactive eplet with the HLA-A2 reactive beads, this being the 62GE eplet present upon the HLA-B*57:03, 58:01 and 59:01. The reactivity on these beads was reduced even further, with MFI levels of 3,827, 3,393 and 2,495 respectively. These epitopes have been previously observed (Mulder *et al.*, 2005).

These results confirm the reactivity of the purified HLA-A2 antibody for the HLA-A2 antigen, and demonstrate the antibody reactivity is directed against two epitopes, identified as the 62GE and 107W eplet.

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ଘଣ୍ଟା ଗ୍ରହଣ କରନ୍ତୁ । ଯଦି କୌଣସି ପ୍ରଶ୍ନ ଥାଏ ତେବେ ଶୁଣାଯାଉ ।
ପ୍ରଶ୍ନ ୧ : ଯଦି ଏକ ବସ୍ତୁର ବସ୍ତୁତ୍ଵ ୧୦ କିଲୋଗ୍ରାମ ହୁଏ ଏବଂ ଏହାର ଆୟତ୍ତ ୨୦ ଘନ ମିଟର ହୁଏ ତେବେ ଏହାର ଘନତ୍ଵ କେତେ ହେବ ?
ପ୍ରଶ୍ନ ୨ : ଯଦି ଏକ ବସ୍ତୁର ବସ୍ତୁତ୍ଵ ୧୦ କିଲୋଗ୍ରାମ ହୁଏ ଏବଂ ଏହାର ଆୟତ୍ତ ୨୦ ଘନ ମିଟର ହୁଏ ତେବେ ଏହାର ଘନତ୍ଵ କେତେ ହେବ ?
ପ୍ରଶ୍ନ ୩ : ଯଦି ଏକ ବସ୍ତୁର ବସ୍ତୁତ୍ଵ ୧୦ କିଲୋଗ୍ରାମ ହୁଏ ଏବଂ ଏହାର ଆୟତ୍ତ ୨୦ ଘନ ମିଟର ହୁଏ ତେବେ ଏହାର ଘନତ୍ଵ କେତେ ହେବ ?
ପ୍ରଶ୍ନ ୪ : ଯଦି ଏକ ବସ୍ତୁର ବସ୍ତୁତ୍ଵ ୧୦ କିଲୋଗ୍ରାମ ହୁଏ ଏବଂ ଏହାର ଆୟତ୍ତ ୨୦ ଘନ ମିଟର ହୁଏ ତେବେ ଏହାର ଘନତ୍ଵ କେତେ ହେବ ?
ପ୍ରଶ୍ନ ୫ : ଯଦି ଏକ ବସ୍ତୁର ବସ୍ତୁତ୍ଵ ୧୦ କିଲୋଗ୍ରାମ ହୁଏ ଏବଂ ଏହାର ଆୟତ୍ତ ୨୦ ଘନ ମିଟର ହୁଏ ତେବେ ଏହାର ଘନତ୍ଵ କେତେ ହେବ ?

HLAMatchmaker output file

| HLA Class | Allele | Gene | Position | Frequency | Population | Match | Match Score | Match Type | Matched Allele |
|-----------|---------|---------|-----------|-----------|------------|-------|-------------|------------|----------------|
| A*01:01 | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| | A*01:01 | A*01:01 | 253200000 | 0.999 | CEU | + | 1000 | + | A*01:01 |
| B*07:02 | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |
| | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |
| | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |
| | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |
| | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |
| | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |
| | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |
| | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |
| | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |
| | B*07:02 | B*07:02 | 253200000 | 0.999 | CEU | + | 1000 | + | B*07:02 |

HLAMatchmaker output file

HLAMatchmaker output file

HLAMatchmaker output file

4.3.3 Identifying the proximity of the identified epitopes to the HLA bound peptide

Using the protein database the crystal structures of HLA-A*02 (PDB no. 3D25) were visualised in Swiss-PdbViewer, and the eplets 62GE and 107W were analysed for proximity to the peptide binding groove (figure 4.7 [A], [B] and [C]).

This reveals that the 62GE eplet is proximal to the peptide bound within HLA-A2. The structural modelling in figure 4.7 [A], showing the 3 dimensional structures of the peptide and 62GE, appearing to touch each other. The 62GE eplet is identified as being upon the side wall of the α -helical peptide binding groove. The 107W eplet however is shown as being distant from the peptide binding groove, shown from the top in figure 4.7 [B], the 107W eplet is located on the side of the HLA-A2 molecule, on the β -pleated sheet outside, the peptide binding groove.

4.3.4 Assessing the variation in antibody binding due to differential peptide bound by HLA-A*02

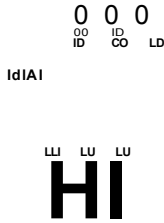
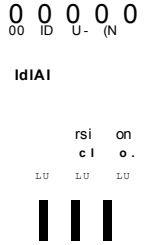
The proximity of the 62GE eplet to the peptide makes 62GE an ideal candidate for assessing if any changes within the peptide can influence the ability of peptide to bind. The assessment of the anti-HLA-A2 antibody's ability to bind to HLA-A2 when loaded with different peptides is shown in figure 4.8. Whereby, the ratios of FL-1 (anti-HLA-A2) and FL-2 (w6/32) MFI were used to demonstrate any change in the ability of the anti-HLA-A2 antibody to bind. Comparison of the two negative controls, the DMSO control and the unstabilized T2 cells, reveal a consistent ratio of around 6, indicating the assay has reproducible results. Comparison of the FL1/FL2 ratios of the T2 cells loaded with different peptides shows variation within the ratios (figure 4.8). The HA-1 peptide, which has a sequence of VLHDDLLEA, has the highest ratio of around 12, indicating the greatest antibody binding was observed when this peptide was bound by HLA-A2, while peptide 7, with a sequence of VMAPRTLIL demonstrated the weakest antibody binding, with a ratio of 8, peptides 13, VLLLLSAAL and 19, ALGFYPAEI, having ratios of around 11 and 10 respectively.

The major difference between the two specific peptides which are associated with the strongest (the VLHDDLLEA peptide) and weakest (the VMAPRTLIL peptide) HLA-A2

Titration of human anti-human IgG against HLA-A*02 specific antibody stained T2 cells

w6/32 titration against the T2 cell line

5 6 7 8



0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

0 15 30 45 60

antibody binding, in terms of the peptide regions which are proximal to the 62GE eplet, is seen in peptide residues 2 and 3. Both of these peptides have V (valine) in position 1, while the strongest antibody binding was seen when Leu (leucine) and His (histidine) were in positions 2 and 3, while the weakest binding was seen when Met (methionine) and Ala (alanine) were in position 2 and 3. In peptides 13 (VLLLLSAAL) and 19 (ALGFYPAEI), leucine was also seen in position 2. Therefore all the peptides which had L in position 2, demonstrated a greater ability to bind the anti-HLA-A2 antibody, than the peptide with Met in position 2. To determine if a change between Met and Leu at position 2 could be responsible for the difference between the antibodies ability to bind, structural modelling of HLA-A2 with peptides which were identical except for containing Leu, and then Met at position 2 was performed (figures 4.9 and 4.10).

4.3.5 Structural modelling of the effect of changing a single amino acid within the HLA bound peptide on an epitope proximal to the peptide binding groove

The structural modelling of the HA-1 peptide containing L at position 2 is shown in figure 4.9. The 62GE eplet consists of both glycine (G or Gly) and glutamic acid (E or Glu), these amino acids are highlighted within figure 4.9, while the position of 62GE in relation to the L amino acid of the peptide can be seen to be separated by another amino acid upon the α -chain, a lysine (K) at position 66. The 62GE eplet does not appear to be obscured by this amino acid at position 66, in terms of its accessibility to be bound by an antibody. The figure also demonstrates the overall electrostatic potential of the HLA-A2 molecule bound with HA-1, with Leu in position 2, for comparison to the electrostatic potential when Met is in position 2. The distance between the Leu and 62GE is 4.081Å while in the modelling of 62GE when Met is in position 2 (shown in figure 4.10), the distance is reduced to 3.341Å. The modelling of Met in position 2 also shows that hydrogen bonds are formed between the 62GE eplet and the Met amino acid, no hydrogen bonds were visible when Leu was in position 2. A comparison of the electrostatic potential and total free energy of the glycine and glutamic acid residues, which form the 62GE eplet is shown in table 4.2 for both the Met and Leu residues within position 2 of the peptide. The data for the Met residue shows a decrease in the total energy (E) on both the Gly and Glu amino acid residues within the 62GE eplet, with a reduction in total energy of -0.7777 and -2.108 E

than -1.6, white 0.00 and blue greater than 1.6. The area circled indicates the 62GE -Leu area. Modelled using Swiss-PdbViewer (Guex & Peitsch,

Modelled using Swiss-PdbViewer (Guex & Peitsch, 1997).

| Residue | Position | Electrostatic Potential (kT/e) with Leu at peptide P2 | Electrostatic Potential (kT/e) with Met at peptide P2 | Total Energy (E) with Leu at peptide P2 | Total Energy (E) with Met at peptide P2 |
|----------------|--------------------|--|--|--|--|
| Gly | α -chain 62 | 0.00 | 0.00 (0) | -14.632 | -15.409 (-0.777) |
| Glu | α -chain 63 | 11.61 | 11.61 (0) | -28.414 | -30.522 (-2.108) |

Table 4.2 The predicted change in total energy and electrostatic potential upon Gly and Glu, due to changing the amino acid within the peptide at position 2 (P2) from Leu to Met.

respectively. There was no difference in the electrostatic potential for either of the Gly or Glu residues when Met or Leu was in position 2.

To determine if any change between the Met and Leu amino acid residues within position 2 of the peptide, altered the accessibility of the 62GE eplet to antibody, their solvent accessibility was calculated for comparison (figure 4.11).

The solvent accessibility demonstrates no variation between the molecules when they have either Leu or Met in the peptide position 2. The area of the 62GE eplet is highlighted within the solvent accessibility structure. A sliding scale of colours is used to determine the accessibility of solvent into the molecule, blue indicating a completely buried residue while red a total exposed residue.

accessible are coloured red while blue indicates completely buried residues. The circle indicates the 62GE and Leu/Met area.

4.4 Discussion

The purified HLA-A2 antibody was shown to be specific for both the 62GE and 107W eplets. The reactivity within the Luminex assay was not limited to HLA-A2 however, but the presence of the 62GE eplet on HLA-B*57:03, 58:01 and 59:01 antigens, explains the reactivity of beads coated with these antigens within the assay, as does the presence of 107W on the HLA-A*69:01 antigen coated bead. Interestingly the 62GE and 107W eplets, which are both present on HLA-A2, being present on separate antigens (62GE on B*57, 58 and 59 and 107W on A*69) allows for an indication of the strength of reactivity to these individual eplets, which cannot be determined from the HLA-A2 reactivity alone. The mean reactivity on the beads which had both 62GE and 107W eplets is 14,309 MFI, which comprises reactivity to both 62GE and 107W. The reactivity on the bead coated solely with 107W however was 12,198, much higher than the reactivity seen on those beads with only the 62GE eplet (mean MFI 3,238). This indicates that the anti-HLA-A2 antibody most likely consists of two anti-HLA-A2 antibodies, at two different strengths. The first and strongest being reactive to 107W, while the second and weaker reactivity being to 62GE. The addition of the individual 62GE and 107W reactivity from beads with only one of these eplets present gives a value similar to that seen on beads which possess both of these eplets.

Mapping the location of these eplets to the surface of HLA-A2, identifies they are located at distinctly different sites on the molecule. The 107W being on the side of the molecule and distant to the peptide binding groove, and therefore unsuitable for assessing the effect of variation within the peptide upon antibody binding. The 62GE eplet however, is shown to be on the α -helical wall of the peptide binding groove, and therefore would be an ideal candidate to assess if changes within the peptide bound within the binding groove, could affect the ability of the antibody to recognise this eplet.

The data showing the ratio of the bound HLA-A2 antibody to the bound w6/32 antibody, shows that there is a variation in the amount of HLA-A2 antibody bound, when the peptides within the HLA-A2 binding groove are changed. However, as mentioned within the introduction to this section, the majority of binding of an

antibody is derived from the 'hot spot' area (Duquesnoy, 2011a), identified in this case as the eplet 62GE, but further binding energy is also provided by the area surrounding the 'hot spot'. Therefore the variability in the HLA-A2 antibody binding HLA-A2, when different peptides are present, could be due to antibody contact directly with the peptide itself as well as to the 62GE eplet. To clarify this further work is required, which could encompass the alteration of a single amino acid residue at a time, within the peptide bound to HLA-A2 and measurement of antibody binding. Furthermore the use of surface plasmon resonance (SPR) to assess the on/off rates of antibodies, when different peptides are present within the HLA-A2 binding groove, could be carried out. However what the data presented here does demonstrate is that anti-HLA-A2 antibody binding, is affected by different peptides being bound within the HLA-A2 molecule, it is the specific mechanism however, which is not fully elucidated here.

Interestingly those peptides which have the highest antibody binding ratios (figure 4.8), all have Leu at position two of the peptide, which fits the reported binding characteristics of the HLA-A2 molecule (Paul, 2013), and is observed as a favourable amino acid for the HLA class I molecules pocket B (discussed in section 1.1.19) within HLA-A2 molecules. Furthermore, another amino acid which reportedly fits the pocket B characteristics is Met amino acid residue, which is observed frequently within this position from peptides eluted from HLA-A2, although less so than Leu (Paul, 2013).

The reported observation of Met and Leu, as favoured amino acid residues at position 2 of peptides bound within the HLA-A2 molecule, combined with the data presented here showing that the peptides with Leu in position 2 were able to bind more HLA-A2 antibody than the peptide with Met at position 2. Highlight these two amino acids as interesting candidates for affecting the binding of anti-HLA-A2 antibody.

The structural analysis of peptides with Leu or Met at position 2 (figure 4.9, 4.10, and 4.11), and the relative effect upon the 62GE eplet, revealed some interesting findings. At the closest point between the Leu and 62GE eplet, the distance is measured as 4.081Å, greater than the 3.0Å radius HLAMatchmaker uses to generate its eplets (Duquesnoy and Marrari, 2009), no bond formation is seen between the 62GE and Leu residue either, in addition a Lys at position 66 appears above both the 62GE and Leu,

although the 62GE does not appear to be directly obscured by the Lys at position 66, it may prevent some interaction between them. Furthermore to some extent this Lys could be expected to shield the Leu residue of the peptide to access by a binding antibody, due to its presence above the Leu residue side chain.

Changing the Leu to a Met residue however, results in hydrogen bond formation between Met and the 62GE eplet. This formation appears to draw the Met residue closer to the 62GE eplet, with a distance of 3.341Å from the 62GE eplet. The Lys at residue 66 is positioned as before, but this time proximal to the Met residue. Potentially the formation of the hydrogen bonds between Met and 62GE could account for the reduced ability of the 62GE specific, purified HLA-A2 antibody, to bind its target by drawing it closer to the Lys residue at position 66 and shielding its access to 62GE specific antibody. This seems unlikely as the majority of 62GE still appears to remain in an antibody accessible position, however to determine any change in accessibility more qualitatively, an assessment of solvent accessible residues was performed, which revealed no discernible variation in terms of antibody accessibility between both the Leu and Met containing peptides.

In an assessment of the Bw6 and Bw4 epitopes, and their ability to bind antibody, Kosmoliaptsis and colleagues (2011), determined the electrostatic potential of Bw4 and Bw6 epitopes upon various HLA-class I molecules and determined that 'changes of critical amino acids that abrogate antibody binding also induce distinct changes in epitope electrostatic potential'. These concepts were used to investigate the binding of the purified HLA-A2 antibody to the 62GE eplet.

A comparison of the electrostatic potential of the Gly and Glu residues, which form the 62GE eplet, with Leu and then Met present at position 2 of the peptide, revealed no variation within the electrostatic potential. However the electrostatic potential calculations performed by the Swiss-PdbViewer utilizes Coulomb calculations, which are reportedly less sensitive than the Poisson-Boltzmann calculations (Guex and Peitsch, 1997) performed by the DELPHI program used by Kosmoliaptsis *et al.*, (2011), and therefore may not be sensitive enough to measure any change. However the additional sensitivity of the Poisson-Boltzmann calculations is derived from their ability

to consider the influence of buried residues within the protein (Guex and Peitsch, 1997), which may be of a lesser significance here, since both the peptide bound by HLA-A2 and the 62GE eplet are present at the protein surface.

Recently, a measure of total free energy has been shown to influence not only the ability of an HLA specific antibody to bind, but also the subsequent ability of this antibody firstly to bind C1q, and secondly, to initiate the complement cascade (Duquesnoy *et al.*, 2013). It is interesting to note then that the total free energy calculated for the Gly and Glu amino acid residues of the 62GE eplet varies dependent upon whether Leu or Met is in position 2 of the peptide bound by the HLA-A2 molecule, with a reduction of total energy when Met is present in comparison to Leu. This could be a result of the formation of the hydrogen bonds when Met is present, which would require energy, and therefore less energy is available for antibody binding. Whether this could explain the reduction in the ability of anti-HLA-A2 antibody to bind is uncertain, and most likely results from a combination of different factors, including reduction in available total energy. An alternative hypothesis could also be developed from the Duquesnoy *et al.*, (2013) paper.

Duquesnoy describes the function of other epitopes, which are within a region of around 6-15Å of the epitope to which the antibody is specific. Clearly this would include much of the peptide, which would be expected to fall within a 6-15Å region of the 62GE eplet (figures 4.9 and 4.10). These epitopes within 6-15Å of the 62GE eplet are capable of providing variation within free energy, depending upon their composition and therefore may influence antibody binding and function. This has not been assessed within the structural modelling carried out here, but offers another explanation for the variation in HLA-A2 (62GE) antibody binding, observed when the peptide loaded onto HLA-A2 is altered within the T2 binding assay.

The impact of the variation in antibody binding as a result of different peptides being bound to the HLA molecule is potentially multifaceted, with implications for the detection of antibodies by routinely employed methodologies within H+I laboratories, as different peptides may be present in different manufacturers solid phase assay HLA antibody detection kits (Duquesnoy *et al.*, 2013), while in a clinical context different

minor histocompatibility antigens could change the ability of antibodies to bind to their target, such that the same antibody could have a different clinical relevance in different individuals.

In summary, chapter 4 has highlighted that peptides are not only capable of inducing alloreactivity through their influence upon the TCR, but also could affect humoral responses through their ability to affect antibody binding to the allograft.

Current concepts concerning the role of peptides within the processes of T cell selection and the establishment of central tolerance, as well as the key role played by peptides in the TCR-pHLA interaction are an area of intense research. Peptides are also fundamental to the function of HLA molecules and are crucial to their formation within the HLA-Class I and Class II processing and presentation pathways. Thus, an appropriate response to pathogens while maintaining a tolerance of self, fundamentally involves peptides as well as HLA molecules and the TCR.

In the context of allotransplantation, the roles of peptides within allorecognition demonstrates a fundamental role for peptides, including mHAGs, within allorecognition. Of most interest in the context of this thesis, is the significant association of HLA mismatching on the outcome of allotransplantation, and the role of peptides derived from the HLA molecules themselves.

The experimental work in chapter 2, investigated the identification of peptides derived from HLA molecules, which could have an influence upon the alloresponse, as a consequence of their presentation by another HLA molecule. This was achieved by the creation of a comprehensive database of peptides predicted to bind to HLA-A*02. Due to the differential binding properties of HLA molecules themselves, limiting the database to HLA-A*02 was necessary. This produced a database of peptides predicted to bind to HLA-A*02 from each of the HLA class I amino acid sequences listed within the IMGT/HLA database.

This database was then utilized to identify peptides bound by HLA-A*02, with the HLA-A*02 molecules being obtained from the THP-1 monocytic cell line. Through the use of MS/MS sequencing and the database of peptides which were predicted to bind to HLA-A*02, two peptides, which were derived from the other HLA molecules expressed upon THP-1 cells, and were bound and presented by the HLA-A*02 molecule, were identified. These were peptides ALGFYPAEI and VMAPRTLIL, encoded for within the HLA type present on THP-1 cells (Battle *et al.*, 2012), specifically within HLA-B*15 and HLA-C*03 HLA molecules respectively.

The consequence of this in a transplant setting is that matching for donor and recipient HLA-A*02 antigens, while having a mismatch on the donor cells of B*15 and/or HLA-C*03 would allow for a recipient's HLA-A*02 restricted T cells, to bind the donor HLA-A*02 molecule, complexed with the ALGFYPAEI or VMAPRTLIL peptide, since the recipient's TCR would not have experienced the ALGFYPAEI or VMAPRTLIL peptide complexed with self HLA-A*02 during development in the thymus, thus it would have the potential to form a response.

Significantly HLA-B*15 and HLA-C*03 are not the only HLA molecules to encode ALGFYPAEI or VMAPRTLIL, the database of peptides generated within chapter 2 also shows that HLA-A*01, A*03, A*11, A*23, A*24, A*30; B*18, B*27, B*35, B*38, B*39, B*40, B*41, B*42, B*44, B*45, B*46, B*47, B*48, B*49, B*50, B*51, B*52, B*53, B*54, B*55, B*56, B*57 alleles encode for ALGFYPAEI, while HLA-C*01, C*04, C*05, C*06, C*08, C*12, C*14, C*16, C*17:02 alleles encode VMAPRTLIL. The significance of this is that mismatches of each of these molecules would be expected to generate the same HLA-A*02 and ALGFYPAEI or VMAPRTLIL bound peptide. However, if the recipient also possessed one of the other HLA molecules which encoded for the peptide, then this potential to generate an alloresponse, as a consequence of the peptide, would be null, as they would possess an HLA type which included the peptide, and thus T cells expressing TCR specific for this self peptide/HLA would have been eliminated in the thymus, as self reactive T cells.

These principles can be proposed analogous to Duquesnoy's system of determining acceptable mismatches, by the identification of epitopes throughout an HLA type, (Duquesnoy, 2011a), whereby instead of comparing whether a donor and recipient share the same HLA molecules, comparison of whether the donor and recipient HLA molecules share the same epitopes is assessed via the HLAMatchmaker program, these epitopes are not necessarily on the same HLA molecules. Using this logic to demonstrate the significance of the work initiated within chapter 2, an HLA molecule could be seen as a group of peptides capable of being bound by other HLA molecules, some of these peptides will be shared between HLA molecules and others not, allowing the identification of permissible peptide mismatches, and identifying potential alloreactive peptide mismatches.

Clearly the work undertaken in chapter 2 can only be seen as the first steps in this approach, and a significant amount of further work needs to be performed. In the first instance, peptides obtained from HLA-A2 expressed in conjunction with other HLA antigens needs assessing, allowing the database of HLA-A*02 encoded alleles, which can present peptides obtained from HLA molecules which were not expressed on THP-1 to be examined. Subsequent to this work the database needs expanding for the prediction of peptides which bind to HLA molecules other than HLA-A2.

To achieve this a significant amount of further work needs to be performed, however this is not as insurmountable as it first appears. Due to the knowledge that although HLA molecules are extensively polymorphic, the HLA molecules can be thought of as being highly polymorphic in specific areas, whilst conserved in others (figure 1.9). This has the effect of generating peptides which are of identical sequences from the non polymorphic areas, limiting the number of peptides which require analysis.

Furthermore, many of the polymorphic sites are shared between some HLA alleles, indeed the ALGFYPAEI peptide identified within chapter 2 was encoded for by over 25 groups of HLA class I alleles.

The peptides derived from non-polymorphic areas should not however be dismissed as unable to generate alloresponses, as, if these conserved peptides are presented by a mismatched HLA molecule, which therefore the recipient has not experienced during the establishment of central tolerance, and this presentation produces novel epitopes as a consequence of variation in the binding of the peptide, due to different properties of the peptide binding groove; then these conserved peptides can also be thought of as having an alloreactive potential.

These concepts can be thought of as an extension of the multiple binary complex theory, in which, donor peptides which are derived from homologous intracellular proteins, which are also present within the recipient, have a different set of peptides presented from the same intracellular proteins, due to variation in the recipient and donor's HLA binding grooves (Bharat and Mohanakumar, 2007).

It is most likely that a combination of peptide related alloresponses are responsible for direct recognition, rather than the single concept outlined in the multiple binary complex theory. This combination would include the peptides generated as described by the multiple binary complex theory, with the addition of the presentation of peptides derived from mismatched HLA and presented by matched HLA, as described in chapter 2, and the presentation of the homologous peptides in a different manner to that experienced during central tolerance, due to variation in the peptide binding groove. Perhaps a more appropriate collective term for these processes is abating negative selection, in which the negative selection process of central tolerance is reduced in its amount, degree and intensity. In support of this more comprehensive description of the involvement of peptides in direct allorecognition is the observation that central tolerance can be re-established post transplant by the intrathymic injection of peptides (Oluwole *et al.*, 2001), and more recently that a chimeric state in renal transplant recipients by simultaneous HSCT has been shown to be capable of providing an environment suitable for renal allograft survival, without maintenance immunosuppression (Kawai *et al.*, 2008). The proposed mechanism of tolerance following chimerism has been suggested to be the elimination of 'reactive cells within the thymus which are reactive to donor antigen' (Kawai *et al.*, 2008). Essentially re-establishing central tolerance, or described another way, removing the impact of abated negative selection by re-establishing it with the presence of donor antigens.

In further work, the impact of the formation of HLA-A*02 molecules complexed with HLA derived peptides, to which a recipient does not encode any antigens for, should be examined in a functional setting. The presence of T cells, which are reactive to HLA-A*02 possessing these specific peptides could be assessed post transplant in peptide mismatched patients. For example, in a post transplant setting, where the recipient and donor both possess HLA-A*02 molecules, while the donor also expresses the VMAPRTLIL peptide encoded for by HLA-C*03 and the recipient does not possess another VMAPRTLIL encoding HLA allele, the patients' post transplant CD8⁺ cells could be stained with HLA tetramers specifically targeting HLA-A*02/ VMAPRTLIL complexes, allowing any CD8⁺ positive cells with a TCR reactive to the HLA-A*02/ VMAPRTLIL complexes to be identified. Once identified, these cells could then be interrogated in terms of their function via intracellular cytokine staining and the cell surface markers

present. Such work could identify the presence of surface expression of CD69⁺, an activation marker on CD8⁺ T cells, while the cytokine profiles of these cells, may further elucidate the mechanisms of allograft damage. The examination of post transplant patients for the non-classical HLA-E molecules complexed with specific peptides using tetramers has already been performed within the literature (Allard *et al.*, 2012) validating this approach to some extent.

The leader peptide sequences of HLA are of particular interest, with only one of the comprehensive list of leader peptide sequences identified within chapter 2, demonstrated as being able to be bound to by HLA-A*02 however. This was confirmed both by binding studies using the T2 cell line, as well as it being identified amongst the peptides bound to the HLA-A*02 molecule encoded on the THP-1 cell line itself. The identification of the leader peptide VMAPRTLIL within chapter 2 initiated the work undertaken in chapter 3. As novel functions have previously been reported for leader peptides such as VMAPRTLIL, in that they are crucial for the formation of HLA-E and their function with NK cell receptors NKG2/CD94 (Petrie *et al.*, 2008).

Given that leader peptides, as shown within chapter 2, can be bound by HLA class I molecules (in this case HLA-A*02) and therefore be presented to CD8⁺ T cells, and that they also form a part of a crucial ligand for NK cell receptors. They therefore cross both the innate and adaptive immune responses and are an interesting target for investigating the role of peptide mismatching within the transplant setting.

The effect of mismatching of HLA class I derived leader peptides was performed within chapter 3 via the assessment of the clinical impact of their mismatches within a cohort of renal transplant patients. A search of the literature indicates this represents the first time a group of endogenously derived peptides had been assessed in such a manner, although the need for the assessment of such peptides in the setting of allotransplantation has been identified previously (D'Orsonga *et al.*, 2013). The assessment of HLA class I derived leader peptide sequence mismatches, demonstrated a striking relationship between a reduction in renal allograft function, as assessed by Δ creatinine, with an increase in the number of mismatches. This function appeared to be independent of HLA matching (Battle *et al.*, 2013). Scrutiny of the work within

chapter 3 reveals several areas for further work. Firstly, the cohort of patients analysed here was relatively small, this is of particular significance when data analysis focuses on the level of the leader peptide mismatches within specific HLA matched groups, such as the ODT matching level. A larger validating cohort of patients should now be assessed to validate this data and confirm the findings within chapter 3. By increasing the size of the cohort, the confounding influence which affected the work assessing the effect of leader peptide mismatches in NHSBT ODT level 3 matched groups may be limited.

Secondly, the exact mechanism by which the leader peptide mismatching is exerting this effect also needs investigating. In the first instance this work could be performed in conjunction with the additional work required from chapter 2. In that tetramer staining of post transplant recipient cells for potential HLA leader peptide binding targets, specifically HLA-E and mismatched HLA antigens could be performed, and if the presence of any cells responding to these specific antigens are identified, then they could be interrogated in terms of their function and cytokine profiles, as described for the further work outlined within chapter 2. Although the effect of presentation of leader peptides via HLA class I molecules directly to CD8⁺ cells will most likely generate alloresponses as described earlier, the evidence within the literature (Tamouza *et al.*, 2006; Danzer *et al.*, 2009; Hosseini *et al.*, 2013; Hoare *et al.*, 2008; Battle *et al.*, 2013) already supports the concept of a strong influence of HLA-E bound leader peptide mismatching upon the Δ creatinine levels described in chapter 3.

The literature reports the presence of HLA-E restricted effector memory cells in patients who are infected with the CMV AD169 virus, which encodes a homologous leader peptide sequence to maintain HLA-E surface expression, as part of an immunoevasion strategy (Mazzarino *et al.*, 2005). The effector memory cells being detected only in patients who possess different leader peptide sequences in their HLA type to the one CMV is mimicking (Mazzarino *et al.*, 2005), and can thus effectively target and kill the viral infection. This demonstrates the specificity of the mechanism by which leader peptides can exert allograft damage, in that instead of CMV introducing a leader peptide sequence to which a response is formed, the leader peptides are introduced via the allograft. Essentially the further work using HLA

tetramers, described above, is similar to that work carried out on CMV infection (Mazzarino *et al.*, 2005), except that responses to mismatched leader peptides on the allograft would be assessed, instead of mismatched CMV leader peptide immune-evasion strategies, in terms of their ability to generate responses.

Considering concepts which relate, instead of to HLA-E presentation to CD8⁺ cells, but to the involvement of HLA-E and innate immune responses to allografts. HLA-E interaction with CD94/NKG2 receptors, as described in chapter 3, has been shown to be affected by the affinity of leader peptides to HLA-E (Hoare *et al.*, 2008). This affinity alters the ability to be bound by HLA-E and has a direct effect upon the expression level of HLA-E (Vales-Gomez *et al.*, 1999), and therefore changes within peptides which have different binding affinities would be expected to alter surface expression of HLA-E, and thus alter the ability of HLA-E to interact with the CD94/NKG2 activating/inhibiting receptors.

It is interesting to note then that the data in chapter 3 demonstrates that Δ creatinine levels increase, when the leader peptide mismatches are amongst those leader peptides which are reported to have different affinities for HLA-E, such as HLA-B derived leader peptides, while in those reported as having the same affinity for HLA-E, such as HLA-A derived leader peptides (Hoare *et al.*, 2008; Lee *et al.*, 1998; Lemberg *et al.*, 2001; Vales-Gomez *et al.*, 1999), there appeared to be no change in Δ creatinine levels. This data strongly indicates that the innate responses generated by HLA-E have a role within the influence of HLA class I leader peptide derived mismatches and the performance of renal allografts, within the first 12 months post transplant (Battle *et al.*, 2013). Again this work needs replicating with a much larger cohort of patients, however another intriguing observation in the literature, which indicates the affinity of leader peptide to HLA-E, may contribute significantly to the results described in chapter 3, is the association with the HLA-E*01:03 allele with reduced TRM post HSCT. The HLA-E*01:03 is expressed at a much higher level than its counterpart HLA-E*01:01, and the explanation for the reduced TRM with HLA-E*01:03, being the increased availability of HLA-E*01:03 to bind to inactivating CD94/NKG2 receptors (Hosseini *et al.*, 2013). This data then does not directly involve leader peptides but demonstrates

that expression of HLA-E, which is a function of the leader peptide's ability to bind to HLA-E, has an influence upon HSCT outcome.

The work in chapter 3, again highlights the complex nature of peptides in the formation of alloresponses, and draws attention to a group of peptides, HLA class I derived leader peptides, as an interesting target for further work.

Chapter 4 focused upon another mechanism by which peptides could influence the response to allografts, specifically by looking at the role of peptides on the binding of HLA specific antibodies to their target epitope. After obtaining and purifying an HLA-A*02 specific antibody, analysis of the antibody's ability to bind to HLA-A*02 on the T2 cell line was shown to vary depending upon the peptide which was bound. Reports within the literature demonstrate that antibodies can be peptide specific (Hülsmeier *et al.*, 2004) so perhaps this finding is not surprising. However such a peptide specific antibody would have several implications in the setting of allotransplantation. Firstly many laboratories supporting transplant programs rely on commercially available kits to detect HLA antibodies, within potential transplant recipients, often going to transplant without a pre-transplant crossmatch where appropriate. The ability to detect any peptide specific HLA antibodies using these techniques is unknown, but given that these kits use recombinant HLA as the target antigens (Susal *et al.*, 2013), it is likely that the peptides, which are bound to the HLA groove are derived from proteins which are not representative of those expressed on the allograft. To some degree this would also be true in a crossmatch, as the T and B lymphocytes used in a crossmatch, although not expressing recombinant proteins, would not be expected to express the same proteins as upon a transplanted kidney or heart. However, the donor derived T and B lymphocytes used in crossmatching would be more likely to represent donor antigens than the recombinant HLA used within the often used luminex assays (Susal *et al.*, 2013).

No such peptide specific antibodies were identified within chapter 4, however the observation that changes within the peptide can influence specific antibody binding, resulting in a greater or lesser amount of antibody binding, may still be of importance in the detection of antibodies within patients awaiting allotransplantation, for example

a different peptide being bound to the HLA molecules within the test assay's HLA antigens, to those expressed upon the transplanted tissue in a recipient means that, an antibody has the potential to bind with different affinity in the test to within the transplanted tissue. Given that current concepts in the understanding of alloantibody reactivity now includes the observation that binding affinity, and available free energy of particular epitopes, can affect the ability of an antibody to bind, fix complement and activate the complement cascade (Duquesnoy *et al.*, 2013), then changes within the peptides could be expected to induce changes within the antibody functions.

Although the structural modelling undertaken in chapter 4 did not discern the functional impact of any observed changes, this work was able to demonstrate that peptides induced conformational variation within epitopes (Battle *et al.*, 2014), and also produced a modest variation within the free energy of the amino acids, which comprised an epitope proximal to the peptide binding groove, namely 62GE.

The work undertaken in chapter 4 then, allows a greater understanding of the potential impact of HLA bound peptides on the ability of HLA antibodies to bind their target, while chapter 3 identifies that HLA derived peptides, specifically the HLA class I derived leader peptides, have an impact upon renal allograft function in the first 12 months post transplant when mismatched. These investigations and findings however would not have been possible if considerable effort had not been undertaken within chapter 2, to establish a database of HLA class I derived peptides, which were capable of being bound by HLA-A*02 molecules.

The overall output from this thesis emphasises the potential role of peptides in the alloimmune response, with the concept of abated negative selection tentatively introduced as a holistic term, for the description of the effect of changing the peptidome, through the introduction of novel proteins through transplantation, and the variation in presentation of peptides introduced via transplantation. This knowledge gives new insights into the matching of HLA types in the context of renal transplantation.

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Web references. *The full URL to the website used is listed here*

The impact of HLA mismatches (mm) upon renal allograft survival over a 20 year period. <http://www.ctstransplant.org/servlet/ArchiveServlet?group=K-21101-0214&archivemode=false&ts=1400939174138>

NCBI website, the source of crystallized HLA molecular models data.

<http://www.ncbi.nlm.nih.gov/protein>

2014 British Society of Histocompatibility and Immunogenetics (BSHI) and British Transplant Society (BTS) joint guidelines for the detection and characterisation of clinically relevant antibodies in allotransplantation

[http://www.bshi.org.uk/BSHI BTS Ab Guidelines Revision June 2014.pdf](http://www.bshi.org.uk/BSHI_BTS_Ab_Guidelines_Revision_June_2014.pdf)

Antigen frequencies in the UK solid organ donor population

<http://www.odt.nhs.uk/transplantation/histocompatibilityv-and-immunogenetics/handi-information/>

The Swiss-PdbViewer

http://spdbv.vital-it.ch/download/binaries/SPDBV_4.10_PC.zip



Sheffield
Hallam University

SHARPENS YOUR THINKING

24 November 2011

To Whom It May Concern

SPONSOR'S SELF DECLARATION

Research Project: Development of immuno-proteomic approaches for assessing transplants

Academic Supervisor: Nicola Woodroffe

Student Researcher: Richard Battle

Institute: Sheffield Hallam University

Sheffield Hallam University will act as the sponsor for the above research project, and take on the responsibilities of the sponsor as set out in the Department of Health Research Governance Framework for Health and Social Care.

Sheffield Hallam University's standard public liability and professional indemnity cover will automatically apply for non-clinical trials.

Yours faithfully

Mr Lloyd Snellgrove
Head of Regional Development
The Enterprise Centre
Sheffield Hallam University

For correspondence: Mr Brian Littlejohn, Research Funding Coordinator
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This project is part-funded by the
European Regional Development Fund

Tracking No: 710134522

Cell Line Authentication Report

Customer: Richard Battle

Sheffield Hallam University, Biomedical Research Centre



Summary of results

Cell line profile has good characteristics

THP-1

T2

Suggest investigation of the provenance of these cell lines.

Cell line profile does not match the available reference profile.

NRES Committee Yorkshire & The Humber - Leeds Central

Yorkshire and Humber REC Office
First Floor, Millside
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LS6 4RA

Telephone: 0113 3050127
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04 January 2012

Mr Richard Battle
Biomedical Research Centre
Sheffield Hallam University
Howard Street, Sheffield
S1 1WB

Dear Mr Battle

Study title: Development of immuno-proteomic approaches to the
assessment of tissue compatibility for transplantation:
Redefining the matching paradigm.
REC reference: 11/YH/0452
Protocol number: NIHR DRF Fellowship DRF

The Research Ethics Committee reviewed the above application at the meeting held on 16 December 2011. Thank you for attending to discuss the study.

Ethical opinion

The Committee asked you why the samples will be stored in a linked anonymised form. You stated that the date and time will be detailed on the sample and consent form to demonstrate that different samples will be used during the study. Members agreed that the only the sample should contain information to identify separate samples. The Committee recommended against using the date and time on the consent form as this could potentially identify participants.

The members of the Committee present gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Ethical review of research sites

NHS Sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

The Leeds Teaching Hospitals **NHS**

NHS Trust

Ref: M021001

Research & Development

03/02/2012

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Dear Mr Richard Battle

Re: NHS Permission at LTHT for: Development of immuno-proteomic approaches to the assessment of tissue compatibility for transplantation: Redefining the matching paradigm.

LTHT R&D Number: IM10/9622 (72228/WY)

REC: 11/YH/0452

I confirm that *NHS Permission for research* has been granted for this project at The Leeds Teaching Hospitals NHS Trust (LTHT). NHS Permission is granted based on the information provided in the documents listed below. All amendments (including changes to the research team) must be submitted in accordance with guidance in IRAS. Any change to the status of the project must be notified to the R&D Department.

Permission is granted on the understanding that the study is conducted in accordance with the *Research Governance Framework for Health and Social Care*, ICH GCP (if applicable) and NHS Trust policies and procedures available at [http://www.leedsth.nhs.uk/sites/research and development/](http://www.leedsth.nhs.uk/sites/research%20and%20development/).

This permission is granted only on the understanding that you comply with the requirements of the *Framework* as listed in the attached sheet "Conditions of Approval".

If you have any queries about this approval please do not hesitate to contact the R&D Department on telephone 0113 392 2878.

Chairman of the Board: Chief Executive Maggie Boyle

The Leeds Teaching Hospitals incorporating:

Chapel Allerton Hospital Leeds Dental Institute Seacroft Hospital

St James's University Hospital The General Infirmary at Leeds Wharfedale Hospital

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18 January 2012

Mr Richard Battle
Biomedical Research Centre
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Howard Street, Sheffield
S1 1WB

Dear Mr Battle

Full title of study: Development of immuno-proteomic approaches to the assessment of tissue compatibility for transplantation: Redefining the matching paradigm.
REC reference number: 11/YH/0452
Protocol number: NIHR DRF Fellowship DRF

Thank you for your letter of 16 January 2012. I can confirm the REC has received the documents listed below as evidence of compliance with the approval conditions detailed in our letter dated 16 December 2011. Please note these documents are for information only and have not been reviewed by the committee.

Documents received

The documents received were as follows:

| <i>Document</i> | <i>Version</i> | <i>Date</i> |
|-------------------------------|----------------|------------------|
| Participant Consent Form | 2.0 | 22 November 2011 |
| Participant Information Sheet | 2 | 22 November 2011 |

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to **R&D** offices at all participating sites

11/YH/0452

Please quote this number on all correspondence

Yours sincerely

Mrs Nicola Mallender-Ward
Committee Co-ordinator

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Letter to the Editor

Identified HLA class I epitopes can undergo conformational induced variation due to changes within HLA bound peptides

HLA specific antibodies have a significant impact upon renal allotransplantation. Preformed alloantibodies prevent or complicate transplants between otherwise compatible donors and recipients [1] and *de novo* post transplant antibodies are associated with poorer overall allograft survival [2]. The identification of such antibodies and their subsequent characterisation in terms of their specific target, has been the focus of much investigation since the association of preformed HLA antibodies and hyperacute rejection was first established over 40 years ago [3].

Current concepts in identifying and characterising HLA specific antibodies no longer focus upon broad definitions of antigenic targets, such as anti-HLA-A1, A2, A3 etc., but instead attempt to define the specific epitope target of the HLA antibody upon each HLA molecule [4,5]. This process has further developed the concepts of cross-reactive epitope groups (CREG), whereby antibodies demonstrate reactivity against differing HLA antigens which possess the

same structural motifs or epitopes. Several researchers have precisely identified the specific epitopes present upon HLA class I and class II molecules against which reactivity is directed [4,5]. This allows the expected profile of antigenic reactivity of an HLA antibody elicited by exposure to an individual antigen, to be predicted with high confidence, and explains why limited exposure can sometimes result in extensive sensitisation.

Most recently this concept has been taken further, showing that the electrostatic potential of epitopes [6] and their available free energy, required to stabilise antigen-antibody complexes, has a fundamental role in the consequences of antibody binding, in terms of the ability to bind C1q and subsequently activate the complement cascade [7]. A further complexity is introduced by the variable nature of the peptide bound within the groove of the HLA molecule. In some cases these peptides are also considered to contribute critical contact sites for antibodies and are believed capable of generating their own epitopes [7].

Our group is particularly interested in the role of HLA bound peptides in the generation of the alloresponse, be it cellular or humoral. In experimentation to examine how changing the bound

Fig. 1. The interaction of the 62GE epitope and the amino acid at peptide I*2. (A) The 62CE eplet in pink and LEU in red, the distance between these at their closest point is shown (in yellow) as being 4.081 Å. (B) 62GE is shown in pink and MET is shown in red, the distance between the two is shown in yellow (3.341 Å). Green dotted lines indicate the formation of hydrogen bonds. The solid green line represents a LYS at position 66 of the α -chain in both (A and B). (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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peptide would affect the ability of a HLA specific antibody to bind to an otherwise identical HLA target, we observed the variation reported by other investigators [8]. However rather than the concepts previously postulated for this observation, namely crucial contact sites being present upon the peptide [7], we believe, through structural modelling *in silico*, that changes within the nature of the HLA bound peptide directly influences epitopes which are present upon the HLA molecules themselves, via conformational induced epitope variation. We utilised the protein databank (PdB) [9] and the PdB-Swissviewer (10) to analyse the introduction of specific amino acid substitutions upon peptides bound within the HLA molecule, and assessed the impact of these substitutions upon previously defined epitopes proximal to the peptide, in terms of electrostatic potential, total energy and any visible conformational change. We identified that the common HLA-A*02:01 encoded epitope 62GE, formed additional hydrogen bonds with the peptide when the amino acid within peptide position P2 was changed from LEU to MET, in a HLA-A2 molecule bound with the HA-1 peptide. The position of the 62GE epitope relative to the P2 peptide amino acid was altered as a result of this substitution; the distance being 4.018 Å for 62GE and LEU (Fig. 1A), but 3.341 Å when MET was in position P2 (Fig. 1B). Furthermore, the total available energy for binding was reduced in the 62GE epitope. The energy loss was spread across both amino acids within 62GE, with a change of -14.632 to -15.409 total energy for GLY, and -28.414 to -30.522 total energy for GLU. How these changes functionally relate to the ability of antibody to bind to the 62GE epitope requires further elucidation in terms of the effect upon strength of binding, the ability to bind C1q and to activate the complement cascade. However our observation [6,7] does demonstrate that changes within the bound peptide are capable of altering structural epitopes of HLA which have been demonstrated as key components of antibody binding [4-7])

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LETTER TO THE EDITOR

The relationship of HLA-class I derived leader peptide mismatch and renal function within the first 12 months post-renal transplant

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Key words: endogenous peptides; human leukocyte antigen class-I leader peptides; kidney transplantation

In a recent review article, D'Orsogna et al. describe the latest insights and clinical implications of endogenous-peptide-dependent alloreactivity (1). Arguing that T-cell allorecognition is highly likely to be endogenous peptide specific, and that, non-T-cell lineage lymphocyte populations, such as natural killer (NK) and B cells, could also be reliant on peptide binding and presentation in the formation of alloresponses. The authors state that 'definition of specific endogenous peptide targets for allorecognition should be a major focus for transplantation research'.

We concur with the authors regarding the importance of endogenous peptides in the formation of a variety of alloresponses, and have been assessing the clinical impact of a group of endogenous peptides in the context of allograft function post-renal transplantation.

Specifically we have been assessing the impact of leader peptide sequences of human leukocyte antigen (HLA) class I molecules. These are relatively conserved regions of the HLA class I α -chain which are cleaved from the molecule at an early timepoint within HLA class I molecule assembly, and are consequently present at a high intracellular abundance. We and others (2) have shown that HLA class I-derived leader sequences can themselves be presented by HLA class I molecules, and would thus provide a target for CD8+ T lymphocytes. Additionally, HLA class I-derived leader sequences have been shown to be crucial for the formation of

the CD94/NKG2 activating/inhibiting receptors ligand, HLA-E (3, 4). These leader peptides, then, are capable of providing an innate and adaptive antigenic determinant which may form a target for alloresponses.

In additional evidence for the immunological importance of HLA class I-derived leader peptides in alloresponses; several cytomegalovirus (CMV) strains have been shown to employ an immuno-evasion strategy by encoding a HLA class I-derived leader peptide homologue (5). Furthermore cross-recognition by CD8+ T cells, responding to a homologue encoding CMV strain, to HLA-E complexed with the HLA class I-derived leader sequence has been observed in an allorecognition setting (6).

Given the remarkable antigenic potential of these peptides, we sought to investigate if mismatches of HLA class I-derived leader sequences between donor and recipient renal transplant pairs influenced renal allograft performance within the first 12 months post-transplant. Our study cohort comprised 139 consecutive renal transplant recipients. Mean age 41.8 (± 17.81 SD), sex 54F/84M, 50 had anti-HLA-IgG of which three were donor specific antibodies, 77 kidneys were from donation following brain stem death, 20 cardiac death and 42 were from live donors. National Health Service Blood and Transplant, Organ Donation and Transplantation (NHSBT ODT) matching levels were 11.5% level 1, 28.8% level 2, 40.3% level 3 and 19.4% level 4. Mean creatinine levels post-transplant were

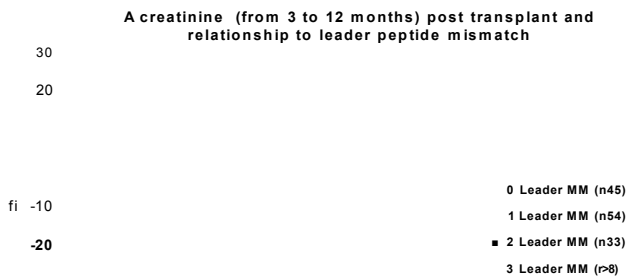


Figure 1 The relationship of human leukocyte antigen (HLA) class I derived leader sequence mismatch and A creatinine post transplant. Confidence intervals of 95% are shown.

Effect of leader peptide mismatch within NHSBT ODT matching levels

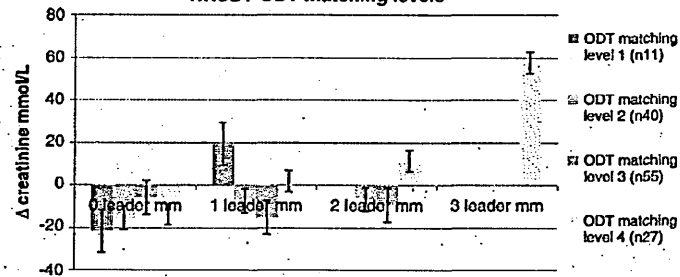


Figure 2 Relationship of human leukocyte antigen (HLA) class I-derived leader sequence mismatch upon creatinine within NHSBT ODT matching groups, confidence intervals of 95% are shown.

135 mmol/l (± 60.9 SD) at 3 months and 131 mmol/l (± 54.4 SD) at 12 months. To determine the HLA class I-derived leader sequence matching between donor and recipient pairs, we utilised the HLA/IMGMT database to identify the encoded amino acid sequence of the leader sequence; found at position 3–11 of the class I α -chain amino acid residues (4), for all listed class I alleles ($n > 6000$). We then created a key allowing a patient, or donor HLA type, to be searched to reveal their HLA class I-derived leader sequence. In total we identified 11 HLA class I-derived leader sequences from the HLA/IMGMT database. We then compared the level of leader mismatch to Δ creatinine calculated from 3 to 12 months with outputs as shown in Figure 1.

This identified a striking relationship of leader peptide mm with Δ creatinine, and a statistically significant association for 0 leader mm vs 3 leader mm patients ($P = < 0.05$). Furthermore, as shown in Figure 2 this influence was seen within patients and donors grouped according to NHSBT ODT HLA matching levels, indicating that our observations were not merely a function of coincidental HLA mismatching. We believe that this is the first example of the effect of multiple endogenous peptide mismatches upon renal allograft functional outcome in a clinical setting, and that it provides some of the requirements for the definition of endogenous peptides highlighted by D'Orsogna and colleagues. Work is ongoing to further elucidate and characterise the role of leader peptides as targets of alloreactive responses.

Conflict of Interest

There are no conflicts of interest.

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Molecular characterisation of the monocytic cell line THP-1 demonstrates a discrepancy with the documented HLA type

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Dear Editor,

The use of the acute monocytic leukemia cell line, THP-1, has been well documented since its establishment was reported more than 30 years ago in the IJC.¹ Since then, the THP-1 cell line has been used in countless research papers and is now a widely recognized resource as a model of monocyte cells within the immunology and oncology research communities.

Routinely applied good cell culture practice of THP-1, as well as all other cell lines, requires quality assurance of the cell line, including assessment of relevant phenotypes and genotypes, state of differentiation and the assessment of any possible crosscontamination.² In line with these requirements, our recent use of the THP-1 cell line revealed an inconsistency with the reported Human Leukocyte Antigen (HLA) type which may have potential consequences for some researchers.

Initially THP-1 was recovered from our bank of frozen cell lines and cultured in RPMI 1640 medium (5% Fetal Calf Serum (FCS), 200 mM L-Glut, 100 µg/ml Pen/Strep) before HLA typing for HLA class I A and B, and the HLA class II DR and DQ antigens, *via* commercially available serological typing trays. These serological typing trays represent the contemporary equivalent of the assay used to determine the HLA type of THP-1 when the cell line was established.¹ The results of these assays conclusively identified HLA-A2. "Weak" reactions against other class I antigens and cross-reactivity, particularly between HLA-B5 and B15 antisera, confounded the identification of a full HLA-A and B type. The HLA class II antigens were entirely inconclusive.

Since the THP-1 cell line was established in 1980, there have been marked advances within the detection methodologies used to determine HLA types, most notably the development of molecular biology-based techniques, such as the polymerase chain reaction (PCR). Therefore, to determine a conclusive HLA type for the THP-1 cell line, an "in-house" PCR using sequence specific primers (PCR-SSP) was performed for HLA-A*, B*, C*, DRB1*, DRB3*, DRB4*, DRB5* and DQB1* genes. In addition, an ABO group was determined using a similar method. Both of these in-house genotyping systems are based on methods which have been previously published.^{3,4}

Using this assay, we identified the following HLA type for THP-1. HLA-A*02; B*15; C*03; DRB1*01, DRB1*15; DRB5*01/02; DQB1*05 and DQB1*06 (and ABO blood group genotype BB).

The reported HLA type for THP-1 is HLA-A2, A9, B5, DRw1 and DRw2. (The HLA nomenclature system used here corresponds to the contemporary system at the time THP-1 was established in 1980).¹

Clearly the HLA class I type did not correspond to the reported HLA type for THP-1, differing at the HLA class I A and B loci (to our knowledge HLA-C* has not previously been reported for THP-1).

The HLA class II type corresponded to the original HLA type of HLA-DRw1 and DRw2, being DRB1*01 and DRB1*15. The expressed product of the HLA-DRB1*15 allele represents a split of the DR2 antigen and demonstrates the increased resolving capabilities of the PCR-SSP assay in comparison to the serological technique. (again to our knowledge HLA-DQB1* has not previously been reported). In contrast to the reported type, THP-1 appeared to be homozygous at the HLA-A, B and C loci by PCR-SSP.

Alterations in the HLA class I phenotype of malignant cells is a frequent event during cancer progression, allowing tumor cells to evade the immune system. Indeed, loss of one major histocompatibility complex class I haplotype in human melanoma cells has been shown not only to allow evasion of immunosurveillance⁵ but also to increase their intrinsic oncogenic potential.⁶ The mechanisms which lead to HLA class I alterations can occur at any step required for HLA synthesis. Most commonly, the alteration represents a structural defect or a regulatory defect on the transcriptional level.⁵ Such alterations would not interfere with the PCR-SSP method used here, although a rarer deletion event upon chromosome 6 would be undetectable.

In addition the "missing" class I HLA antigens from the original report show strong crossreactivity with the confirmed HLA type, and most likely account for this discrepancy, indeed our serological assay also demonstrated crossreactivity at the HLA-B5 and B15 antigens, which were proved erroneous by the PCR-SSP.

Interestingly, the HLA-B*15 identified within the haplotype HLA-A*02; B*15; C*03, is unusual in itself, corresponding to the serological equivalent of B75 (15). The PCR-SSP was capable of resolving the B*15 allele to B*15:08 or B*15:11.

A search on the Allele* Frequencies in World Populations website⁷ for each of these alleles revealed their frequency within specific populations. Of the two alleles B*15:11 appeared to be the more common, as, although extremely rare within the majority of populations, it is seen in the

RECEIVED 10 JULY 2001

Asian Pacific Islander population.⁸ The HLA-B*15:11 allele would therefore correlate with the reported ethnic origin of the THP-1 cell line.¹

In order to confirm these findings and eliminate the possibility that our frozen THP-1 cells were incorrectly identified as such, a frozen vial of THP-1 cells was ordered from the American Type Culture Collection (ATCC), ATCC Number TIB-202TM. The ATCC THP-1 cell line depositor was the author of the original paper identifying the development of the cell line.¹ The ATCC-supplied THP-1 cells were HLA typed as described above, by both serology and PCR-SSP.

The determination of the ATCC-supplied THP-1 HLA type confirmed our original findings from our frozen stock supply, for both serological and molecular assays. The THP-1 HLA type therefore is not, HLA-A2, A9; B5; DRw1 and DRw2 as reported. The correct type being, HLA-A*02; B*15; C*03; DRB1*01, DRB1*15; DRB5*01/02; DQB1*05, DQB1*06.

This discrepancy is not extraordinary when considering the assay systems originally used to determine the THP-1 HLA type. Indeed the error rate for HLA-B antigen assignment by serology has previously been demonstrated to be as high as 22.5% when compared to DNA based typing.⁹ Greater precision has also been shown for PCR-SSP HLA typing in comparison to serological typing for both HLA-A¹⁰ and HLA-DR.¹¹

Further HLA typing to determine which HLA-B*15 allele/s were present on THP-1 was carried out using commercial high resolution HLA-A and B typing kits. The results of these tests confirmed the presence of HLA-B*15:11, as the frequency data had suggested. The HLA-B*15:11 allele has also previously been documented to be in association with C*03:03, as it is here in THP-1.

In conclusion, we present here a revised HLA type for THP-1 using molecular-based typing methods, this type being HLA-A*02; B*15; C*03; DRB1*01, DRB1*15; DRB5*01/02; DQB1*05, DQB1*06. The presence of the HLA-B*15:11 allele was also confirmed within this type.

The homozygosity of the HLA class I antigens could perhaps represent a mechanism by which the leukemic cells evaded detection within the individual from whom the THP-1 cell line was established, following a deletion event.⁵ This tumour escape mechanism may have possibly arisen from the selective pressure of a patient-specific graft-versus-leukaemia reaction,¹² although it is unknown if the patient received a haematopoietic stem cell transplant prior to THP-1 being developed from their leukemic cells.¹ However, this cannot be investigated further due to the lack of normal autologous leukocytes.

It should be noted that the revised type presented here confirms the utility of THP-1 cells in coculture assays with HLA-A*02-expressing responder cells, as described recently in this journal.¹³ However, due to HLA restriction, the use of THP-1 cells in coculture with HLA-B*05-expressing re-

sponder cells would produce false negative results and therefore should be avoided.

Yours sincerely,

Richard Battle
Katherine Poole
Sarah Haywood-Small
Brendan Clark
M. Nicola Woodrooffe

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Key words: THP-1, HLA, revised HLA type, PCR SSP

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