Influence of antigen processing in the shaping of an autoimmune T cell repertoire

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PhD

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Declaration

This thesis has been composed by myself and the work it describes has been carried out by myself except where specific reference is made to other individuals. This work has not been submitted for any other degree or professional qualification except as specified.

Lorna Henderson

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certain high DR15-affinity DA peptides, and could be a factor in corresponding autoreactive T cells escaping self-tolerance.

This work has provided strong evidence supporting the hypothesis that destructive processing may account for the survival of self reactive T cells in the mature peripheral repertoire. It has, in addition, provided a powerful tool in which to dissect the influences of processing on the generation or destruction of α3(IV)NC1 peptides recognised by patients' T cells and may be used to examine other factors postulated to influence antigen presentation such as co-expression of non-DR15 HLA molecules on human antigen presenting cells.
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Abbreviations

α3(IV)NC1  The NC1 domain of the α3 chain of type IV collagen, the Goodpasture antigen
āa  Amino acids or residue
ACN  Acetonitrile
AEP  Asparginyl endopeptidase
APC  Antigen presenting cell
BSA  Bovine serum albumin
CLIP  Class II-associated invariant chain peptide
DC  Dendritic cell
DMSO  Dimethyl sulfoxide
DTT  Dithiothreitol
EBV  Epstein Barr Virus
EDTA  Ethylenediaminetetraacetic acid
ELISA  Enzyme linked immunosorbant assay
ER  Endoplasmic reticulum
FCS  Heat inactivated fetal calf serum
GBM  Glomerular basement membrane
GILT  IFNγ inducible lysosomal thiol reductase
HAT  hypoxanthine aminopterin thymidine medium
HT  hypoxanthine thymidine medium
HLA  Human leukocyte antigen
li  Invariant chain
kD  kilodaltons
mAb  Murine monoclonal antibody
MALDI-TOF  Matrix-Assisted,Laser-Desorption/Ionisation Time-Of-Flight
MBP  Myelin basic protein
MHC  Major histocompatibility complex
MHCII  MHC class II compartment
MS  Mass spectrometer
NC1  Carboxyl terminal non collagenous domain of collagen chains
PBS  Phosphate buffered saline
SDS  Sodium lauryl sulphate
tris  tris(hydroxymethyl)aminomethane base
Chapter 1

Introduction

1.1 Autoimmunity

1.1.1 Introduction

Autoimmune diseases are characterised by immune responses that cause self-injury that are, to varying degrees, targeted at self antigen. More specifically, disease may be caused by the activation of T cells, B cells or both in the absence of ongoing infection. With the exception of Rheumatoid arthritis and autoimmune thyroiditis, most autoimmune diseases are individually rare however together they affect greater than 5% of the Western population (Sinha, 1990, Jacobson, 1997).

The immune system has developed complex ways of maintaining tolerance (immunological unresponsiveness to self antigen) to combat a vast diversity of foreign proteins whilst avoiding damaging self and causing autoimmune disease. For many years immunologists believed the principal mechanism of tolerance was the clonal deletion of autoreactive cells leaving a repertoire of B and T cells that recognise foreign antigen alone. More recent work acknowledges that a low level of autoreactivity is physiologic (Dighiero, 1999) and in fact crucial to normal immune function. Autoantigen helps to form the repertoire of mature lymphocytes and the survival of naïve T and B cells in the periphery requires continuous low level exposure to autoantigens.

Some autoimmune diseases have been linked to altered peptide selection and presentation suggesting that deviations from normal antigen processing pathways may be sufficient to trigger pathologic situations (Manoury, 2002).
As there is no fundamental difference between the structure of self and foreign antigen it is now thought that lymphocytes have evolved not to distinguish self from foreign but to respond to antigen only in certain microenvironments, generally in the presence of certain inflammatory cytokines. Therefore if autoreactivity is in fact physiologic, the challenge is therefore to understand how it becomes a pathologic process and how T and B cells contribute to injury.

T cells recognise peptides that are associated with major histocompatibility complex (MHC) on the surface of antigen presenting cells. Antigen recognition by T cells is the initiating stimulus for their activation. The process by which peptides from internalised exogenous antigen are processed within the antigen presenting cell (APC) and subsequently presented to T cells is central in immunity. Immune responses that protect us from infectious disease as well as those causing transplant rejection and autoimmune disease are all driven by lymphocytes that recognise components of protein derived from micro-organisms, foreign tissue such as in transplants or self antigen, (for example joint tissue) to identify targets for immune attack.

The nature of these targets is clearly of immense importance in manipulating the immune response (making new vaccines) and understanding what goes wrong in autoimmune disease.

My work sought to unravel the process by which targets (usually peptides) are generated or destroyed from intact antigen using a well characterised antigen that is attacked in an autoimmune kidney disease as a model. In doing this I hoped to account for how this process may lead to the survival of self-reactive T cells into the peripheral T cell repertoire as a possible mechanism of tolerance breakdown and the development of autoimmune disease.

In the following sections I will introduce the basic concept of tolerance and discuss the possible mechanisms leading to the breakdown of tolerance and development of autoimmunity. Key to my work is the understanding of how antigen is processed and recognised by T cells. This will be covered in detail before considering how alterations in the processing pathway may lead to deviations in the immune response to self. The
second section of this chapter will deal with the model of autoimmune kidney disease used for my research, Goodpasture’s disease. I hope to highlight its strengths as a model of autoimmunity and cover in some detail what is already known about the immunopathogenesis of the disease including previous research within our own group. I will conclude with how this work lead on to my own research, detailing the aims of the study including an outline of the research approach used.

1.1.2 Mechanisms of tolerance

Immunological self-tolerance consists of all mechanisms which prevent the immune system reacting to self, in other words, it denotes the absence of autoimmunity. This state may be achieved passively (by the absence of activation of auto-reactive lymphocytes) or actively (via mechanisms that eliminate or inactivate self-reactive lymphocytes).

The induction and maintenance of self-tolerance in the developing and mature T cell repertoire is mediated by several mechanisms and controlled at numerous levels. Crucial to self-tolerance is the interaction between APC and lymphocytes and occurs in both the thymus (important in central tolerance) and peripheral lymphoid tissue (important in peripheral tolerance) (Sprent, 1995, Schwartz, 1993).

1.1.3 Central tolerance

T cells first encounter the potential for self-reactivity as soon as they express their antigen-specific receptor during intra-thymic development. If they recognise self-antigen with high avidity at this stage, apoptotic cell death follows, a process defined as negative selection (Sprent 1995, Schwartz, 1993).

Thymic selection had long been considered an effective tolerogenic mechanism only for widely expressed self-molecules. This assumption was based on the belief that proteins with tissue-restricted expression would not be available for presentation in the thymus and that tolerance to these proteins could only be achieved through mechanisms of peripheral tolerance. Recent evidence demonstrates, however, that numerous otherwise tissue-restricted antigens are expressed in the thymus (Klein, 2000, Pugliese, 2002,
Hanahan, 1998). This discovery along with studies demonstrating expression of self-molecules in peripheral lymphoid tissues elevates the importance of the role of self-antigen presentation in determining tolerance.

T cells recognise proteolytically cleaved peptide fragments (peptide epitopes) bound to major histocompatibility complex (MHC) rather than intact protein. The efficiency of antigen processing and presentation is now known to be a major limiting factor in the generation of self-epitope/MHC ligands on thymic APC with implications in turn for the survival or deletion of potentially auto-reactive T lymphocytes.

1.1.4 Peripheral tolerance

For reasons described above, many tissue specific proteins are not expressed at sufficient levels to induce deletion of auto-reactive T cells in the thymus. Several mutually non-exclusive mechanisms under the heading of peripheral tolerance provide a complimentary safety net whereby potentially autoreactive T cells that have survived into the periphery are kept in check.

Deletion

In analogy to negative selection in the thymus, auto reactive T lymphocytes may be eliminated on encounter of self-antigen in the periphery. Whether deletion in the periphery represents a distinct tolerance mechanism involving a specialised type of APC for instance, or merely reflects depletion due to chronic stimulation of T cells after differentiation into effectors in the lymph node without causing tissue damage, is not known. This “exhaustive” stimulation has been observed in the late stages of chronic infections (Moskophidis, 1993).

Anergy

The two-signal model of T cell activation suggests that in addition to the TCR signal (signal 1) a second signal is required to fully activate the naïve T cell. The CD28 receptor and its ligand B7 have been identified as the first set of molecules capable of delivering signal 2 although other pairs have been described (Mueller, 2000). Tolerance may be
induced if the APC presenting self antigen bears insufficient co-stimulatory signals. Triggering TCR in the absence of co-stimulation (signal 2) results in functional inactivation of T cells a condition termed “T cell anergy” (Schwartz, 1990). This term refers to a T cell state of inactivity but retaining the potential to become active.

Ignorance

This refers to the “harmless” co-existence of naïve autoreactive T cells and their respective target peptide antigen on peripheral tissues (Miller, 1993). Ignorance can be described, in contrast to the above mechanisms, as a passive phenomenon. Ignorant cells are readily activated on encounter with antigen in appropriate conditions such as immunisation or viral infection but otherwise exist in the naïve pool without contacting their cognate antigens due to spatial separation (Ohashi, 1991, Barker, 1994)

Regulatory T cells

The “suppressor” T cell was the subject of heated debate for many years and for some time fell into disrepute. This concept has been, however, resurrected with the unequivocal demonstration of thymus-dependant T cells capable of modulating the function of “conventional” T cells in vitro and to control immunopathogenesis in vivo (Saoudi, 1996, Modigliani, 1996, Seddon, 1996 & 2000, Thornton, 1998, Mason, 1998, Itoh, 1999). The molecular and cellular mechanisms involved in the thymic generation and selection of these regulatory T cells remains poorly understood as does their mode of action which depends on cell to cell contact rather than soluble factors. In addition to the thymus-dependant regulatory T cells, T cells with similar functional characteristics have been generated in the periphery using various experimental procedures including intranasal or oral antigen administration. The regulatory effect of these T cells seems to be at least in part related to the secretion of “anti-inflammatory” cytokine transforming growth factor β (Miller, 1992, Chen, 1994).
1.1.5 Breakdown of tolerance with development of autoimmunity

Altered signaling and APC maturation state

For decades it has been known that an adjuvant is required to induce an effective immune response and that pathogens can break tolerance and induce autoimmunity (Dresser, 1961, Brooke, 1965, Chiller, 1973, Ohashi, 1991, Röcken, 1992). Current data is generally consistent with a model whereby T cell interaction with immature APC lead to induction of tolerogenic mechanisms and T cell interaction with pathogens stimulate mature APC leading to efficient activation of the immune response. For example, work by Nussenzweig et al compared T cell response to antigen presented by un-stimulated and stimulated APC (Hawiger, 2001). Antigen was targeted to dendritic cells (DCs) in vivo.
leading to tolerance via abortive expansion, deletion and anergy of antigen specific T cells. If antigen was targeted to DCs with anti-CD40 antibody which promotes APC maturation, a prolonged immune response was seen. Interactions with other members of the TNF receptor family have been demonstrated to influence tolerance versus immunity. (Bansal-Pakala, 2001).

In general, the majority of the literature is consistent with a model whereby factors that promote APC maturation such as adjuvants and pathogens are crucial for converting tolerogenic signals to activating signals and therefore have the potential to break tolerance and cause an immune response to self.

There have been a number of recent studies uncovering the multiple molecular pathways that must be triggered to induce APC maturation and optimal immune response and they have in turn shed light on how pathogens may break tolerance. APC such as DCs and macrophages are equipped with various receptors that are able to detect the presence of different components of pathogens connecting the innate immune recognition to the adaptive immune response. Pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) are an example. Direct recognition of pathogens by TLRs and other immune receptors is an important mechanism where TLR signaling induces DC maturation promoting activation of T cells and potential breakdown of tolerance.

Danger: molecules and receptors guiding APC function

Whereas direct recognition of pathogens by TLRs and other innate immune receptors is an important mechanism whereby APC are stimulated to promote activation of T cells, other situations such as organ transplantation and tumours may trigger the immune response. Injuries damaging epithelial layer are often associated with infection and so the innate immune system may have evolved to recognise molecular events associated with trauma as danger signals promoting activation of the adaptive immune response. Research revealed the immune system is programmed to sense this danger via TLRs. One possible source of 'danger' is heat shock proteins (HSPs) which are released from dying cells. These are abundant intracellular proteins which make up to 15% of total protein after cells have been exposed to certain stress such as toxins, infection or heat.

**Altered presentation**

The induction of peripheral T cell tolerance is also influenced by the concentration of self antigen. If antigen is expressed at low levels, minimal presentation occurs and the self-reactive T cells remain tolerised. Events modulating self antigen expression such as tissue necrosis or tumour development alter the level of presentation. Increased presentation of self antigen may allow ignorant autoreactive T cells to detect the self antigen. The fate of the T cell will then be coupled with the events that lead to APC maturation. If increased levels of antigen occur in the absence of signals promoting maturation then tolerance will occur. If however self-antigen is detected in the presence of pro-inflammatory signals that promote APC maturation, then tolerance will be broken and autoimmunity will arise. Therefore events which lead to a sudden release of self-antigen if coupled with signals that induce maturation of APC may break tolerance by activating ignorant T cells (Ohashi, 2002).

**Molecular mimicry**

Cross reactivity between microbial and self-determinants recognised by the adaptive immune system as a means of breaking tolerance and inducing autoimmune disease is an intriguing hypothesis. Definite proof is however difficult to achieve and evidence has often been circumstantial. Complex series of DNA rearrangement and other genetic modifications generate a large diversity of TCR. Therefore the repertoire of the immune system appears large enough to adapt to every possible antigen component in the realm of foreign and self. However, there is much degeneracy. It is well known that these cross-reactive receptors have the potential to interact with several ligands that share a certain degree of similarity. The concept of molecular mimicry is based on this finding and requires that two or more components (foreign or self) are structurally so similar, the T cells cannot distinguish between them.
In recent years, cross-reactive T cells or antibodies have been found in situations where association between an infectious agent and autoimmune disease have been known. For example, a large number of patients with myasthenia gravis carry antibody to the acetyl choline receptor which shares a cross reactive epitope to herpes simplex virus (HSV) glycoprotein D (Schwimmbeck, 1989). Type I diabetes has been associated with a number of viruses including coxsackie B (Gamble, 1980, Notkins and Yoon 1984, Yoon, 1979).

However, despite all the indications for molecular mimicry as a mechanism involved in the aetiology of autoimmune disease, no direct evidence for molecular mimicry as an initiator or accelerator of autoimmunity in humans could be demonstrated to date. Some propose that molecular mimicry more likely impacts on an already existing autoimmune process rather than precipitating de novo disease. (Christen, 2004).

1.1.6 Altered antigen processing as means of tolerance breakdown

Both central and peripheral tolerance mechanisms are influenced by constitutively expressed self-proteins. As detailed, auto reactive T cells capable of recognising epitopes from self peptide that are not presented in sufficient amounts to stimulate T cell deletion ("cryptic epitopes") must clearly be restrained from driving a deleterious autoimmune response by peripheral tolerance (Elson, 1995, Gammon, 1989). There are now a number of animal models of autoimmunity where pathogenic T cells have been demonstrated to recognise cryptic self-epitopes (Moudgil, 2000, Sercaz, 2002, Watts, 2003).

Constitutive expression of self antigen was previously thought most strongly influenced by affinity for HLA class II. However, the discovery of self-reactive T cells specific for high affinity peptides in the peripheral repertoire in both animal models of autoimmunity and human disease, has led to an interest in alternative mechanisms which may influence presentation of self-peptides.

One such mechanism is destructive processing (Watts et al 2003, Trombetta, 2005) whereby processing occurs such that specific epitopes are consistently destroyed and
therefore, despite high affinity for HLA molecules, never presented to T cells. These autoreactive T cells then escape irreversible self-tolerance and persist into the peripheral repertoire with the potential to drive autoimmune disease in the appropriate environment.

1.2 Antigen processing and presentation

1.2.1 Introduction

Section 1 has already demonstrated that T cells play a critical role in the adaptive immune response to both foreign and self protein antigens. The physical form of antigen that is recognised by T cells differs however from those recognised by B cells. T cells recognise peptide fragments derived from protein antigen bound directly to cell surface proteins encoded by genes of the major histocompatibility complex (MHC). MHC molecules thus serve to display or present peptides to T cells. T cells in turn recognise antigens through their clonotypic immunoglobulin-like T cell receptor which engages the peptide/MHC complex. This resultant tri-molecular complex is the basis of antigen-specific T cell responses (Figure 1.2) Usually peptides derived from cytosolic proteins bind to class I MHC molecules and are recognised by CD8 T cells (including cytolytic T cells). In contrast peptides derived from extracellular proteins that are endocytosed by specialist antigen presenting cells (APC) bind to class II MHC molecules and are recognised by CD4+ T cells which include T helper cells. These cells are often required for the induction of humoral and cell mediated responses which are most efficient in eliminating extracellular and phagocytosed pathogens.
Figure 1.2 Antigen presentation to CD4+ T cells. The boxed figure represents the tri-molecular complex: MHC class II molecule, bound peptide and T cell receptor. This complex forms the basis of CD4 T cell recognition of antigen. However, many other cell surface and soluble signals may participate and influence the type of T cell response generated following recognition of antigen.
In this next section, I will review how complex protein antigens are internalised into APC and processed into peptide fragments and how these fragments in turn are exposed to and incorporated into the peptide binding groove allowing MHC/peptide complexes to be displayed for T cell recognition.

1.2.2 Uptake of extracellular proteins into vesicular compartments of APC

Antigen uptake is a crucial initial first step in the processing and presentation of antigens in the class II processing pathway with the possible exception of some peptide antigens. Different APC utilise different mechanisms of uptake with varying degrees of specificity and efficiency (Lanzavecchia, 1996). The rate at which antigen is internalised can be a determinant in the efficiency of antigen presentation. (Lanzavecchia, 1990). All APC are capable of endocytosis of extracellular fluid and plasma membrane. Endocytosis is the process whereby invaginations of the cell membrane develop and pinch off as pre-endosomal vesicles which in turn fuse with early endosomes. A number of alternative mechanisms of uptake also exist. Macrophages internalise many different proteins with limited or no specificity to surface molecules and internalise them by the process of ligand-induced endocytosis or phagocytosis. Macropinocytosis is a distinct mechanism in dendritic cells which internalises large amounts of extracellular fluid.

Special cases of more specific binding by well characterised surface molecules on the APC can mediate binding and internalisation of antigen via receptor-mediated endocytosis in clathrin-coated vesicles (van Deurs, 1989, Watts, 1992). Clathrin is capable of binding a range of membrane proteins by interactions with their cytoplasmic domains. Proteins that bind, are concentrated at sites of pit formation and subsequently internalised into pre-endosomal vacuoles. Examples of this process include the specific receptors for the Fc portions of immunoglobulin and receptors for the complement protein C3b, both of which are present on the surface of macrophages. These receptors can bind opsonised antigens and enhance their internalisation. Other receptors internalised by clathrin-dependant endocytosis include the transferrin and LDL receptor. Endocytosis may also occur via clathrin-independant mechanisms. Both clathrin-dependant and independent mechanisms have been demonstrated to deliver pre-endosomal vacuoles to
the early endosomes. (Hansen, 1993, Tran, 1987). Receptor mediated endocytosis is the most efficient uptake mechanism for low abundancy extracellular antigens but is only possible for antigens that can be captured via cell surface receptors so is relatively specific.

Phagocytosis is a ligand-induced form of endocytosis where cell surface receptors bind ligands such as immunoglobulin Fc, resulting in cytoskeleton rearrangements and envacuolation of the ligand-coated antigen into phagosomes. The fate of these phagosomes has been demonstrated to be determined by the receptors involved (Falkow, 1992). Usually phagosomes fuse with lysosomes which leads to proteolysis and peptide generation which are in turn efficiently loaded onto class II molecules.

Macropinocytosis is a distinct mechanism used by dendritic cells. Soluble protein antigen is internalised into APC without actually binding to the cell surface by marked membrane ruffling followed by envacuolation of the extracellular fluid within large vesicles. Dendritic cells can use macropinocytosis to internalise large volumes of extracellular fluid (Sallusto, 1995). This leads to highly efficient presentation of fluid phase antigens. Macropinosomes however do not exchange contents with early endosomes which suggests a distinct and alternative pathway for antigen presentation (Hewlett, 1994).

1.2.3 Efficiency of antigen uptake

The efficiency by which antigen is internalised into APC varies considerably depending on the mechanism of uptake and the abundance of antigen available. The most efficient mechanism for low abundance extracellular antigen is that of receptor-mediated endocytosis. This pathway is however specific and selects antigens that are captured by cell surface receptors. The specificity of these receptors can vary from high specificity such as the B cell mlg which allows a higher efficiency of uptake for a limited range of antigens to a range of receptors which although allow uptake of a wider range of ligand-associated antigens (Chu, 1993) e.g. dendritic cells internalise a wide range of mannose-associated bacterial proteins using a mannose-fucosyl C-type lectin receptor (Sallusto, 1995).
The efficiency of receptor mediated endocytosis is influenced also by the fate of the internalised antigen-receptor mediated complexes. The mlg receptor and Fcγ receptor are proteolytically degraded with antigen and internalisation can be limited in some cases by available receptor. Mannose receptor, in contrast, recycles to the cell surface for reuse after it has released bound antigen, (Sallusto, 1995).

Fluid phase antigens which do not bind to surface receptors are internalised by pinocytosis as described. Most cell types including B cell types internalise fluid by micropinocytosis at 25-250 fl/cell/hr (Selby, 1995, Steinman, 1972). The efficiency of antigen uptake therefore is dependant on the concentration of antigen in the extracellular fluid. Only antigens at higher concentrations such as $10^{-6}$-$10^{-8}$M are presented at levels that induce a T cell response. Dendritic cells are capable of internalising fluid at much higher rates, 1000-2,400 fl/hr (Sallusto, 1995) allowing much more efficient presentation of low abundancy fluid phase antigens.

1.2.4 Antigen uptake and immunogenicity

The enhancement of antigen uptake which in turn influences the efficiency of antigen presentation, has a marked effect on immune response *in vitro* and *in vivo*. This has been demonstrated by conjugating antigens that are usually internalised by fluid phase micropinocytosis to ligands for cell surface receptors. T cell responses can be induced at 500-1000 fold lower antigen concentrations if antigen is conjugated and targeted to surface immunoglobulin (Barnes, 1995, Lakey, 1988, Snider, 1989). Enhancement can also be demonstrated for conjugates targeting other cell surface receptors such as the transferrin receptor, Fcγ receptors and mannose receptor. The increased immunogenicity of cationised antigens has also been demonstrated to be a result of enhanced antigen uptake (Faergeman, 1982).

1.2.5 Antigen processing

Different techniques have been utilised to study the pathway followed by internalised antigen. Antigen has been radiolabelled and SDS PAGE lysates made of APC pulsed with labelled antigen. Other techniques include the labelling of antigen with gold and
tracking with electron microscopy. C\textsuperscript{13} labelling allows detection of peptide fragments from labelled antigen using mass spectrometry.

Label has been detected within early endosomes within 5 minutes of exposure to antigen and at later timepoints in late endosomes and lysosomes (Rabinovitz, 1992). SDS PAGE of lysates made of APC incubated with labelled antigen demonstrate that antigen fragmentation is occurring within 30 minutes of antigen exposure as a result of proteolysis in early and late endosomes (Watts, 1989, West, 1994).

The environment in which protein antigen migrates within structures of the endocytic path becomes increasingly acidic and reducing from early endosomes to lysosomes. These conditions unfold and denature protein antigens allowing access of endosomal proteases (Jensen, 1995) and in the later compartments, for binding to class II molecules.

Clearly APC exhibit a well developed capacity for lysosomal proteolysis. However it is acknowledged that different APC may utilise distinct strategies for processing upon antigen encounter. Macrophages contain high levels of lysosomal proteases and degrade internalised protein antigen rapidly. Dendritic cells and B cells in contrast are protease poor and have a limited capacity for lysosomal degradation.

1.3 Class II processing

1.3.1 Overview

Although perhaps an oversimplification, antigen processing and presentation can be divided into two separate pathways which involve the two major classes of MHC; class I and class II. This in turn leads to presentation to 2 distinct subsets of T cells (Germain, 1993).

Class II molecules, in contrast to Class I molecules which are expressed on all cells, are only expressed by professional antigen presenting cells (APC) such as thymic epithelial cells, dendritic cells, macrophages and B cells as well as certain other activated cell types. The molecules present peptides of variable length (12-30 residues) to CD4\textsuperscript{+} T
cells. Class II molecules are capable of binding peptides at several different locations within the endosomal system and indeed on the surface of APC (reviewed by Robinson, 2002). Therefore most class II peptides are derived from self proteins present at high concentration within endosomes although a proportion are derived from extracellular proteins internalised into the endocytic system.

There is evidence that a degree of overlap can occur between the two pathways (e.g. in some circumstances extracellular antigen is effectively presented to class I molecules).

1.3.2 Class II processing pathway

In almost 3 decades of research it has been established that T cell antigen receptors recognise fragments of antigens or peptides bound to antigen presenting molecules encoded mainly by genes of the major histocompatibility complex or Human Leukocyte Antigen (HLA).

Extensive literature has established that the majority of peptides recognised by CD4 T cells are presented by a common pathway which is used by both professional and non-professional APC.

The peptide epitopes recognised by CD4 T cells are mainly derived from exogenous proteins that require some form of processing before they are accessible to bind to MHC class II molecules.

In addition this pathway is dependant upon a type II membrane protein, the invariant chain, li which engages class II α and β-chains and serves as both targeting subunit and a means of stabilising the class II peptide binding site.

1.3.3 Structure and function of MHC class II molecules

All class II molecules are composed of 2 non-covalently associated polypeptide chains (figure 1.3). Typically the two class II chains are similar to each other in terms of structure however the α-chain is slightly larger (32-34kD) than the β-chain (29-32kD) due to more extensive glycosylation. In class II molecules both polypeptide chains contain N-linked
oligosaccharide groups, both have extracellular amino termini and intracellular carboxy termini and over two thirds of each chain is located in the extracellular space. The two chains are encoded by different MHC genes that are usually polymorphic.

**Figure 1.3 Schematic diagram of MHC of a class II MHC complex molecule.** NH$_2$ and COOH represent the amino and carboxy termini of the polypeptide chains respectively. The red lines represent disulphide bonds and CHO, carbohydrate.

The three-dimensional structure of class II molecules has been solved by x-ray crystallography (Stern, 1994) and has demonstrated a similar structure to Class I MHC.
Similarities are most striking in the peptide binding cleft (Figure 1.4) (Madden, 1995). Both have an antigen binding groove with a beta pleated sheet floor bordered on 2 sides by alpha helices allowing the secure binding of peptides and the concomitant exposure to T cells. It is useful to divide class II molecules into a peptide binding domain, an Ig-like domain, a transmembrane domain and a cytoplasmic domain (Figure 1.3).

In class II, the extracellular portions of both α and β chains are subdivided into two segments of around 90 amino acid residues called α1 and α2 or β1 and β2 respectively. The peptide binding region of class II is formed by an interaction of both chains involving the α1 and β1 segments. This differs from class I where only the α chain is involved in forming the peptide binding cleft. Class II α1 and β1 fold to form an eight stranded, antiparallel β-pleated sheet platform supporting two α helices fours strands of the β-pleated sheet and one α helix formed by α1 and β1 equally.

Both the α2 and β2 segments of class molecules contain internal disulfide bonds and belong to the Ig superfamily. These segments like class I α3 and β2 microglobulin are folded into Ig domains within the native MHC molecule. Class II α2 and β2 segments are essentially nonpolymorphic among various alleles of particular class II genes but some show differences in different class II loci.

The correlation of CD4 expression on T cells with specificity for class II MHC molecules arises from the binding of CD4 with a projecting loop of the Ig-like non-polymorphic β2 domain of the class II molecule similar to the interaction of CD8 with the α3 of the class I heavy chain (see Figure 1.2).

The Ig-like regions of class II are probably important for non-covalent interactions between the two chains although other portions of the polypeptide chains are likely to contribute.

The carboxy terminal ends of the α2 and β2 segments continue into short connecting regions followed by approximately 25 amino acid stretches of hydrophobic residues that span the membrane. In both class I and II the hydrophobic transmembrane region ends.
with a cluster of basic amino acid residues which are followed by the carboxy terminal ends of the polypeptides forming short hydrophilic cytoplasmic tails.

**A Structure of class II molecule.** Ribbon diagram of MHC class II. Class II peptides are not bound at termini and therefore are free to extend beyond the groove. The length of the peptide is usually 13-17 amino acids long. The ends of the peptide are not constrained, the protrusion of the side chains into small cavities along the peptide binding hold the peptide in the groove. These cavities are known as pockets and may be deep or shallow.

**B Pockets in peptide binding groove of an MHC class II molecule.** A core region of nine amino acids are known to be essential for binding. The pockets that interact with the residue at positions 1, 4, 6 and 9 in the peptide are the most significant to the MHC Class II-peptide interaction.

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*Figure 1.4 Structural basis of peptide binding to MHC molecules*
Before considering the structural features of the binding of peptides to class II MHC, there are a number of properties of this interaction which have been deduced from biochemical studies.

The association of antigenic peptides and MHC molecules is a saturable low affinity interaction ($K_d$ ranging from $10^{-5}$ to $10^{-8}$M) with a slow "on rate" that is regulated by DM and a often a very slow "off rate" (although this may as fast as 60 minutes for low affinity peptides). The affinity of peptide-MHC interaction is much lower than that of antigen-antibody binding ($K_d$ of $10^{-7}$ to $10^{-11}$M) (Abbas, 1991). Once bound peptides may stay associated for hours to weeks.

X-ray crystallography has demonstrated that each class II molecule binds only one peptide at a time. Functional assays and direct binding studies have provided definitive evidence however that multiple different peptides are capable of binding to the same MHC molecule (Reviewed by Madden, 1995). There are structural constraints that prohibit peptides from binding. This together with the limited number of MHC alleles expressed in individuals supports the hypothesis that MHC molecules have a broad specificity for peptide binding but the fine specificity of antigen recognition is determined the antigen receptors of T lymphocytes.

There are distinct differences in the nature of peptides binding to class I and II most importantly peptides eluted from class I molecules are 9-11 amino acid residues in length whereas peptides eluted from class II can range from 10-30 residues or more (Figure 1.4A).

In contrast to class I which has a tight limitation on the length of peptide capable of binding to the groove, the α helical sides of the cleft in class II MHC molecules do not converge allowing bound peptides to extend outwards from the ends of the cleft. This ensures that there is no maximum length of peptide allowed to bind to class II. (Madden, 1995)

Most peptides binding to a particular allelic form of an MHC molecule demonstrate certain common features. Examples of shared features are a hydrophobic residue at position 2.
or a positively charged residue at position 7. Mutagenesis studies have confirmed that motifs like these can be crucial for peptide binding to particular allelic forms of MHC molecules.

In class II peptides bind with 12-13 residues held in the peptide binding groove with longer peptides protruding at either end. This bound segment is often termed the core binding sequence and lies in the groove in an extended conformation with a type II polyproline twist which causes about every 2 in 3 of the peptide side chain residues to be directed into the walls and floor of the peptide binding groove and every 1 in 3 of the side chains to be directed upwards where they contribute to the surface of the class II peptide complex seen by the T cell receptor. Thus amino acid side chains from both peptide and MHC molecules contribute to T cell recognition.

The interactions most involved in stabilising binding between peptide and class II occur between backbone atoms and conserved residues/main chain atoms in the areas of class II molecules flanking the peptide binding groove (Stern, 1994). Therefore binding is stabilised mainly by interactions independent of the bound peptide’s sequence.

The characteristically conserved features of peptides binding to MHC class II are complemented by specific structural features of MHC such as the presence of pockets in the floor of the cleft. These pockets are spaces between the peptide backbones of the β-pleated strands and are numbered according to the peptide side chain residue they accommodate (figure 1.4B). These pockets determine the nature of the peptide side chain that can fit into the pocket (e.g., hydrophobic, charged etc) and in some cases are critical for attaching peptide the peptide to the MHC molecule ("anchor residues").

1.3.4 Synthesis, trafficking and loading

MHC class IIα and β chains are synthesized de novo in the endoplasmic reticulum (ER) where they are assembled and transported to the golgi under the regulation of chaperones including the invariant chain (Ii). Ii acts as both targeting subunit and guardian of the peptide binding groove. Trimerisation of Ii brings in three MHC class II molecules to form a nonameric complex (Iiαβ)₃ which are then sorted to endosomes
including multilamellar and multi-vesicular compartments (Neefjes, 1999, Pond, 1999, Geuze, 1998) or to phagosomes (Ramachandra, 2000). li as well as the MHC class II-like chaperones DM and DO regulate the peptide loading of MHC class II molecules (Nordeng, 1998 Busch, 2000, Vogt, 1999, Alfonzo, 1999). The li-derived class II invariant chain peptide CLIP remains associated with the MHC class II peptide binding cleft until DM catalyses the dissociation of CLIP and binding of processed antigen peptides which is performed optimally at low pH (Kropshofer, 1999). DO regualte DM activity probably by enhancing the association of DM with MHC class II. MHC class II molecules bind the peptides generated in endosomes and the complexes are then transported to the cell surface for presentation to the CD4 T cells. Endogenous proteins and peptides may also bind to MHC class II molecules following transport into the endosomal pathway. (Harding, 1997 Lechler, 1996). Cytosol to endosome transport may lead to the processing of proteins translocated into the host cytosol by intracellular pathogens such as Listeria monocytogenes.

Antigen processing occurs in roughly the same compartments as li processing (Watts, 1997) but the late endosomes are thought to be the usual location where the antigenic peptides and the MHC class II molecules are able to interact. There is accumulating evidence however that peptide binding occurs in other cellular compartments (discussed below). In addition MHC molecules display peptides of varying length (often including a core sequence) which is variably extended in both N and C terminal directions (Engelhard, 1994). Peptide-loaded MHC class II molecules travel to the cell surface after li removal and peptide binding.

The majority of evidence indicates that MHC class II-li complexes enter the early endosomes, capture peptides at various points along the processing pathway and can probably exit at any point (Hiltbold, 2002). Once the cell surface is reached, the existence of the class II-peptide complexes is generally long. Some T cell epitopes generated in early endosomes can still engage “recycling” MHC class II molecules which have most likely exchanged their peptide or possibly reached the cell surface. (Robinson, 2002).
1.3.5 Invariant chain

The association of MHC class II with invariant chain has important influences on the assembly, transport and function of class II molecules (Ceman, 1995, Cresswell, 1994, Germain, 1995). Although the genes encoding li are non-polymorphic, the molecule occurs in several forms as a result of splicing of one exon, p41. And the use of 2 different start codons. The major forms are 33,35,41,and 43 kD (which includes 2 kD added through glycosylation) and are termed, p33, p35, p41 and p43 respectively. All variants perform 3 important functions; they contain a trimerisation domain and spontaneously associate into trimers to provide a framework in which 3 class II αβ heterodimers bind, they have a class II binding domain which includes a sequence that binds to the class II binding groove, the class II-associated invariant chain peptide, CLIP. CLIP once in the peptide binding groove stabilizes the αβ heterodimer and prevents early binding of peptide or denatured protein in the ER. Finally they have targeting motifs in their cytoplasmic domain that directs (liαβ)3 which are exiting the trans-golgi network towards cytoplasmic vesicles.

In macrophages, B cells and dendritic cells, newly synthesized αβli enters the MHC class II compartment, MIIC, which is quite distinct from the compartments of the classical endocytic pathway. It has a density similar to late endosomes but has distinct multivesicular morphology on EM and lacks late endosomal (mannose-6-phosphate receptor) and early endosomal (transferrin receptor) markers. It contains molecules important for the formation of class II/peptide complexes which include leupeptin sensitive proteases which cleave li in a staged manner as discussed below. MIIC also contains HLA-DM which promotes the dissociation of CLIP (discussed below). Most nascent αβli reaches endosomes without visiting the cell surface. The exact route is not certain however some is detectable at the cell surface but is rapidly recycled into endosomes.
1.3.6 li processing

Studies using chemical and genetic ablation of lysosomal proteases have demonstrated that li processing is a staged process which moves in a C-terminal to N-terminal direction. The cysteine protease inhibitor, leupeptin causes the accumulation of a 22kDa or 10kDa li product in human or mouse cells respectively (Blum, 1988, Amigorena, 1995). The leupeptin insensitive enzyme responsible for initiating li processing in these conditions is Asparginyl endopeptidase (AEP) which targets asparagine residue 155 (Asn 155) and probably Asn 132 on the outside of the trimerized C-terminal domain (Manoury, 2003). Processing of the mouse li proceeds further because of an additional more N-terminally located asparagine residue. However li processing can be initiated by proteases other than AEP. Maehr et al have addressed li processing in AEP null mice and found the absence of AEP had no effect on li processing at any stage suggesting li processing can also be initiated by enzymes other than AEP (Maehr, 2005). This is not unexpected given the importance of li removal. Although lack of AEP does not impair invariant chain processing in the murine model, AEP inhibition has been shown to delay the initiation of li cleavage in some human EBV transformed B cell lines and monocyte-derived dendritic cells. The specific cleavage by AEP immediately C-terminal to asparagine residues (an action which is insensitive to cysteine protease inhibitors, leupeptin and E-64) has been implicated in li processing. In humans there are at least two subsequent steps in li processing.

The use of specific inhibitors and the study of gene knockout mice have been utilised to identify putative proteases involved in li and antigen processing. Blockade of the progressive cleavage of li results in the accumulation of li intermediates and a decrease in the surface expression of class II MHC products (Neefjes, 1992, Brachet 1997).

Experiments in human EBV B cells treated with a cathepsin S-specific inhibitor and characterization of cathepsin S knock out mice have implicated cathepsin S in the terminal cleavage to yield CLIP. (Busch, 2005) Further studies have demonstrated cell-specific cleavage of p10 by cathepsin L (CatL) in thymic epithelial cells (Nakagawa, 1998) and cathepsin F in macrophages (Shi, 2000) Inhibition of cathepsin S leads to the
accumulation of a 10kDa processing intermediate, p10 (Riese, 1996). Processing of this intermediate is less redundant and the terminal stages of ll processing are cathepsin S-dependant, at least in respect to some HLA haplotypes. In its absence or in the presence of leupeptin, MHC class II I-Ab-p10 complexes accumulate, slowing but not completely inhibiting transport to the cell surface. (Brachet, 1997, Driessen, 1997). Although transport can still occur in the absence of cathepsin S, class II peptide loaded is prevented.

**Figure 1.5 Class II antigen processing and presentation** This diagram demonstrates an APC presenting peptides from extracellular antigen to a T cell. Sequential aspects of this pathway are
1. Internalisation of protein by endocytosis into endosome
2. Proteolytic processing of protein in endosome or lysosome generating peptides
3. Class II MHC polypeptide synthesis
4. Fusion of endosomal derived CIIV/MIIC vesicle containing class II MHC
5a. Degradation of li and DM mediated release of CLIP
5b. Binding of peptide antigen to class II MHC
6. Fusion of vesicle with plasma membrane; expression of peptide-class II MHC complexes on cell surface
7. Presentation of peptide-class II MHC complex to CD4 helper T cell.

1.4 Proteolytic enzymes and their importance in antigen processing

1.4.1 Introduction

The capture of peptides by class II molecules is remarkable given the unfavourable circumstances in which this occurs. Class II molecules and their li chaperone are delivered to the endosome/lysosome system and exposed to an environment rich in proteolytic enzymes. Despite this, processed peptides derived from protein antigens which are usually present in very small amounts weather this aggressive proteolytic environment and survive to bind to the peptide binding groove of newly synthesised and (once the li is removed, potentially unstable) class II molecules. DM stabilises this complex and assisted by DO in some cells, guides peptide loading in immunological desirable directions.

1.4.2 Antigen processing and processing enzymes

The list of proteases found within the endosome/lysosomal compartment continues to increase (reviewed by Watts, 2001, Chapman, 2006). There are at least a dozen enzymes related to the cysteine protease, papain. Of these cathepsins B,H,L,S,F,Z,V,O,C and possibly K are expressed in APC of different types. In addition, APC contain other classes of proteolytic enzymes, including the aspartic proteases, cathepsins D and E/G and asparaginyl endopeptidase (AEP). Most of these proteases operate in an acidic pH optima and exhibit rather broad substrate specificity. Cathepsin S
is the exception to the former and AEP an exception to the latter. Other enzymes such as cathepsins H,C,B and Z have structural features that limit access to the active site and function as amino (cathepsins H and C) or carboxyl (cathepsins B and Z) exopeptidases (Figure 1.1).

Reduction of disulphide bonds has been shown to be important for the presentation of certain T cell epitopes and to depend on a reducing environment in endosomes and lysosomes. GILT, is an IFN-γ-inducible lysosomal thiol reductase identified in endosomes that were positive for class II MHC. GILT reduces protein disulphide bonds at an acidic pH optimum and is therefore be involved in the reduction and possibly the unfolding of protein antigens. (Phan, 2000)

Exopeptidases are likely to partly account for the heterogeneous termini of naturally processed T cell epitopes found bound to class II MHC. Many of these enzymes have homologues in plants or simple animals. It would appear therefore that the mammalian immune system has taken advantage of ancient proteolytic systems to perform essential steps in antigen presentation. Expression in APC of the most useful enzymes and regulation of the expression of some of these with cytokines such as IFN-γ tailor the processing machinery to the requirements of the APC.
A number of studies have identified proteases that generate T cell epitopes from protein antigens *in vitro* but it has been more difficult to establish which enzymes are responsible *in vivo* (Watts, 1997). A number of approaches have been utilised in an attempt to shed more light on which enzymes are involved in processing including the identification and mutagenesis of processing sites in protein antigens, the use of specific inhibitors (Puri, 1988) and the use of protease-gene-targeted mice (Nakagawa, 1999, Shi, 1999).

Other factors apart from proteases are involved in the generation of suitable substrates for loading onto class II molecules.

The open ends of the class II MHC peptide binding groove appears well suited to the capture of unfolded and extended antigen domains. Indeed this feature could be essential in avoiding over-digestion and potential destruction of T cell epitopes. Peptides eluted from bulk class II MHC populations usually have a maximum size of around 25 residues but a number of studies have suggested that longer processing products might be

<table>
<thead>
<tr>
<th>Protease</th>
<th>Example</th>
<th>Cleavage preferences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initiating protease</td>
<td>AEP, Cathepsin B, D, S, GILT</td>
<td>Preference for polar/charged side chains. Relatively non-redundant. Initiate processing</td>
</tr>
<tr>
<td>Secondary protease</td>
<td>Cathepsin D, E, others?</td>
<td>Preference for cleavage at hydrophobic sites. Some redundancy. GILT/other chaperones may aid with disulphide bond cleavage &amp; unfolding</td>
</tr>
<tr>
<td>Exopeptidase</td>
<td>Cathepsins H, C, Z, B</td>
<td>Generate short but heterogeneous peptides</td>
</tr>
</tbody>
</table>

Table 1.1 Proteases involved in endosomal processing (From Watts, 2001)
captured by class II MHC (Davidson, 1991, Lindner, 1996). A study performed on HEL-pulsed splenocytes identified a class II MHC dimer made up of one I-E^k and one I-A^k class II MHC molecule. This association was only seen in HEL-pulsed cells and appeared to be due to a 7kDa processed fragment of HEL linking the two class II MHC molecules (Castellino, 1998). Intermediates of this type are difficult to detect due to their transient nature; the I-E^k-HEL-I-A^k complex was detected probably because of its unusual longevity and stability. However, capture of unfolded, extended sequences by class II is probably important for capture of vulnerable peptides in an aggressive proteolytic environment because once bound they are protected.

After uptake, protein antigens undergo unfolding and disulphide reduction followed by degradation by cysteiny1 and aspartinyl proteinases with optimal activity in an acidic environment. A number of recent studies have shown that epitopes within denatured intact proteins can bind to MHC class II without any prior degradation (Germain, 1994)

1.4.3 Variability of antigen processing

Accessibility of antigen

How much processing is needed before binding to class II MHC occurs? As detailed above, earlier studies have shown that class II MHC can bind long peptides and even intact proteins (Germain, 1994). However it has been more difficult to determine whether this actually happens during normal antigen processing. Both functional and biochemical data now exist to support the notion that class II MHC can capture long peptides. (Davidson, 1991, Lindner, 1996) Work from Moss et al indicates that in the case of the tetanus toxin C fragment (TTCF) substrate, very limited processing by AEP is sufficient for generation of a form of antigen that is able to bind to available class II molecules. Capture by class II MHC in the early stages of the processing can involve long unfolded stretches of antigen substrate. The open-ended peptide binding groove of the class II molecule allows extended peptides to bind (Castellino, 1998) This may have evolved to allow early capture of extended peptides thereby allowing protection from further proteolysis. This has in turn led to a hypothesis by Watt to explain how the problem of
epitope destruction can be solved in the class II MHC pathway. Here 3 different types of proteases are described: (1) initiating or unlocking proteases; (2) endoproteases that preferentially act on partially processed and unfolded antigen; and (3) exopeptidases that trim long MHC-bound peptides to their normal size range (12-25 residues). (Watts, 2001) (See Table 1.1)

**Quaternary structure**

Quaternary structure may affect presentation of exogenous oliguric protein by influencing a) uptake of protein by APC b) the endocytic pathway c) proteolytic degradation into peptides.

Several reports have demonstrated that conformation of protein antigen influences processing in turn affecting antigenic potency (Vacchio, 1989, Del Val, 1991). Glimcher et al described the tertiary conformation of the insulin molecule was important in the formation of an antigenic determinant recognised by insulin-specific T cells (Glimcher, 1983). Atassi et al (Atassi, 1989) reported T cells specific for regions in the interface between α and β subunits of HbA recognised isolated monomeric but not haemoglobin tetramer. Rouas et al demonstrated that dimerisation of hCG subunits failed to stimulate T cells generated against peptides derived from the α monomer subunit of hCG, suggesting dimerization of subunits may affect the nature of processing (Rouas, 1993). Janssen et al inserted a T cell epitope containing 72-85 of MBP (the critical T cell epitope in experimental autoimmune encephalomyelitis in Lewis rats) in different sites of outer membrane protein of PhOE of e.Coli and examined responses of MBP72-85- specific T cells in denatured monomeric and native trimeric forms of chimeric PhOE constructs. They found that monomeric but not native trimeric forms of PhOE constructs induced proliferation of T cells. This discrepancy was independent of site of epitope insertion in PhOE. Immunisation with monomeric form induced priming of MBP72-85 T cells but trimeric forms were much less efficient in priming cells. Differences were more apparent in induction of EAE studies where monomer was encephalitogenic and trimer was not (Janssen, 1996).
Antibody binding

Several reports confirm the influence of antibody on antigen presentation in vitro for model antigen such as HEL (Guermonprez, 1999) and for putative autoantigen such as GAD 65 and thyroglobulin (Reijonen, 2000, Banga, 2004, Dai, 1999 & 2005) as well as viral antigen (Fournier, 1996). In all cases the effectors of antigen could be attributed similarly to enhanced FcR-mediated uptake but was more likely a combination of uptake and antibody-modulated (autoantigen) processing. In vivo studies have demonstrated enhancing and inhibitory effects on presentation depending on combination of antibody and T cells specificity. Studies on processing of tetanus toxin (TT) processing in the human system using tetanus toxin C fragment (TTCF) demonstrated a striking enhancement of presentation of some but not all T cell epitopes from TTCF with antibody (Antoniou, 2002).

Extracellular processing

There is abundant evidence that MHC class II molecules on the surface of fixed APC can directly bind and present synthetic peptide epitopes although requiring higher concentrations than fixed cells (Lindner, 1996, Aoi, 1997, Harris, 1996). Fixed APC have also been shown to present denatured protein (Jensen, 1991, 1993, Carrasco-Marin, 1998). Therefore class II molecules on the surface of APC are capable of binding protein or peptides denatured or degraded by the activity of extracellular enzymes in the inflammatory exudate.

A number of proteins have been demonstrated to bypass intracellular processing and influence presentation by surface MHC class II including MBP68-86 from guinea pig MBP (Mannie, 1990). Cleavage by serum proteinases implicated in the serum-dependant extracellular processing of soluble Hep δ antigen (HDAg) leading to the presentation of HDAg 106-122 at the surface of B cells (Accapezzato, 1998). Immature dendritic cells (DC) have been shown to express ectoproteinases at the cell surface (Amoscato, 1998) and secrete serine proteinases which cleave proteins into antigenic peptides (Santambrogio, 1999).
Peptide generated by extracellular processing may bind cell surface MHC class II molecules under the control of DM expressed on the surface of professional APC such as B cells or immature DC (as shown for MBP111-129 and MBP87-99 or type II collagen COL259-276) (Arndt, 2000). It has been suggested that B cells and immature DC by virtue of high DM expression on cell surface, facilitate loading of peptides at sites of inflammation, rescuing peptides that are easily degraded which are processed by endosomal proteases.

1.4.4 How may diversity in antigen processing influence the immune response to self?

T cells are implicated in the pathogenesis of organ-specific and systemic autoimmunity, leading to the identification of candidate autoantigens and mechanisms precipitating diseases. An altered array of peptides to which the T-cell repertoire has not been exposed by whatever mechanism, could potentially break self tolerance. Possible mechanisms by which processing might give variable results include:

1.4.5 Non-professional APC

It has been hypothesised that self-tolerance could be broken if an altered array of self peptides to which the T cell repertoire has not been previously exposed, is presented on the cell surface in association with MHC class II in the absence of li and DM regulation therefore potentially breaking self tolerance (Lechler, 1996). The loading of MHC class II molecules in early endosomes is less strictly regulated by li or DM which permits association between MHC class II molecules and unfolded endogenous proteins. In this way self-epitopes may be generated in some non-professional APC, which lack li chain expression. Therefore it is possible that cellular compartments such as early endosomes that permit li chain and DM-independent loading of MHC class II molecules, play an important role in the generation of autoepitopes implicated in the pathogenesis of autoimmune diseases. The hypothesis is supported by studies of myelin basic protein (MBP) a major autoantigen implicated in the pathogenesis of multiple sclerosis (MS) (Stinissen, 1998).
It has been shown that APC from β2 chain-deficient MBP84-105 TG mice could present additional self-epitopes from the MBP fragment compared to T cells from β2-chain sufficient MBP84-105 TG mice. (Bodmer, 1994) MBP 84-105 has been shown to load surface and recycling MHC class II molecules independent of DM (Pinet, 1995, Vergelli, 1997). Two human DR4-restricted immunodominant MBP autoepitopes MBP 111-129 and MBP 87-99 involved in MS (Zamvil, 1988) were also shown to be presented on MHC class II molecules in a DM-independent pathway in immature DC and B cells (Arndt, 2000). DM was found to inhibit presentation of an immunodominant autoepitope from type II collagen COL259-273 implicated in RA (Arndt, 2000).

Therefore considerable interest should be focused on antigen processing itself and in particular, factors that could account for certain autoantigen-derived peptides being constitutively presented at low abundance despite good affinity for MHC class II.

1.4.6 Aberrant processing

Several other autoantigens have been described as being processed independently of newly synthesized MHC class II molecules and endosomal acidification. The presentation of the retinal S-antigen, involved in the pathogenesis of endogenous posterior uveoretinitis was resistant to cyclohexamide and chloroquine. In addition processing of S antigen was not influenced by inhibitors of cysteine proteinases and other lysosomal enzymes except the serine proteinase plasmin. These observations suggest that S-antigen is not processed for binding to newly synthesized MHC class II in late endosomes. It has been speculated that since 2 out of the 3 conserved putative uviotogenic epitopes include a cathepsin B cleavage site, these epitopes are likely to be forwarded for destructive processing in the late endosomes unless rescued by MHC class II molecules recycling from the cell surface. (Liversidge, 1992)

1.4.7 Creative and destructive processing

In the MHC class I processing pathway the proteolytic machinery that generates peptides primarily in the proteosome is separated from the machinery that catalyzes the loading of peptides onto nascent class I molecules. The key events of peptide generation and
peptide loading are separated by the membrane of the ER. The situation is quite different from that which occurs in the MHC class II processing pathway where the entire process of antigen processing and peptide capture by class II MHC takes place within the same compartmental system. (Watts, 2003) In addition many of the enzymes present within the endosomes and lysosomes of APC are rather non-specific in terms of their cleavage specificity. The question arises then how can T cell epitopes survive and be loaded onto MHC class II in such an aggressive proteolytic environment? Are specific enzymes required for optimal processing and loading of T cell epitopes-i.e how redundant is antigen processing in the class II pathway?

Possibly professional APC such as dendritic cells (DC) have specialised mechanisms to overcome this difficulty. DC function is carefully regulated by a process of terminal differentiation called “maturity”. DCs can alter their ability to form and accumulate peptide-MHC complexes depending on their maturity (Mellman, 1998). Trombetta et al demonstrated that a key mechanism responsible for this alteration was the generalised activation of lysosomal function where immature DC slowly and inefficiently degraded internalised antigen. In contrast, maturation-induced activation of the vacuolar proton pump that enhanced lysosomal acidification and antigen proteolysis, facilitated the efficient formation of peptide-MHC complexes (Trombetta, 2003).

Some potential T cell epitopes will contain protease cleavage sites and will be destroyed during antigen processing. The immunological consequences of this have not yet been established. In the case of self-proteins this destructive processing may have serious consequences and proteolytic enzymes in turn may have a profound influence on the establishment and potential breakdown of tolerance.

Proteases perform 2 key roles in the MHC class II processing pathway. They initiate removal of invariant chain chaperone for MHC class II and also generate peptides from foreign and self proteins for capture and display to T cells. It is however difficult to understand how a balance is achieved between generation of peptides and their destruction in such an aggressive proteolytic environment. Nor is it known in most cases which proteases are involved in antigen processing.
Moreover little is known about the processing of putative human autoantigens and why tolerance is achieved to some T cell epitopes and not others.

Recent work by Watts et al studied the processing of the candidate human autoantigen in multiple sclerosis, myelin basic protein (MBP) which can also induce the MS-like disease, experimental allergic encephalomyelitis (EAE) in susceptible strains of mice and rats. They demonstrated that a principal human HLA-DR2-restricted epitope-amino acids 85-99 of MBP ‘MBP(85-99)’ contained a processing site for the cysteine protease asparagine endopeptidase (AEP) after Asn 94. Presentation of this epitope by human antigen presenting cells is inversely proportional to the amount of cellular AEP activity where inhibition of AEP in living cells greatly enhanced presentation of the MBP(85-99) protein whereas over expression of AEP diminished presentation. The results indicated that central tolerance to the encephalitogenic MBP epitope may not be established because destructive processing limits its display in the thymus (Manoury, 2002). Supporting this hypothesis, they also demonstrated that AEP is expressed abundantly in thymic antigen presenting cells.

How this epitope, rendered “cryptic” through destructive processing is in turn presented as a target for autoreactive T cells, is not yet known. It may be related to the levels of the enzyme responsible for destruction (AEP expression in the central nervous system, for example in the case of MBP, where MBP 85-99 reactive T cells are thought to be responsible for the immunopathology)

1.5 Goodpasture’s disease as a model of autoimmunity

1.5.1 Introduction

Autoimmunity is thought to be important in the pathogenesis of most forms of glomerulonephritis which together account for approximately 25% of patients with end stage renal failure. Although rare, Goodpasture’s disease provides a unique opportunity for the study of renal autoimmunity as it is the only glomerulonephritis in which the target of autoimmunity has been characterised.
Anti-glomerular basement membrane (anti-GBM) disease or Goodpasture’s disease is the best defined renal organ-specific autoimmune disease. The disease is tightly associated with autoantibody formation to a specific target found in the glomerular and alveolar basement membranes and is characterised by a rapidly progressive glomerulonephritis which is often associated with pulmonary haemorrhage though either may occur alone.

In 1919 Ernest Goodpasture first described a patient with pulmonary hemorrhage associated with glomerulonephritis in an 18 year old man during the 1918 influenza pandemic (Goodpasture, 1919). Stanton and Tange described in 1958 a group of 9 patients with similar findings and gave Goodpasture’s name to the syndrome (Stanton, 1958).

There are multiple causes of the association between glomerulonephritis and pulmonary haemorrhage. The term Goodpasture’s disease is reserved for individuals who have disease in association with antibodies to the glomerular basement membrane (GBM) either bound directly to the target organ or in the circulation. In early series mortality was very high and irreversible renal failure a common outcome. The introduction of cyclophosphamide and plasma exchange however transformed therapy, and this became the first unequivocally effective therapy for inflammatory renal disease (Lockwood, 1976).

1.5.2 Epidemiology

Goodpasture’s syndrome is uncommon with an incidence of up to 1 case per million population per annum in the UK based on the identification of anti-GBM antibodies by immunoassay and biopsy specimens. It has been reported to account for up to 5% of cases of glomerulonephritis (Wilson and Dixon, 1973; New Zealand GN Study group, 1989) but 10-20% of crescentic glomerulonephritis (Couser, 1988, Andrassy 1991).

The distribution by gender is approximately equal with a slight preponderance of males to females. The age at presentation can vary from first to ninth decade. There is however a bimodal distribution with a greater number of individuals presenting around 30 years and a second peak at around 60 (Savage, 1986). The youngest case presented at 11 months.
old and several patients in their 9th decade have been described although presentations at such extremes are very rare. This age and gender distribution is somewhat dissimilar to those of other organ-specific autoimmune disorders.

The disease is more common in white and certain other racial groups including the indigenous population of New Zealand (Teague, 1978). It seems to be rare in South Asian and Black races but cases have been reported in Japan and China.

Pulmonary haemorrhage appears more common in the young and in males and glomerulonephritis alone is more common in older patients and in women. The disease can present at any time but its incidence appears to increase in the spring and early summer.

Clustering of cases has been described in 3 reports (Simpson, 1982, Perez 1974, Williams, 1988) and many anecdotes. For example, within our own unit in Edinburgh, 2 cases presenting within a few months of each other were unrelated individuals who were in fact next door neighbours. Such reports lend support to the hypothesis that exposure to an infective or other exogenous agent is involved in the pathogenesis (see later). To date, no such pathogen has been identified. One of the greatest difficulties in analyzing potential exogenous agents is the likelihood that any infective agent may exacerbate pre-existing inflammation leading to its clinical presentation without necessarily causing it.

Disease associations may give some clues to etiology. Several are disorders in which there may be increased production or destruction of the target antigen in GBM (Table 1.3). Associations with other autoimmune disorders are uncommon unlike other AID such as SLE and autoimmune thyroiditis, suggesting a general breakdown of tolerance is not the underlying pathogenic mechanism of this particular disease.
<table>
<thead>
<tr>
<th>Disease</th>
<th>Number of reports (approx)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANCA-associated vasculitis (mostly with anti-myeloperoxidase ANCA)</td>
<td>Hundreds</td>
</tr>
<tr>
<td>Membranous nephropathy</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>ten</td>
</tr>
<tr>
<td>Lithotripsy to intrarenal stones</td>
<td>3</td>
</tr>
<tr>
<td>(Alport’s syndrome following renal transplantation)</td>
<td>(tens)</td>
</tr>
</tbody>
</table>

Table 1.2 Disease associations Diseases recurrently associated with Goodpasture’s disease. Alport’s syndrome following transplantation is bracketed as it has a distinct aetiology and is not ‘true’ Goodpasture’s disease.
A strong class II MHC association has been described, with over 80% of patients carrying HLA-DR15 (Phelps, 2000). A negative association exists with DR7 and possibly DR1 (Phelps, 2000). Class II MHC associations are found to varying degrees in almost all autoimmune diseases and this is discussed further below.

1.5.3 Aetiology

The clinical outline presented above gives some clues that are helpful in approaching the pathogenesis of disease. It illustrates that

- Breaking of tolerance is rare
- Association with other autoimmune diseases is rare
- Increased turnover or destruction of basement membrane may be implicated in disease initiation suggesting a possible change in presentation due to increased turnover a factor in immunopathogenesis
- Clustering of cases suggests a potential role for exogenous infection/toxin in breaking tolerance
- Antibody formation is a consistent finding and likely to be a central part of the pathogenesis of the disease
- HLA is a strongly predisposing factor in disease susceptibility

The clinical syndromes of autoimmune disease caused by activation of T cells, B cells or both in the absence of infection is the consequence of the failure of a number of safety mechanisms established under the heading of tolerance.

The challenge then is to understand how this physiological process becomes pathological. More is known about Goodpasture's disease than any other autoimmune renal disorder. Some information comes from animal studies, but in this disease there are also invaluable clinical studies and results from laboratory investigations of the human antigen, antibodies and lymphocytes.
1.5.4 Antigen

Basement membranes are specialized and complex structures found usually at the boundary between cells and connective tissue stroma. The membranes themselves can influence the fate of cells, the polarization of subcellular constituents and the location of cell receptors and transporters.

The majority of the components of the basement membrane are ubiquitous to all basement membranes but others have a more restricted distribution probably related to more specific function within the respective basement membrane (Miner, 1999). Within the kidney the GBM is a key component of the ultrafiltration barrier. It is assembled through interweaving of IV collagen with laminins, nidogen and sulphated proteoglycans.

Collagen IV (Figures 1.6 and 1.7) was first isolated by Kefalides from GBM in 1966. It belongs to a family of collagenous proteins with at least 25 members. Six genes encode collagen IV chains: α1(IV)-α6(IV), COL4A1 to COL4A6. Collagen IV is composed of triple helical (protomers), α1α1α2, α3α4α5 and α5α5α6 which are selectively expressed in different membranes at different stages of embryonic development. These protomers create fishnet-like collagenous networks by covalent associations as dimers head-to-head and tetramers tail-to-tail. The α1α1α2 network is present in almost all basement membranes while the other networks have a much more tissue-specific distribution with that of α3α4α5 being particularly limited. It is found especially in basement membranes formed by fusion of epithelial and endothelial cell basement membranes or those involved in gas or fluid exchange or transfer. These networks occur in the GBM and some tubular basement membrane; the kidneys, lungs testis, cochlea and eye (Cashman, 1988).
Figure 1.6 Type IV collagen is the major protein of basement membranes. Its triple helical protomers have multiple non-collagenous interruptions that make the fibres non-linear, with kinks and tertiary coils and the head-to-head and tail-to-tail interactions form an open meshwork. The Goodpasture antigen lies in the C-terminal, non-collagenous domain.
C-terminals of adjacent collagen (IV) molecules

**Figure 1.7** The NC1 domains of adjacent α3α4α5 protomers are tightly associated as hexamers. Digestion with enzymes that break down collagens releases hexamers intact and this process is likely to be part of normal basement membrane turnover and of destructive proteolysis during inflammation. Dissociation of NC1 hexamers (e.g. for SDS-PAGE chromatography or by low pH) releases dimers and monomers of the constituent alpha chain NC1 domains and exposes further antibody-binding epitopes. Persisting dimers are composed of monomers covalently linked by disulfide and other covalent linkages.

Hudson and co-workers identified that most of the circulating antibodies in patients with anti-GBM disease were directed against a non-collagenous (NC1) domain of a new type IV collagen chain that they termed α3 (Wieslander, 1984, Saus, 1988). This was confirmed by cDNA cloning, which showed that the 230 amino acid carboxy terminal domain α3(IV)NC1 was the consistent target of autoantibodies in all patients with anti-
GBM disease (Turner, 1992). Glomerular epithelial cells seem to be responsible for the synthesis of α3α4α5 collagens in the formation of GBM.

1.5.5 Antibodies and B cell epitopes

In detailed studies with recombinant proteins some patients show additional, usually minor reactivity to other NC1 domains but reactivity with α3(IV)NC1 is consistent and specific. Much of the reactivity of any one patient’s autoantibodies can be blocked by other patients’ sera or even by single monoclonal antibodies (Hellmark, 1994) suggesting consistent specificity between patients. Most patients’ anti-α3(IV)NC1 antibodies include a high proportion specific for epitopes involving residues 17-31 (Eₐ) towards the N-terminal end of the NC1 domain close to the triple helical region, and a smaller population specific for Eₐ in the second hemi domain at 127-141 (Ryan, 1998; Netzer, 1999; Hellmark, 1999, Borza, 2000, David, 2001). The epitope is highly conformational, rendering early studies using synthetic peptides or bacterially produced antigen unreliable. There is a degree of concealment or crypticity of the B cell epitope in the fully folded hexamer, which can be purified from GBM as a hexamer containing the NC1 domains of two adjacent α3α4α5 protomers (Borza, 2000, Wieslander, 1985) (Figure 1.7). However circulating antibodies bind to the normal GBM of experimental animals including primates in vivo, and the apparently normal GBM of humans in vivo. Detailed studies suggest that subtle differences in the covalent bonding between monomers of adjacent fibrils may influence the accessibility of the epitope, with many more sites becoming available when the molecule is dissociated (Borza, 2005).

1.5.6 Regulatory and effector T cell epitopes and their generation

Tolerance to self antigens is maintained by regulatory T cells as well as by deletion of cells with a high affinity for presented peptides of self antigens. The α3 chain of type IV collagen has been demonstrated to be ‘promiscuously’ expressed in the thymus, in common with many other known autoantigens (Salama, 2001, Wong, 2001). Although the percentage of T cells capable of recognising the alpha chain that are deleted in the thymus is not clear, it is known that a small percentage do survive into the periphery.
Peripheral blood T lymphocytes in both healthy individuals and patients with disease are capable of proliferating to \( \alpha 3(IV) \)NC1 in vitro (Derry, 1995, Salama, 2001). T cells that proliferate in healthy individuals are much less abundant than in those with active disease or those in the recovery stage of disease (Salama, 2001). Cairns et al demonstrated that patients' T cells consistently recognised certain epitopes in \( \alpha 3(IV) \)NC1 with greater abundance in comparison with healthy controls and this is discussed further below. The relative proportions of anergic and regulatory cells are not yet known (Cairns, 2003).

1.5.7 Initiation of the autoimmune response

In experimental animals, initiating an autoimmune response requires several actions, and these are highly informative when considering how autoimmune diseases may be initiated in man:

- Choose a susceptible strain – where genetic susceptibility has been mapped, multiple loci are usually involved, for example in the Wistar-Kyoto rat which is particularly susceptible to crescentic nephritis and vasculitis (Reynolds, 2002). It is clear that this is also true in human autoimmune diseases.

The only well established genetic association in Goodpasture's disease so far is HLA linkage. There are reports of discordance in monozygotic twins, and this is generally true for autoimmune diseases, showing that at least second if not third hit is required on a background of genetic susceptibility. Reports of clusters of cases mentioned above provide strong circumstantial evidence for an exogenous factor – but no factor has been identified in any of these clusters.

- Immunize with a slightly modified antigen – to induce an immune response that is to a slightly different molecule that can cross-react with a self protein. The most common strategy is to use antigen prepared from another species so that there are some sequence differences, but post-translational or chemical or structural alterations may also suffice. B lymphocytes may often be important in generating this type of cross-reactivity. It is frequently hypothesized, though rarely proven, that molecular mimicry by microorganisms might be an important initiating element in autoimmune diseases.
(reviewed by Wucherpfennig 2001). Looking for linear sequence similarities is probably an oversimplification as T cells see shapes, not sequences, and T cell clones may be stimulated by peptides of quite different sequence (Wucherpfennig, 1995).

- Use an adjuvant – without an adjuvant, immune responses in animals are frequently absent or benign. The stimulation of toll-like receptors and possibly other pathways can critically influence the outcome of the interaction between an antigen presenting cell and interacting lymphocytes, to shift the response from the default response of tolerance to one of active immunity – the ‘danger hypothesis’ (Gallucci and Matzinger, 2001). If the mimicry hypothesis is favoured, the same microorganism might provide the adjuvant stimulus too. However there is no demonstrable consistent relationship between specific infections and most autoimmune diseases.

The outcome of interactions is likely to be highly contingent on quantitative aspects of each of these three factors.

The associations of Goodpasture's disease with small vessel vasculitis, lithotripsy, and to a lesser extent membranous nephropathy and possibly diabetes support the idea that a quantitative or qualitative change in antigen presentation may be important. Lithotripsy is known to cause renal trauma; as the kidney moves with respiration it is almost impossible to prevent some exposure to shock waves and potential tissue damage when fragmenting intrarenal stones (Evan, 1991, Jaeger, 1995). In diabetes and membranous nephropathy the thickened and abnormal GBM contains an increased amount of Goodpasture antigen. As discussed above, there is good reason to suspect that a change in antigen presentation must occur in disease initiation, to permit generation of disease-associated peptides which are not normally presented, and to which there is therefore no (or less) tolerance.

1.5.8 Mechanisms of Injury

Though opinion on the predominant mechanism of injury has swung forward and back, it is now widely accepted that both cell-mediated and antibody mechanisms are integral to the damage seen in anti-GBM disease.
1.5.9 Injury caused by antibodies

In 1967, Lerner, Glassock and Dixon demonstrated that anti-GBM antibodies were nephrotoxic. Nephritic kidneys from patients with anti-GBM disease were acid-eluted and antibody purified in order to obtain purified antibody. The antibody was then injected into the unilaterally nephrectomised squirrel monkeys which then went on to demonstrate non-crescentic glomerulonephritis and renal failure. In humans, pathogenicity of circulating anti-GBM antibodies was suggested by the close association of circulating antibodies and disease as well as the titre of antibody and severity and subsequent resolution of disease (Simpson, 1982, Savage, 1986, Herody, 1993).

In genetically engineered mice that produce human IgG antibodies, immunization with α3(IV) NC1 domains results in the production of human anti-GBM antibodies and a proliferative glomerulonephritis (Meyers, 2002). It should be noted that in this model the changes produced by antibodies alone were not of a crescentic nephritis. In the WKY rat, transfer of antibodies has been demonstrated to transfer disease (discussed further below).

Human anti-GBM antibodies usually of the IgG-complement-fixing class or rarely IgA are of particularly high affinity and remain attached to the GBM for prolonged periods (Rutgers, 2000) (Figure 1.8).

When anti-GBM antibodies bind GBM they activate complement leading to cell injury and inflammation. The cleavage of C3 leads to C3 fragments and ultimately to the formation of the membrane attack complex (C5b-9) which inserts into cell membranes where it can kill cells or activate them to secrete cytokines: recruitment of cellular effectors is critical to further damage.
1.5.10 Injury caused by T lymphocytes

The strong evidence to suggest that cell mediated immunity has an important role to play in aggressive glomerulonephritis has been summarised by Bolton (Bolton 2002). Lymphocytes can be seen in biopsies, and the IgG subclass distribution of autoantibodies IgG1 and IgG4 suggest a T cell dependent process. The strong association of disease with HLA Class II alleles, in vitro evidence of cell mediated immunity and the observation that effective treatments also act on cell mediated immunity provide additional support.

In animal models of injury caused by anti-GBM disease CD4+ and CD8+ T cells and intrinsic renal epithelium induce the migration of macrophages and neutrophils into the kidney (Tipping, 1997, Wu 2002, Timoshanko, 2001). IL-12 and IFN-γ mediate crescent formation. (Timoshanko, 2001, 2002). The initial inflammatory reaction in the glomerulus produces proteinuria and the attendant downstream consequences for tubular epithelium the development of interstitial nephritis and the subsequent appearance of fibrosis.
Studies of murine models show the importance of inducing a Th1 response if pathology is to develop and suggests T cells may be important not only in providing help for autoantibody production but in driving glomerular inflammation (Huang, 1997). In particular disease could only be passively transferred with anti-α3(IV)NC1 antibody from nephritogenic strains to syngeneic recipients but not to TCR deficient mice suggesting crescentic nephritis requires cell mediated immunity. (Tipping, 2006)

In the last few years, a model of experimental allergic glomerulonephritis (EAG) in the rat has allowed a greater understanding of the potential role for T cells in the immunopathogenesis in disease. The Wistar Kyoto (WKY) rat model of experimental allergic glomerulonephritis (EAG) originally described by Sado (Sado, 1998) demonstrates that EAG can be generated in animals without antibody production, that disease can be transferred by T cells alone, and that disease can be induced by peptide immunization where antibodies are undetectable on the GBM (Robertson, 2005). Interestingly, transfer of antibodies can transfer disease in the WKY rat perhaps through altered antigen presentation leading to generation of new aggressive T cells in the recipient animal – though antibody independent disease can also be shown in this model.

The T cell epitope responsible for EAG in WKY rats is in the amino-terminal third of α3(IV)NC1 where the human antibody epitope is to be found (Bolton, 2005), but it is distinct from the T cell epitopes identified in patients (Cairns, 2003). Remarkably, immunization with a minimal peptide can induce disease in which antibodies are formed as a secondary phenomenon, or without antibody generation at all. The length of the peptide influences the formation of anti-GBM antibody and the presence of certain amino acid residues at position 19 appears to be critical for disease induction (Bolton, 2005).

The potential for immunotherapy directed at T cells is highlighted by the success of tolerogenic intervention by oral α3(IV)NC1 administration in attenuating nephritis and the associated Th1 response in disease susceptible rats (Reynolds, 2005).
1.5.11 Factors modulating injury

Intercurrent infection has long been known to increase the intensity of the inflammatory response and end organ damage (Rees, 1978). These clinical observations are supported by experimental evidence (e.g. Karkar and Rees, 1997).

1.5.12 Genetic influences

Epidemiological studies have demonstrated that genetic factors are crucial determinants of susceptibility to autoimmune disease. Most autoimmune diseases are multigenic with multiple susceptibility genes working to create susceptibility. On their own these polymorphisms or mutations are usually subclinical. Only when present with other susceptibility genes do they contribute to autoimmunity, and even then, some kind of 'second hit' is required to initiate the disease process.

In common with other human autoimmune diseases, Goodpasture's has been associated with inheritance of specific class II HLA alleles (Phelps, 2000) HLA class II molecules consist of an alpha and beta chain with the greatest variability in the beta chain. They are encoded at the DR, DP and DQ loci on chromosome 6. DR specificities are determined exclusively by differences in the β chain encoded by the DRB1 locus. DR2 requires DRB1*15 or DRB1*16 at the B1 locus, the former of which accounts for >80% of the DR specificity in the northern European caucasoids that are most commonly affected by this disease.

Meta-analysis has confirmed that anti-GBM disease is strongly associated with DRB1*1501 and has clarified weaker associations with alleles DRB1*04 and DRB1*03. Indeed weaker negative associations are also seen with DRB1*07 and DRB1*01 (Phelps, 1999).

Susceptibility to Goodpasture disease is shown to be influenced by a combination of DRB1 alleles inherited on maternal and paternal chromosomes. Gene dosage does not affect susceptibility as it is similarly increased in homozygous or heterozygous individuals for DRB1*1501. The effect also includes neutralization of the susceptibility enhancing
effect of DRB1*1501 by co-inherited DRB1*07. Thus DRB1 and 7 confer dominant protection against anti-GBM disease. This strongly suggests an interaction most likely occurring between the class II molecules they encode. The obvious assumption is that there might be competition for the disease-causing peptide. However the explanation could also lie in the opposite interpretation, that a tolerance-inducing (or regulatory cell-stimulating) peptide is particularly well presented by the protective alleles and poorly presented by DR15.

1.6 Processing of the Goodpasture autoantigen

Work within my supervisors’ group has focused on Goodpasture’s disease as a model for autoimmunity for a number of reasons;

- the target of autoimmunity has been characterised and is known to reside in the 230aa COOH-terminal of the non-collagenous domain of the $\alpha3$ chain of type IV collagen ($\alpha3$(IV)NC1), a constituent of the GBM (Turner, 1992, Saus, 1988, Hellmark, 1999, Leinonen, 1999, Ryan, 1998).

- This can be produced in a number of recombinant forms, some with conformations similar or even identical to that of the native antigen, as detected by binding of conformation-sensitive antibodies.

- As with the majority of autoimmune diseases and as detailed above, it has a strong HLA association with a positive association with HLA DRB*1501 and a negative association with HLA DRB1*0701, which is dominant in the presence of DRB1*1501 (Phelps, 1999)

Extensive work in our laboratory has used biochemical approaches to identify the major peptides that are bound to HLA-DR15 on the surface of B cells that have processed recombinant Goodpasture antigen ($\alpha3$(IV)NC1). This avoids the confounding effects of tolerance in using conventional lymphocyte responsiveness assays to define peptides presented. This work has lead to a number of key observations:
• The major naturally processed peptides are three nested sets of peptides, each centred on core sequences of 9 amino acid long MHC-binding motifs. The peptides have only intermediate affinity for the presenting HLA-DR15 molecules, confirming that antigen processing pathways exert a strong influence the peptides presented (Phelps, 1996 & 1998).

• Importantly, data investigating what α3(IV)NC1-derived peptides are recognised by T cells from patients with active Goodpasture's disease found that autoreactive T cells in 6/6 patients with active disease recognised two α3(IV)NC1 derived peptides, dap79-91 (VNDVCNFASRNDYSYWLSTPA) and dap131-150 (IPPCPHGWISLWKGFSFIMF) (Cairns, 2003), which were not efficiently presented, as detected using biochemical studies despite a much higher affinity for DR15 than detected peptides (Phelps, 1998).

• Recent observations suggest that the disease-associated peptides might be impeded from tolerising self-reactive T cells because in normal antigen processing they are destroyed very early in an obligatory first 'unlocking' step in proteolysis by Cathepsin D/E (Zou, 2007). This dependence on a specific unlocking step has been described for other globular proteins including another potential autoantigen, myelin basic protein (Manoury, 2002), but with dependence on a different proteolytic enzyme, asparaginyl endopeptidase, AEP). The dependence of processing on a specific and consistent unlocking step could be immunologically important, as it should help antigen presenting cells to generate a predictable rather than random set of peptides in early antigen processing.

1.7 Purpose and design of study

These observations raised the question I set out to study. My question was:

Could low presentation of disease associated peptides (dap) in Goodpasture's disease such as dap79-91 and dap131-150, despite high affinity for HLA DR15, be a consequence of processing factors and of so, how can processing be perturbed so that disease associated peptides are presented at all?
When I joined the lab, another worker was attempting to answer the question by adopting a biochemical approach using techniques already described by Watts et al (Antoniou, 2000, Watts, 2005). This approach involved endolysosomal processing of recombinant \( \alpha_3(IV)\)NC1 whereby antigen was incubated with either freshly disrupted lysosomes from human EBV B cells or specific proteases. Degradation was then followed by SDS page gel and mass spectrometric analysis. This first approach affords the potential to detect all peptides generated as a result of processing but generates a complex array of peaks in the mass spectrogram which in turn influences specificity.

As described above, 6/6 patients with disease have T cells with specificity for two \( \alpha_3(IV)\)NC1 derived peptides peptides, dap79-91 and dap131-150, which are not efficiently presented despite high DR15 affinity. Biochemical studies have shown both these epitopes are exquisitely sensitive to endosomal proteases, but does this sensitivity have any bearing on presentation to T cells by living APC?

Since it would be necessary to quantify low level presentation, I needed an alternative approach to the biochemical studies which had already shown itself only able to detect most abundant peptides. Cellular approaches have been used to determine the amount of specific peptide complexes on APC, but have always involved the use human T cell clones and this technique is limited by the difficulties in production and maintenance of cell lines. I thought I might use T cell hybridomas, which have a number of advantages over T cell clones in the study of APC function. They are reliable, reproducible and convenient to use, they can also be grown to unlimited supply for large antigen presentation experiments. In addition they function well with fixed APC allowing for kinetics analysis and the use of inhibitors for antigen processing. Most importantly however they have the advantage of detecting specific peptide-MHC complexes on the surface of APC. An alternative approach is the use of transgenic mice with human MHC genes to produce "humanised" murine T cells and subsequent T cell hybridomas that are restricted by human HLA alleles and therefore respond to peptide presented on human APC.
I chose to adopt a cellular approach, which would allow me to follow the consequences of processing factors on specific α3(IV)NC1 peptides of interest. With this approach, presentation of α3(IV)NC1 peptides by human APC was determined using α3(IV)NC1 peptide-specific DR15 restricted murine T cell hybridomas in mice engineered to express HLA DR15 (donated by Professor Altmann, Altmann, 1995) to study the influence and consequences of processing.

On this background, I addressed my question by:

1. Developing murine DR15-restricted T cell hybridomas specific for the two major epitopes in α3(IV)NC1 that are recognised by patients with Goodpasture’s disease.

2. By pulsing recombinant α3(IV)NC1 into different DR15 expressing human antigen presenting cells, I proposed to examine whether propensity of lysosomal aspartate protease activity to destroy α3(IV)NC1 T cell epitopes in vitro had discernible consequences in live APC.

3. Using these hybridomas as peptide detectors, I aimed to probe the effects of various factors, in particular focussing on the influence of aspartate proteases (with the use of specific protease inhibitors), on the processing and presentation of peptides dap79-91 and dap131-150. This would in turn allow me to address the specific hypotheses that the disease-associated peptides in Goodpasture’s disease are particularly susceptible to proteolytic destruction in APC.

If I was successful in generating DR15-restricted and peptide specific T cell hybridomas, I would have a powerful tool with which to examine what other factors thought to alter the processing of α3(IV)NC1 so as to influence its presentation.

Specifically, I hoped to investigate the effect of dominant protection observed with the co-expression of HLA DRB1*0701 and HLA DRB1*1501 on the presentation of the same disease associated peptides shown to be destroyed in early processing.
The first and most straightforward approach would utilise EBV transformed B cells from patients known to express either DR15,7 or DR15,15 and compare ability to present either dap79-91 and dap131-150 to the DR15-restricted T cell hybridomas already generated. Differences in presentation could also potentially be compared with other DR15 heterozygotes demonstrated to influence disease susceptibility.
Chapter 2

Materials and Methods

2.1 Molecular biology

2.1.1 Materials

a) General chemicals

All materials, unless otherwise mentioned, were obtained from either Sigma-Aldrich Company Limited, UK; Fisher Scientific, UK; BDH Laboratory Supplies (VWR International); or Scientific Laboratory Supplies Ltd, UK.

b) Molecular biology stock solutions

The following stock solutions were formed and stored at -20°C

- Ampicillin 50mg/ml in dH2O
- Carbenicillin (CN Bioscience) 50mg/ml in dH2O
- Chloramphenicol (Transgenomic Limited) 34mg/ml in 100% ethanol
- IPTG (Transgenomic Limited) 1M in dH2O

c) Plastics / Glassware

All materials, unless otherwise mentioned, were obtained from either Nalge Nunc International (Denmark), Sarstedt Inc. (USA), or Techne (Cambridge) Ltd (UK).
d) Cell Lines

All cell lines, unless otherwise mentioned, were obtained from either Invitrogen Life Technologies (Holland).

e) Media

All media, unless otherwise mentioned, were obtained from either Invitrogen Life Technologies (Holland) or PAA Laboratories (UK).

2.1.2 Solutions

a) Agarose mini gels

All solutions were produced following the methods laid out in Sambrook et al (Sambrook, 1989).

b) SDS-PAGE gels

All solutions were produced following the methods laid out in Sambrook et al (Sambrook, 1989).

c) Triton wash solution

0.5% Triton X-100
1 mM EDTA

The solution was made up to 1 litre and stored at room temperature

d) Inclusion body resuspension solution

6M Urea
50mM Hepes pH8
1M NaCl

The solution was made up in dH₂O

e) Nickel chelation chromatography

All solutions were passed through a 0.45μm filter (PAL Gelman Corporation) prior to use

f) Column stripping solution

100mm EDTA

1M NaCl

The solution was made up in dH₂O, adjusted to pH 8.0 using NaOH to dissolve EDTA

g) Column metal loading solution

100mM NiCl₂

10mM sodium acetate

The solution was made up in dH₂O, adjusted to pH 4.5 using 1M HCl and stored at room temperature.

h) Column wash solution

6M GnHcl

50mM Tris

20mM imidazole

Solution made up to 250mls in distilled H₂O at pH 7.5
i) Column Elution solution

6M GnHcl
50mM Tris
0.1M EDTA

Solution made up to 250mls in distilled H₂O adjusted to pH 7.5

j) 12.5% acrylamide/Bis gel

4.2mls Acrylamide/Bis 30%
3.2mls dH₂O
2.5mls 1.5M pH 8.7
100μl 10% SDS
40μl 10% Ammonium persulphate
7μl TEMED

k) Running buffer x5

93.75g Glycine
15.1g Tris Base
50mls 10% SDS

Solution made up to 1litre with dH₂O and adjusted to pH 8.3 using 1M HCl

l) Sample Buffer

10mls SDS (10%)
830μl Tris pH6.8 (1.5M)

0.3ml Bromophenol blue (0.2%)

10g Sucrose

Solution diluted to 50mls dH₂O

m) Agar plates

SOB/LB agar plates were prepared by adding 1.5% agar (7.5g) to 500mls dH₂O. The bacretial growth medium was autoclaved in a microwave before pouring onto 90mm plates (Bibby Sterilin). If adding antibiotics, the solution was left to cool to approximately 55°C before adding the antibiotic and then poured as before. The solution was then left to cool and set before drying overnight at 37°C.

2.1.3 Bacterial protein production methods

Production of recombinant α3(IV)NC1

a) Transforming BL21(DE3) Cells

BL21(DE3) (CN Bioscience) cells were transformed using the constructs for α3PHisBirA ligated into pET25b (CN Bioscience) as previously described (Phelps, 1996). These transformed cells were stored as glycerol stock and used for future protein production.

Transformed bacteria were plated onto LB agar plates containing 100μg/ml carbenicillin by streaking the plate with the still frozen glycerol stock of pET25ba3PHisBirA using a sterile applicator (Nalge Nunc Ltd.) The plate was incubated overnight at 37°C.

b) Small scale production and analysis

At least 5 isolated colonies were then selected for inoculation into 5-10mls LB broth containing carbenicillin 200μg/ml. Inoculations were grown at 37°C in an orbital shaker at 250rpm until starter cultures had an OD of approximately 0.6 then stored overnight at 4°C.
1 ml of each starter culture was then inoculated into 15mls of LB broth containing 400µg/ml carbenicillin and grown at 37°C in an orbital shaker at 250rpm for approximately 3-4 hours.

The use of high concentrations of carbenicillin helps prevent the overgrowth of cells that have lost the transformed plasmid which may otherwise occur due to bacterial β-lactamase production.

The optical density at 600nm (OD₆₀₀) was measured. Cultures were allowed to grow to an OD of approximately 0.5-0.6. At this stage 1ml from each culture was then induced with 0.8mM IPTG for 4 hours at 37°C in an orbital shaker at 250rpm before storing overnight at 4°C. The following day, samples were spun alongside 5 x 1ml of non-induced cultures at 10,000rpm for 1 minute and supernatant removed. 100µl of ice cold PBS and 100µl 2x SB was then added mixed and samples sonicated for approximately 15 seconds on ice at duty cycle 30% (Ultrasonic Processor – Jenkons Scientific Ltd). At this stage a sample from each suspension was analysed using an SDS PAGE gel and Coomassie Brilliant Blue stain as described in section 2AVa. α3PHisBirA was identified by having the molecular weight of 31kD and could be present as either a dimer or monomer.

Colonies were selected for large scale protein production according to SDS PAGE analysis where the colony with the greatest protein production was used.

c) Large scale production of recombinant protein

Once the colony with the highest protein yield had been selected from the small scale preparation, a larger scale preparation was made. 500mls LB with 300ug/ml carbenicillin was inoculated with 10mls of starter culture in a 2 litre baffled flask. The suspensions were incubated at 37°C in an orbital shaker as before at 250rpm until the OD₆₀₀ had reached 0.5-0.6. Cell suspensions were induced with 0.8mM IPTG for 3 hours and transferred to oakridge tubes. Suspensions were pelleted by spinning for 25 minutes at 5000rpm. Pellets were stored at -20°C until required.
d) Bug busting and inclusion body preparation

Frozen pellets were defrosted and resuspended in 10mls ice cold PBS by sonicating on ice at 30% duty cycle for 80 seconds. Following this, 0.2mg/ml Hen egg lysozyme (HEL) was added and samples left on ice for 30 minutes before adding 0.1% deoxycholate, 2mM magnesium chloride (MgCl₂) and DNase I to a final concentration of 2μg/ml. The inclusion body preparation was left at room temperature for 15 minutes. Samples were then sonicated on ice until liquid (60 seconds) and spun at 10000rpm for 7 minutes at 4°C. Supernatant was removed and spun again to collect any further pellet. Pellets were then washed in 10mls PBS and 0.1% Triton and sonicated for another 80 seconds. Samples were pelleted as before resuspended in 10mls PBS before sonicating and spinning as before. Pellets were then resuspended in 2M urea Tris 50mM pH 7.5. This solution was sonicated and spun and pellet resuspended in 6M GnHCl Tris 50mM pH 7.5. The pellet was sonicated for approximately 160 seconds and left rolling at 4°C.

e) His Tag purification of recombinant protein, α3HBirA

20ml columns were packed with approximately 4mls IAA beads. The column was initially washed twice in 50mM Tris pH7.5 before running 5mls NiCl₂ through the column. This was repeated with 10 mls NiCl₂ until the beads had turned blue (approximately three times in total). Any unbound nickel was washed off by washing the column twice with 50mM Tris pH 7.5 and a further three times with 6M GnHCl, 50mM Tris pH7.5, 20mM imidazole. Sample solution was then added directly to the column and the beads agitated gently. The column was clamped at the end and the solution allowed to settle for a few minutes before being allowed to run through. This first collection was labelled the post load. Three washes were carried out with wash solution (GnHCl,Tris,imidazole) and bound α3HBirA then eluted by sequentially running 2mls GnHCL,Tris,imidazole,EDTA through the column three times, agitating the beads as above before collecting the eluate. 10mls 100% EtOH was added to each 2ml sample before storing at -20°C overnight. Samples were then washed twice in 70% EtOH before resuspending the pellets in 6M urea, 50mM Tris pH 7.5.
Representative samples (50-200μl) from pre and post load as well as all samples from washes and eluates were precipitated in 4x EtOH, left overnight and washed on 70% EtOH before being resuspended in 40μl SB (7-8M urea, 10mM DTT, 50mM Tris) and analysed using SDS PAGE gel and Coomassie Brilliant blue stain.

f) Quantification of recombinant α3(IV)NC1

100μl of stock solution was diluted 1 in 5 in 6M urea/50mM Tris pH 7.5. Absorbance was measured at 260nm and 280nm. Concentration was calculated using the equation

\[ \text{Concentration} = \frac{(1.55 \times A_{280}) - (0.76 \times A_{260}) \times 5}{5} \]


g) Testing of recombinant α3(IV)NC1

Prior to use in any experiments, purified recombinant α3(IV)NC1 was used to pulse antigen presenting cells at 10, 50, 100 & 200 μg/ml as described in section 2.3.4.f

2.1.4 Protein identification methods

a) SDS-PAGE

All solutions were produced following the methods laid out in Sambrook et al (Sambrook, 1989).

Once set, the gels were placed in an electrophoresis tank which was subsequently filled with running buffer. Samples were mixed 50:50 with SDS sample buffer and the protein allowed to run at 120V,20mA (per gel) for approximately 1.5 hours (consort E834). 10ul of perfect protein™ marker (CN Bioscience) was loaded in the first well as a means of identifying protein by molecular weight.

b) Coomassie Brilliant Blue

To identify proteins, gels were stained overnight using GelCode® Blue Stain Reagent (Perbio Science) and washed thereafter in distilled water.
c) Drying SDS-Page gels

Gels were washed in dH2O as above and carefully sandwiched between two sheets of gel drying film that had been soaked in a solution of 40% methanol, 10% glycerol and 7.5% acetic acid. The films were stretched and clamped between two drying frames and dried at room temperature.

2.2 Cellular Biology

2.2.1 Materials

a) Synthetic peptides

All synthetic peptides were 20mers generated using standard F-moc chemistry by G. Bloomberg (University of Bristol U.K.). The composition and purity of these peptides had been confirmed by mass spectrometry.

DerP1 was used as a negative control in peptide recognition assays. It is a major mite faecal antigen belonging to the peptidase C1 family with a molecular weight of approximately 36kD.

All peptides were dissolved in DMSO (Sigma) at 20mg/ml and further diluted to 0.5mg/ml in sterile PBS. All peptides were stored at -20°C.
b) Bacterial protein production of recombinant α3(IV)NC1

See section 2.1.3.1

c) Mice

These mice were originally a kind gift from Professor D.M. Altmann, Imperial College, London as described previously, (Altmann, 1993)

d) Enzymes and inhibitors

Cathepsin D

The recombinant antigen was digested with Cathepsin D purified from human liver and was obtained from Athen Research & Technology. Cathepsin D was in phosphate buffer and reconstituted in water i.e. 25ug lyophilized from 27.3µl NaPO4_2, pH 6.5 reconstituted in H_2O).
Pepstatin A (Sigma)

Pepstatin A is a highly selective inhibitor of aspartate proteases (see Chapter 6). Preparation of this inhibitor was initially problematic due to problems with solubility which is in turn related to preparation purity. It is only sparingly soluble in water but can be dissolved in 10% v/v acetic acid in methanol (9:1 methanol: acetic acid). The later is not desirable due to unknown and potentially toxic effects on cells. Attempts to dissolve the inhibitor in ethanol were also abandoned. Heat is often required for complete solution and temperatures of up to 60°C can be used without any decomposition of the peptide but in my hands the peptide did not dissolve.

Initial samples of pepstatin A were obtained from Sigma but subsequent samples were obtained from Calbiochem which was ≥ 95% pure by HPLC and reported to be soluble at 5mg/ml in DMSO. This allowed easier and more reliable means of preparation. Stock was therefore made in DMSO at 5mg/ml. Samples were further diluted 1 in 10 in R10 to 0.5 mg/ml and aliquoted and stored at -20°C

Chloroquine

This is a member of the quinolone family with a number of applications. It was used in processing experiments to assess the influence of increasing pH. Stock solution was dissolved in water at a concentration of 10mM and stored at -20°C prior to use.

2.2.2 Solutions

a) media

All media and solutions unless otherwise stated were from Gibco or Sigma-aldrich. Solutions were prepared under sterile conditions in a laminar flow Hood and stored at 4°C.

R10 (murine splenocytes, T cell clones, T cell hybridomas, THP-1 cells, BW5147 cells)
RPMI

10% heat inactivated fetal bovine or fetal calf serum
L-glutamine 2mM
Penicillin 10,000U/ml/streptomycin 10mg/ml
5x10^{-5} M β2-mercaptoethanol

**B cell media (EBV transformed B cells)**

RPMI

10% heat inactivated fetal bovine or fetal calf serum
L-glutamine 2mM
Penicillin 100U/streptomycin 0.1mg

**HAT**

RPMI

10% heat inactivated fetal bovine or fetal calf serum
L-glutamine 2mM
Penicillin 100U/streptomycin 0.1mg

HAT (50x) reconstituted in 10ml sterile R10 and added to 500ml RPMI. Working solution
100μM hypoxanthine, 0.4μM aminopterin, 16μM thymidine

**HT**
RPMI

10% heat inactivated fetal bovine or fetal calf serum

L-glutamine 2mM

Penicillin 100U/streptomycin 0.1mg

HT (50x) Reconstituted as with HAT

b) Solutions for tissue culture

RBC lysis solution

Solution A HN₄Cl 8.26g/100ml

Solution B KHCO₃ 1g/100ml

Solution C Na₄EDTA 0.37g/100ml

Working solution

Solution A 10ml

Solution B 10ml

Solution C 1ml

dH₂O 79ml

Freeze Brew

Heat inactivated Fetal calf serum (9 mls)

Sterile DMSO (1 ml)

Stored at -20°C
c) Solutions for FACS

**FACS buffer**

PBS

1% Heat inactivated FCS

0.2% sodium azide

**FACS fix**

PBS

1% FCS

0.2% sodium azide

Paraformaldehyde

d) **Solutions and reagents for ELISA**

All solutions and reagents unless otherwise stated were from the mouse DuoSet® IL-2 development kit (R&D)

**PBS**

137mM NaCl

2.7mM KCl

8.1mM Na₂HPO₄

1.5mM KH₂PO₄

pH 7.2-7.4
Solution was filtered through 0.2\(\mu\)m filter

Alternatively PBS solution was purchased directly from GIBCO

**Wash Buffer**

0.05% Tween\(^\circ\) 20

PBS

pH 7.2-7.4

**Block Buffer**

1% BSA

0.05% NaN\(_3\)

Solution was made up to 500mls PBS

**Reagent Diluent**

0.1% BSA

0.05% Tween\(^\circ\) 20

The above solution were made up to 500mls in Tris-buffered Saline and pH adjusted to 7.2-7.4. The solution was then filtered through a 0.2\(\mu\)m filter.

**Tris-buffered saline**

20mM Trizma base

150mM NaCl

Adjusted to pH 7.2-7.4 and filtered through a 0.2\(\mu\)m filter.
Detection system

Streptavidin-HRP (R&D systems)

1.0ml of streptavidin conjugated to horse-radish peroxidase was stored at -4°C. Samples were diluted to a working concentration in Reagent Diluent.

Substrate Solution

1:1 mixture of Color Reagent A (H₂O₂) and Color Reagent B (Tetramethylbenzidine,TMB), (R&D systems).

Stop solution

2N H₂SO₄

Capture antibody

180µg/ml of rat-anti mouse IL-2 was reconstituted in 1.0ml PBS. Samples were aliquoted and stored at -20°C. Samples were diluted to a working concentration of 1 µg/ml in PBS, without carrier protein.

Detection antibody

72µg/ml of biotinylated goat anti-mouse IL-2 was generated when reconstituted in 1.0ml Reagent Diluent. Samples were aliquoted and stored at -20°C after reconstitution. These samples were diluted to a working concentration of 400ng/ml in Reagent Diluent.

Standards

65ng/ml of recombinant mouse IL-2 was reconstituted in 0.5ml of Reagent Diluent. Standards were allowed to sit for a minimum of 15 minutes with gentle agitation prior to making the dilutions. The reconstituted standard was aliquoted and stored at -20°C. A
seven point standard curve using 2-fold serial dilutions in Reagent Diluent was made with a high standard of 1000pg/ml.

2.3 Tissue culture materials and methods

2.3.1 General methods

a) Thawing cell lines

Cells were thawed rapidly at 37°C and added drop wise to 9mls pre-warmed R10 in a 15ml falcon tube. This was spun 1200rpm for 5 minutes, supernatant removed and the pellet resuspended in 1ml R10. This was transferred to a medium (6-10ml) or small (3-5ml) flask and incubated at 37°C 5%CO₂. Cells were examined using light microscopy initially to assess general health and viability was formally assessed by staining 1:1 with trypan blue and counting using a Neubauer haemocytometer.

b) Freezing cell lines

Approximately 1x10⁶ cells were pelleted and re-suspended in 0.5ml R10 and added to 0.5ml freeze brew before transferring to NUNC cryotubes and freezing at -80°C for 24hours. Cells were thereafter transferred to liquid nitrogen.

c) Maintenance of long term cell lines

Passage of cells varied depending on cell lines as described below. In general lines underwent no more than 20 passages.

2.3.2 Methods specific to cell lines

a) EBV transformed B cells

Cells were grown in suspension (flasks) in B cell media and split ~2-3 times per week to a cell count of 3 x10⁶/ml
b) THP-1 cells

THP-1 cells have monocyte characteristics and were derived from a 1 year old with acute monocytic leukaemia (Auwerx, 1991) and express HLA A2, A9; B5; DRw1, DRw2 (DR1 & DR15). They can be differentiated into a macrophage phenotype using a number of stimulants such as PMA, DMSO, IFNγ and vit D. In their monocyte phenotype, THP-1 cells have low constitutive expression of class II which can be induced by incubating with IFN-γ. After a number of optimisation experiments, THP-1 cells were routinely incubated with 100U IFNγ and 10ng/ml PMA for 24 hours before use in processing experiments.

Non-stimulated cells were grown in R10 and split 2-3 times a week to a cell count of 3x10⁵/ml. Prior to use in antigen presentation experiments cells were stimulated with PMA 10ng/ml and IFNγ 100U/ml in 10mls R10 for 24 hours. Following stimulation, adherent cells were detached by removing media from flask, washing cells gently with 10mls sterile pre-warmed PBS and adding 1ml trypsin/EDTA x1 and leaving for ~5 minutes until cells were seen to detach. 10mls R10 was added and the flask was tapped firmly to encourage further detachment. Cells were then spun at 12000rpm for 6 minutes and the pellet resuspended in R10.

c) BW5147 cells

BW5147 cells are an immortal T cell fusion partner derived from a myeloma (TcRαβ) cell line (a gift from D. Anderton, King's buildings, Edinburgh). They are grown in 24 well plates or small flask in R10 and are split 2-3 times a week. When using for fusion cells were thawed and set up approximately one week before planned fusion and split at different concentrations 48 hours pre-fusion to ensure cells were healthy and optimized for fusion.

d) T cell hybridomas

Cells were grown in suspension (flask) in R10 and split 1 in 10 to 1 in 20 three times weekly.
2.3.3 Establishment and maintenance of T cell lines

a) Priming mice

Crude peptides were dissolved in DMSO then PBS as described above to a final concentration of 0.5mg/ml. 50μg of peptide/mouse in 100μl PBS was added to an equal volume of complete freund’s adjuvant (CFA) ensuring complete resuspension of CFA first. The mixture was emulsified by repeatedly exchanging the solution between 2 glass 2.5ml syringes attached by means of a 3 way tap. The resultant emulsion was then carefully transferred into a 1ml syringe ensuring no air bubbles were present.

50μg peptide was used to prime each mouse. 100μl of emulsion was injected subcutaneously into the left and right inguinal region for subsequent retrieval of splenocytes. Alternatively, 50μl was injected intradermally into the base of the tail and 50 μl subcutaneously into the scruff of the neck and left and right inguinal region for subsequent retrieval of lymph nodes and splenocytes.

b) Setting up T cell lines

Spleens and lymph nodes were retrieved after 10-14 days or 7 days respectively

Spleen was injected with 5mls red cell lysis solution and passed through a filter using a rubber plunger into 50mls R10. Cells were spun 1200rpm for 5 minutes and counted. 2x10⁶ cells were aliquoted into 24 well plates and 20μg/ml peptide added into an end volume of 2 mls. Lymph nodes were retrieved from the inguinal, para-aortic and axillary regions). Nodes were emulsified using a rubber plunger through a sieve into 10mls R10 and spun and aliquoted as with spleocytes.

Cell lines from individual mice were cultures separately.

c) In vitro recall response

2x10⁵ splenocytes were aliquoted into 96 well flat bottomed plate and 20μg/ml (7.5μM) relevant or control peptide or 300μg/ml (9μM) r-α3(IV)NC1 added into an end volume of
200μl R10. Samples were set up in triplicate. Plates were incubated at 37°C 5% CO₂ for a total of 72 hours. Supernatant was removed at 24 and 72 hours (stored at -20°C for later cytokine analysis using ELISA as described below). Cells were subsequently pulsed with 0.5μCi ³H thymidine in 10μl R10 media. Following pulsation cells were incubated for a further 18 hours and proliferation measured using a liquid scintillation β-counter.

**d) Maintenance of T cell lines**

Only cell lines derived from splenocytes and lymph nodes in mice shown to respond to peptide were selected to continue T cell lines for potential fusion (Figure 3.9 Chapter 3). After 3 days re-stimulation with peptide as described above, cells were retrieved and any dead feeder cells removed by spinning the cells through histopaque at 2000rpm for 25 minutes. Resultant interface was removed and washed in 10mls R10, cells counted and aliquoted at 2-4x10⁵ in 2mls media containing 20U/ml rIL-2.

After 4 days expansion with 20U IL-2, 1ml of media was removed and replaced with 1ml R10 + rIL-2 to give a final concentration of 1U/ml. Cells were then rested for 3 days before replacing with a further 1ml of fresh R10 + rIL-2 1U/ml and incubated for a further 4 days (rested for a total of 7 days).

Cell lines could then be rested for a further 7 days or passed through another cycle of re-stimulation, expansion and resting phases.

In further rounds of re-stimulation and in subsequent *in vitro* recall responses¹, fresh splenocytes from DR15+ transgenic mice pre-treated with mitomycin c were used as feeder cells*.  

¹Cell lines were checked for specificity prior to fusion and after each round of *in-vitro* re-stimulation to ensure antigen specificity of the lines was maintained.
2.3.4 Generation and selection of T cell hybridomas

a) Generation of T cell blasts

Only T cells from primed mice shown to have recognised peptide using in vitro re-call assays were used to fuse with BW5147 cells.

Activated T cells were required for optimal fusion and were usually obtained at day 4.

b) Fusion

2x10⁶ T cell blasts and healthy BW5147 cells were washed separately 3 times in 10ml pre-warmed HBSS in order to remove all the serum from the cells which may inhibit the fusion process. Before the last wash cells were counted and mixed at a ratio of 1:1 using 2-3x10⁶ of each. After the last wash all the HBSS was removed from the pellet using suction and the pellet resuspended gently but thoroughly.

0.7ml PEG-1500 (pre-warmed to 37°C) was added gently to the pellet with constant gentle agitation over 90 seconds to ensure all the pellet was disrupted. 5mls HBSS (pre-warmed to 37°C) was added over 2 minutes and 15ml warmed HBSS then added over 3 minutes again with constant gentle agitation ensuring complete mixing.

The cells were then spun at 200g for 5 minutes and the pellet very gently resuspended in an end volume of 30ml pre-warmed R10 over 3 minutes. Cells were then plated out in 100μl volumes in 2x 96-well flat-bottomed plates. The remaining 10mls was diluted 1:3 and 1:9 respectively in R10.

A total therefore of 6 plates duplicated in top dilution, 1:3 and 1:9 dilutions were incubated for 24 hours at 37°C 5% CO₂ before adding 100μl R10 with 2x HAT.

c) Isolation and cloning of Hybridomas

Hybridomas were generally found from day 5 or 6 after fusion and were identified by examining the 96-well plate from underneath and confirming with an inverted microscope.
Healthy clones were easily distinguished growing usually to one side of the well and positive wells were marked. Individual colonies were selected for picking when seen to be growing to about one third of the surface area of the well-if more than this the chance of the colonies not being clonal increased.

As fusion frequency is in the order of 1 in $10^4$ the top dilution plate was often unusable. The growing clones were transferred in 200μl to a 24 well plate and 800μl R10 1x HAT added. Most clones adapted well to this. Transferring 250-500μl of well contents was then used to further seed new wells.

Once the cells were growing well in 1x HAT seeding was then carried out into a well containing only HT to wean them off aminopterin. Wells were then seeded into R10 only and when growing well, the entire well contents transferred into a 25cm² flask containing 5ml R10.

d) Screening and selection

Expression of CD4 and TcR

All potential T cell hybridomas were initially screened at the 24-well stage for the expression of TcR and CD4 by FACS.

Briefly approximately $1x10^6$ cells were taken from the contents of 1 well and resuspended in 200μl R10/FACS buffer. 1μl PE-labelled anti-mouse TcR β-chain and 1μl APC-labelled anti-mouse CD4 (giving 1 in 200 dilution, Pharmingen). Cells were left at 4°C in dark for 30 minutes before washing x 2 in FACS buffer and resuspending in either 100μl FACS buffer (if analysing within 24 hours) or FACS fix.

Only cell populations demonstrated to be clonal and expressing both surface antigens were selected for further screening.

All subsequently selected T cell clones frozen at -80°C, were assessed for expression of CD4 and TCR routinely after thaw as loss of expression of either surface marker can occur at any point in the course of cell culture and storage.
**Peptide recognition**

Resultant CD4+ TCR+ clonal populations after fusion were screened for peptide specificity.

Initial screening of candidate T cell hybridomas was set up by pulsing 2x10^5 mitomycin C treated splenocytes from DR15 transgenic mice and control IA-E6/DR15 mice with 1, 5, and 20μg/ml test or control peptide. After incubation with the candidate hybridoma for 24 hours, the supernatant was removed and frozen and later analysed for IL-2 production determined by ELISA (Duoset-details) as described below. Control wells containing either splenocytes or hybridoma in R10 or in the presence of peptide were also set up to ensure any IL-2 detected was produced solely by hybridomas stimulated by peptide-MHC complexes presented by APC.

**DR15 restriction**

As the initial mice used to generate T cell clones expressed both human and mouse class II it was crucial to ensure any specific recognition of test peptide by hybridoma was restricted to DR15. A number of different human DR15 expressing antigen presenting cells (APC) were co-cultured potential T cell hybridomas and relevant or control peptide. The method used was identical to that with DR15 splenocytes although the human cell line were not pre-treated with mitomycin c prior to co-culture. Results are discussed in more detail in chapter 3.

**e) Peptide binding assays**

In these experiments antigen presenting cells included various murine splenocytes, EBV transformed B cells and IFNγ/PMA stimulated THP-1 cells.

APC (0.5 - 2 x10^5) were pulsed with 1,5 or 20μg/ml test or control peptide for 4-6 hours before incubated with 0.25 - 1x10^5 hybridoma in an end volume of 200μl for 24 hours at 37°C 5%CO₂. The ratio of APC to hybridoma was regardless of actual numbers, always in the ratio of 2.1 100μl of supernatant was then analysed in duplicate or triplicate for the
presence of IL-2 or IFNγ using an enzyme-linked immunosorbent assay kit (R&D/BD biosciences) as described below. Analysis was performed using Stingray software and results were presented as the mean concentration of duplicate wells.

f) Antigen processing assays

APC were pulsed (0.5 -2 x10⁹) with 50, 100, 200 or 300 μg/ml recombinant α3(IV)NC1 (mention molarities here). α3(IV)NC1 was dissolved in 6M urea 50mM Tris pH7.5 as described. Stock solutions ranged from 5-16mg/ml depending on the production and purification of protein antigen. Stock was dissolved directly into 100μl R10 media (allowing for a further 1 in 2 dilution) before adding to a further 100μl R10 media containing APC. Wells were set up in duplicate or triplicate. THP-1 cells that had been pre-treated with PMA and IFNγ were aliquoted at room temperature and antigen added directly. EBV transformed B cells were aliquoted and left on ice for 30 minutes before antigen was added. (discussed further in chapter 3). Murine splenocytes were pre-treated with mitomycin c before pulsing with antigen. In preliminary experiments APC were left for 4-6 hours before adding hybridomas directly to the wells in an end volume of 10μl. (the ratio of APC: hybridoma was 2:1 as in the peptide binding assays). The cells were sealed and co-cultured for 24 hours at 37°C in 5% CO₂ before collecting and analysing the supernatant as above.

Ideally supernatant would have been removed from individual wells and each test well in turn set up in duplicate or triplicate in ELISA. Due to evaporation during the 24 hour incubation, the media was usually reduced by 30-50μl per well making it impossible to set each test well even in duplicate. Therefore supernatant from duplicate wells was pooled and samples set out in duplicate for ELISA as described.

f) Cathepsin D inhibition

THP-1 cells or EBV transformed B cells were aliquoted as before in 100μl R10. Prior to pulsing with peptide or antigen, Pepstatin A was added to the culture at concentrations of 0.1-100μM. Serial dilutions of pepstatin A were made fresh from stock in RPMI allowing for a further 1 in 2 dilution on adding to APC. If B cells were used, pepstatin A was added
to the cells and left on ice for 30 minutes. THP-1 cells were left at room temperature. All cell lines were pretreated for 30 minutes before adding peptide or alpha 3 directly into wells. As 96 well plates can only hold just over 200ul and any further significant volume would alter dilutions, peptide and alpha 3 were added directly from stock. Cells were incubated at 37°C 5% CO₂ for varying timepoints. There was considerable variation in method of fixation or washing of APC before adding hybridoma. This is discussed in detail in chapter 6. Essentially however cells were fixed with FACS fix or washed at different timepoints. Briefly at each timepoint the plate was spun at 1200rpm for 4 minutes, supernatant removed and 200uL FACS Fix added (pre-warmed to 37°C). The plates were left at room temperature for 30 minutes before washing the cells twice in RPMI. After three washes 200μl RPMI was added containing hybridoma ha3p132.3. Plates were incubated for another 24 hours at 37°C 5% CO₂ before analyzing supernatant for IL-2 production as before.

g) Chloroquine

Experiments were carried as described with pepstatin A. Pre-incubation time with respective inhibitors varied however. APC were incubated with chloroquine for 15 minutes before washing or fixing.

2.4 FACS methods and analysis

a) Expression of HLA DR DP DQ on THP-1 cells

The expression of human class II molecules on THP-1 cells after stimulation was determined using a FITC-conjugated mouse anti-human HLA DP DQ DR mAb. (Serotec). The corresponding Isotype control was a FITC-conjugated mouse IgG2a.

b) Expression of CD4/TCR on T cell hybridomas

T cell hybridoma studies used a mouse APC-conjugated anti-mouse CD4 (mAb) and mouse PE-conjugated anti-mouse TcRαβ. Isotype controls were APC-conjugated mouse IgG1κ and mouse IgMκ respectively. (PharMingen, San Diego, USA).
All working dilutions of antibodies were 1 in 100.

**Method**

Cells were counted and aliquoted at $1 \times 10^5$ - $1 \times 10^6$ in FACS buffer. Either test or isotype antibody was added in an end volume of 100ul in a non-sterile 96 well plate. For potential T cell hybridomas cells were stained as follows:

- **Sample 1** APC conjugated IgG1κ/ PE conjugated IgMκ
- **Sample 2** APC conjugated CD4/ PE conjugated IgMκ
- **Sample 3** PE-conjugated TcR/ APC conjugated IgG1κ
- **Sample 4** APC conjugated CD4/ PE conjugated TcR.

The plate was left for 30 minutes at 4°C in the dark. Cells were then washed twice in PBS supplemented with 1% FBS and 0.2% sodium azide (FACS Buffer). After a final wash cells were resuspended in 100μl FACS fix and 150μl PBS and analysed either immediately or stored in the dark at 4°C and analysed within 48 hours.

**Analysis**

Flow cytometry analysis was performed using FACsCalibur flow cytometer and CellQuest software. Unstained populations were always run first to gate on the healthy cell population first before analysis.

**2.5 Phagocytosis by activated THP-1 cells**

To demonstrate THP-1 cells ability to phagocytose following IFNγ treatment, 10 μl fluorescent paramagnetic particles (Polysciences Inc., Warrington, PA) were washed three times in media before resuspending in an end volume of 100μl media. 5μl of this suspension was added to $1 \times 10^6$ THP-1 cells pre-treated with PMA and/or IFNγ as described above in an end volume of 3 mls media. Cells were incubated for a further 24 hours with beads and adherent cells detached using Trypsin/EDTA and washed x 3 in
fresh media before examining for uptake of fluorescent beads using UV light microscopy and OpenLab software.

2.6 ELISA

Method

Corning Costar 9018 high protein-binding capacity polystyrene 96-well ELISA plates were prepared by aliquoting 100µl of capture antibody (diluted to a working concentration of 1µg/ml in PBS) to a 96 well ELISA plate. The plate was sealed and left overnight at room temperature (-4°C). Each well was then aspirated and washed with wash buffer three times by forcefully filling each well using a squirt bottle. Complete removal of liquid was ensured at each stage by inverting the plate and blotting it against clean paper toweling.

The plates were blocked by adding 300µl of blocking buffer to each well and incubating at room temperature for 1 hour. The aspiration and washing steps were repeated as before.

Serial dilutions of standards were carried out in polypropylene tubes. Samples were vortexed between each dilution to ensure the samples were mixed properly. 100µl of sample or standards was added in diluent to each well. The plate was then sealed with an adhesive strip and left at room temperature for 2 hours. The aspiration and washing step was repeated as before. 100µl of Streptavidin-HRP was diluted in reagent diluent and added to each well. The plate was then sealed and incubated at room temperature for 20 minutes avoiding direct sunlight. After further aspiration and washing, 100µl of substrate solution was added to each well (50µl each of color reagent A and B) and incubated for 20-30 minutes at room temperature avoiding direct light. 50µl of stop solution was added to each well and tapped gently to ensure thorough mixing. The optical density (O.D) of each well was determined within 30 minutes using a plate reader set to test filter at wavelength 450nm and reference filter 560nm. To correct for optical imperfections in the plates and allowing more accurate readings.

Analysis was automated using stingray software. This allowed various methods of curve fitting to be assessed including linear, semi-log, log/log and 4 parameter logistic, to
determine which curve best fitted the data. The results from experiments in this work were determined using a semi-log sigmoid fitting curve with tails. Essentially the standard curve was generated using computer software by plotting the mean absorbance (OD) for each standard on the y-axis against the concentration (in pg) on the x-axis. The best fit curve was generated through the points on the graph. Results were expressed as concentration in pg/ml. If samples were diluted, the concentration read from the standard curve was multiplied by the dilution factor. A standard curve was generated for each set of samples assayed.

All standards and samples were plated out in duplicate and results expressed as the mean of two wells.

2.7 Digestion of α3(IV)NC1

Digestion of α3(IV)NC1 used a zip tip method. This is a method by which the tip contains hydrophobic beads which are dry. Organic solvents are better at wetting hydrophobic beads therefore the tip is washed in 70% then 35% acetonitrile (+dH2O) then H2O. This allows uptake of peptide and enzyme onto the tip and resultant peptide can be removed by exposing to organic solvent.

Using the zip tip, 10ul 70% acetonitrile was taken and removed then the process was repeated with 35% acetonitrile and dH2O. Finally, after expelling the dH2O, 10ul peptide/buffer mix (2ul peptide in 30ul citrate buffer (10x)) was taken up and mixed into the tip. The tip was washed in 10uL dH2Ox2 (if sample is in urea this is repeated again). To remove the peptides, 70% ACN+matrix mix was taken up and pipetted several times on the matrix plate to ensure all peptide was expelled then allowed to dry on the plate (TE matrix was diluted 1:1 in 70% CAN as if use saturated TE matrix, crystals may form resulting in decreased sensitivity).

1ul of Cathepsin D was added to the remainder of the peptide/CB sample and left at 37°C for 4 minutes before repeating the above process to elute resultant peptides. Remaining sample was left in the hot plate for another 25 minutes allowing 2 Cathepsin D digestion timepoints: 5 and 30 minutes.
All samples were dried on the plate reader before analysing

2.8 Mass spectrometry

Peptides were sequenced by a number of mass spectrometric techniques including MALDI TOF with MAC analysis (Zou, 2003) followed by data base and manual interpretation of daughter ion spectra.

2.9 Cathepsin D assay

The assay used was innoZyme™ Cathepsin D Immunocapture Activity Assay Kit (Calbiochem®). This assay is designed specifically to measure human cathepsin D in tissue extracts and cell lysates as well as the screening of cathepsin D inhibitors.

All components were brought to room temperature prior to use.

The standards were made up by preparing serial dilutions of the standard (5μg/ml stock) ranging from a concentration of 50ng/ml to 3.125ng/ml. 495μl of the sample buffer was aliquoted into the first tube and 250μl into the remaining tubes. 5μl of the standard was added to the first tube (50ng/ml), the sample vortexed and 250μl of this mixture transferred to the second test tube (25ng/ml). This continued until tube 5. Tube 6 contained sample buffer only.

Due to the small cell numbers, samples were not diluted with sample buffer. Cell lysates were prepared by washing cell pellets with ice-cold PBS. 500μl cell lysis buffer (CytoBuster™ Protein Extraction Reagent) was added and cells incubated for 30-60 minutes. Samples were then vortexed and spun in the centrifuge at 14,000xg (should have been a pre-cooled tabletop microcentrifuge). Sample supernatants were immediately transferred into a new microcentrifuge tube and protein concentration estimated via the BCA assay (see below).

100μl of standard or sample was added to each well. All samples were set up in duplicate. The plate was closed with sealer and incubated for 30 minutes at room
temperature with gentle shaking. The plate was then washed 4 times with 300μl wash buffer and any liquid removed by tapping the plate gently on paper towels. 50μl assay buffer was added to each well followed by 50μl substrate solution. The plate was sealed tightly and incubated for one hour at 37°C.

Fluorescence was measured by on a fluorescence plate reader (FLUOstar OPTIMA) at excitation wavelength 328nm emission wavelength 393nm.

2.10 BCA protein assay

50μl of each standard or protein sample was pipetted into a test tube. 1.0ml of BCA working reagent (Novagen®) was added and the sample mixed with gentle vortexing. Reactions were then incubated at 60°C for 15 minutes. Samples were allowed to cool to room temperature before recording the absorbance.

1ml water was added to a clean cuvette and absorbance adjusted at 562nm to zero. Each sample was transferred to a clean cuvette and absorbance (A562) of all reactions read immediately. The correct absorbance was calculated by subtracting the absorbance of the blank standard from the absorbance measurement of the standard and protein samples. The corrected absorbance was then plotted against the known mass of the BSA standards to generate a standard curve. This curve was then used to interpolate recorded corrected absorbance for samples. The amount of protein present was calculated by correcting for the dilution and sample volume.

2.11 Statistical analysis

ELISA test samples were run in duplicate or triplicate and results expressed as a mean of all samples (SD included in graphs). Where comparisons between 2 variables (such as the comparison of hybridoma reponse to peptide compared with media control), a student unpaired t -test was undertaken. Where experiments were pooled together, either a 2 way ANOVA or Mann Whitney test was used to compare the two groups. In all analysis p values of < 0.05 was taken to represent a significant difference between variables.
Chapter 3

Generation of DR15-restricted murine T cell hybridomas

3.1 Introduction

The fine specificity and sensitivity of peptide detection offered by a cellular approach justifies why I selected this method to study the effects of α3(IV)NC1 processing on the presentation of peptides in Goodpasture’s disease.

Previous cellular approaches used to determine the presence of specific peptide complexes on APC have involved the use human T cell clones but their production and maintenance is difficult. The generation of human T cell clones is a lengthy process requiring the re-stimulation of cell lines every few weeks usually with autologous feeder cells. The ethical issues which prevent the vaccination of humans with a potential epitopes of interest preclude this approach as a potential avenue of investigation. In addition, many human T cell clones eventually lose antigen specificity and sensitivity and often do not respond to fixed human APC which are required for use with inhibitors of antigen presentation. Experiments may also be limited by the number of cells from a T cell clone that can be produced.

A different approach in the study of APC function is to use polyclonal T cell lines. These however lack the same fine specificity. A technique that overcomes the challenging culture requirements of lymphocyte clones is hybridoma formation.

Fusion of B cells with an immortal fusion partner was developed in the 1970s to generate B-cell hybridomas and monoclonal antibodies (Kohler and Milstein 1975). Shortly thereafter this same process was applied to produce T-cell hybridomas that secrete IL-2 after T-cell receptor (TCR) signaling (Kappler, 1981, Rock, 1990).
T cell hybridomas have a number of advantages over T cell clones in the study of APC function. They are more reliable, reproducible and convenient to use and they can often be grown to an unlimited supply for large antigen presentation experiments. In addition, they usually function well with fixed APC allowing for kinetics analysis and the use of inhibitors for antigen processing. They are much less influenced by co-stimulation and so can examine non-professional antigen presenting cells. Most importantly, however, they have the advantage of still detecting specific peptide-MHC complexes on the surface of APC.

There is a need for HLA-restricted T-cell hybridomas to study human APC but human antigen-specific immortalised T cell lines have not been successfully generated.

An alternative approach, however, is the use of transgenic mice with human HLA genes to produce human HLA-specific murine T cells and subsequent T cell hybridomas that are restricted by human HLA alleles and therefore respond to peptide presented on human APC.

Mice transgenic for HLA molecules have been produced for a number of HLA class I and MHC class II alleles. HLA-A2 transgenic mice have been used by a number of groups to study HLA-A2 epitopes in humans. There was a strong correlation between immune responses in HLA-A2 transgenic mice and humans (Wentworth, 1996). HLA-DR1 and HLA-DR4 mice have been studied as models of human autoimmune disease (Boyton, 2002). HLA-A2 transgenic mice used in the studies by Canaday et al have human α1 and α2 chains in the peptide binding domain but a murine α3 chain in the CD8 docking region of the MHC molecule (Le, 1989). This complex allows for a better T-cell response than in mice which have a human α3 chain (Newberg, 1996). Some HLA-DR transgenic mice similarly have a human α1 and β1 chains in the peptide binding domain but murine α2 and β2 chains in the CD4 docking domains (Itoh, 1996).

Since the early reports, a number of technical advances have been applied to ease production of T cell-hybridoma clones and maximise chances of successfully generating an antigen specific hybridoma. The fusion partner is a T thymoma line, which cannot survive in hypoxanthine/aminopterin/thymidine (HAT) selection medium unless it is fused
with a normal T cell (see Figure 3.8 in appendix). In addition, the fusion partner does not express endogenous TcR α and β chains to combine with a normal T cell's TCR (Born, 1988).

When an HLA-DR4 transgenic mouse was initially described, T-cell hybridomas from this mouse were found to recognise human peripheral blood mononuclear cells (Woods, 1994). This technique has been further characterised by only a handful of groups since then (Bäcklund, 2002, Manoury, 2002). Canaday et al were the first to report the systematic characterisation and validation of use of HLA-transgenic mice to make both MHC-I and MHC-II restricted T cell hybridomas that respond to peptide-MHC complexes on human APC (Canaday, 2003).

In this chapter I will describe the generation of DR15 restricted T cell hybridomas specific for α3(IV)NC1 antigen derived peptides, their screening and selection and the initial experiments that demonstrated that the hybridomas were capable of recognising the disease associated peptides dap131-150 (ISLWKGFSFI) and dap71-90 (AS345) (Table 3.2) on the surface of human DR15 expressing APC pulsed with recombinant α3(IV)NC1.

3.2 Results: Generation of T cell Hybridomas

3.2.1 Generation of T cell lines

I initially elected to generate T cell hybridomas capable of recognising the two α3(IV)NC1 peptides that were recognised by T cells from all patients in the study by Cairns et al (Cairns, 2003). Mouse class II/HLA-DR15+ transgenic mice were a gift from Professor D.M. Altmann, Imperial College, London. These mice were used to generate T cell hybridomas which were used in all subsequent experiments described.

3.2.2 Priming of transgenic mice and establishment of T cell lines

The priming of transgenic mice and subsequent establishment of T cell lines is described in detail in chapter 2.
3.2.3 Cell lines generated from HLA-DR15/IA-E transgenic mice demonstrate an in vitro recall response to peptide

To ensure the primed mice had in fact responded to peptide, an in vitro recall assay was set up and only cell lines from mice subsequently shown to respond to peptide were propagated further as T cell lines (Figure 3.1).

Figure 3.1 Example of an in vitro re-call response by splenocytes retrieved from DR15 transgenic mice primed with AS345. 2x10⁵ splenocytes were incubated with a3(IV)NC1, relevant or control (DerP1) peptide added into an end volume of 200μl R10. Splenocytes contain a combination of both feeder and T cells allowing for the presentation of peptide to potentially peptide responsive T cells. Plates were incubated for 72 hours before pulsing with 0.5μCi ^3^H thymidine and measuring proliferation at 18 hours with a beta-scintillation counter. Note that cells from mouse 2 but not mouse 1 demonstrated a recall response. However, mouse 2 proliferated only in response to AS345 and less strongly to recombinant antigen, a3(IV)NC1 but not to WISL or control peptide, Derp1, confirming a specific response to the peptide originally used to prime the mouse.
3.2.4 Cell line maintenance

Cell lines shown to respond to peptide specifically were selected to create T cell lines for potential fusion. Further details on cell line maintenance are described in Chapter 2 and illustrated in Figure 3.2. Different approaches were used in the maintenance of cell lines prior to fusion with the myeloma cell line BW5147. All were in an effort to achieve a T cell line that was not only specific for the peptide of interest but in a healthy state and adequate number in order to optimise the chances of a successful fusion. These approaches differed mainly in the number of cycles of re-stimulation, expansion and resting phases prior to fusion. One approach involved numerous cycles (>4) prior to fusion. This had the disadvantage of large cell loss in the early stages of the cycle process. Providing the line could be maintained after the 5th or 6th cycle, however, the cell line would eventually expand and, in turn, contained a much higher proportion of peptide-specific T cells. An alternative approach involved one cycle prior to fusion, which had the advantage lessening the risk of complete loss of the cell line. The disadvantage was that cells available for fusion included a much higher proportion of non-specific cells which led to more labour intensive line maintenance and screening after fusion.
Production of T cell lines

Peptide + CFA
(\text{Ha3p132-154(AS346)} \text{ or })
(\text{Ha3p64-82 (AS345)})

\text{DR15/IA-E TG}

\begin{itemize}
  \item Resting with low dose IL-2 (7-14 days)
  \item Restimulation with peptide (3 days)
  \item Expansion with high dose IL-2 (4 days)
\end{itemize}

AS346/AS345 specific T cells lines

Figure 3.2 Generation of T cell lines

3.2.5 Selection of T cell hybridomas as determined by surface expression of CD4 and TCR

The selection media was controlled so as not to support the original T cell lines and the myeloma fusion partner. All surviving cell lines were expected to be successfully fused clonal hybridomas. However, a number of problems can arise at the stage of clone selection. Firstly, clonal populations may not express both TCR and CD4 as hybrids throw out chromosomes over time. Secondly, populations may not in fact be clonal despite serial dilutions at the time of fusion (see chapter 2). Clones were selected by visual examination of the bottom of 96 well plates and subsequent selection of wells where isolated populations covered less than one third of the well surface area. To select
out clones expressing both cell surface markers, TCR and CD4, cells were stained for expression of TCR and CD4 using PE-labelled anti-CD4 and APC labelled anti-TCR and analysed using FACS analysis as described in chapter 2.

Only cell populations expressing CD4 and TCR were selected for further analysis. (Figure 3.3) This was often carried out at the 24 well stage to avoid unnecessary and labour intensive cell line maintenance.

![Figure 3.3 Examples of clonal and non-clonal T cell populations](image)

Figure 3.3 Examples of clonal and non-clonal T cell populations Cells were stained with PE-labelled anti-mouse TcR β-chain (FL2-Height channel) and APC-labelled anti-mouse CD4. A shows a homogeneous population of cells likely to be of a single clone. B shows two populations indicative of at least two clones. (All test samples were tested with isotype controls; APC-
conjugated mouse IgG1κ and mouse IgMκ respectively. In test samples represented above, gates were placed according to isotype controls.)

3.2.6 T cell hybridomas recognise peptide presented on murine DR15 expressing splenocytes

CD4⁺TCR⁺ T cell hybridomas were screened for peptide specificity by measuring IL-2 production when incubated with same peptide used to prime the mouse from which the clone had been derived. In these studies IL-2 concentration was determined by ELISA as described in chapter 2. I considered assessing IL-2 concentration using a bioassay that measures proliferation of the IL-2 dependent CTLL-2 line. CTLL-2 assays can be very sensitive but are notoriously difficult to establish in inexperienced hands and for this reason I selected the former method.

Initial screening of candidate T cell hybridomas was set up by pulsing mitomycin c-treated splenocytes from DR15 transgenic mice with either ISLWKGFSFI, AS345 or control peptide, DerP1 (Figure 3.4). After incubation with the candidate hybridoma for 24 hours, the supernatant was removed and frozen and later analysed for IL-2 production using the ELISA technique as described (see Chapter 2). Although not demonstrated in Figure 3.4, control wells containing either splenocytes or hybridoma alone or in the presence of peptide were also set up to ensure any IL-2 detected was produced solely by hybridomas stimulated by peptide-MHC complexes presented by APC.
Figure 3.4 T cell hybridoma IL-2 response to ISLWKGF5FSFI presentation on splenocytes from DR15 expressing transgenic mice. Representative experiment examining 7 of the final 11 T cell hybridomas derived from ISLWKGF5FSFI primed DR15/mouse class II TG mice already demonstrated to express CD4/TCR. Shaded bars represent production of IL-2 by respective hybridomas in a 24 hour co-culture supernatant as determined by ELISA. All ELISA test wells were set up in duplicate and results expressed as the mean of 2 wells. Error bars represent the standard deviation of 2 wells. Statistical analysis of peptide response compared to medium is expressed for each sample (unpaired t-test) The graph demonstrates that the T cell hybridomas make IL-2 in response to ISLWKGF5FSFI using mitomycin treated DR15+ve splenocytes as APC. No response was seen in the in absence of peptide or control peptide, DerP1. Response was dose dependant at 5 and 20µg/ml.
3.2.7 T cell hybridomas recognise specific peptide presented on human DR15 expressing APC

As future experiments which would eventually involve manipulation of the class II processing pathway, the use of splenocytes from a DR15 TG mouse would be difficult to use for two reasons. Firstly, it is impossible to isolate splenocytes in similar states of maturity from the spleen and these cells are a mixed population of dendritic cells, macrophages and lymphocytes. Secondly, their short life span once stimulated and the impossibility of ensuring consistency in the health and stage of maturation between experiments. For this reason it was elected that human DR15 expressing APC were used as well. Human cell lines have the advantage in being more representative of the human system of class II processing and presentation of peptide as well as offering consistency in phenotype, health and state of maturation.

Initially 2 DR15 expressing APC, DR2lum (HLA-DR15, DR1) and CRC036 (DR15,15), an EBV transformed B cell line (kind donation from Professor Dorothy Crawford) were selected as both expressed DR15. They were easily cultured and reliable cell lines. Either DR2lum or CRC036 were pulsed with increasing concentrations of peptide as described in chapter 2. Cells were then co-cultured with different clones already screened for dual positivity for CD4 and TCR. Cells were co-cultured for 24 hours before supernatant removed and analysed for expression of IL-2 as described. An example of one experiment using either cell line CRC036 or DR2lum as APC is shown in Figure 3.5. Controls (not demonstrated here) included APC or hybridoma cultured in isolation or in the presence of peptide and APC and hybridoma co-cultured in the absence of peptide to ensure IL-2 produced was as a result of peptide/DR15 recognition by the hybridoma. It should be noted that at a later stage fixed APC pulsed with peptide were used to stimulate hybridomas ensuring that APC themselves were not producing IL-2. (see Chapter 4)
Figure 3.5 T cell hybridomas recognise specific peptide presented on human DR15 expressing APC. Response of 11 different T cell hybridomas derived from T cell lines specific for ISLWKGFSFI. DR15 expressing EBV transformed cell lines, CRC036 or DR2 lum were used as antigen presenting cells and pulsed with 20µg/ml ISLWKGFSFI, 20µg/ml control peptide Der P1 or media alone. Shaded bars represent production of IL-2 by hybridomas in a 24 hour co-culture supernatant as determined by ELISA. Samples were set up in duplicate. Error bars represent the standard deviation of the mean of the 2 samples. 8 of the 11 hybridomas demonstrated a response by producing IL-2 when co-cultured with ISLWKGFSFI-pulsed CRC036 B cells. 7 of the 11 hybridomas produced IL-2 when co-cultured with ISLWKGFSFI-pulsed DR2 lum cells. 6 of the 11 responded to peptide expressed on either APC. 3 hybridomas, ha3p132.3, ha3p132.5 and ha3p132.11 failed to demonstrate any response to either ISLWKGFSFI-pulsed APC. This may reflect that these hybridomas were not DR15 restricted as earlier selection methods (represented in Figure 3.4) could potentially have identified mouse class II responders by using murine splenocytes from DR15/IA-E TG mice. When repeated, hybridoma ha3p132.3 subsequently consistently demonstrated one of the strongest responses to ISLWKGFSFI.
3.2.8 T cell hybridoma recognition of peptide is restricted to presentation by DR15

As the transgenic mice used to make initial T cell hybridomas were HLA-DR15/I-A/E+, it was crucial to demonstrate that the hybridomas were not only peptide specific but that recognition was restricted to presentation of peptide by DR15. A number of human EBV-transformed B cells expressing either DR15 or DR7 were pulsed with either control peptide DerP1 or ISLWKGFSFI and their ability to stimulate the T cell hybridomas assessed as before (Figure 3.6).

![Figure 3.6](image)

**Figure 3.6** Only APC expressing DR15 are capable of stimulating ISLWKGFSFI-specific hybridomas. The ISLWKGFSFI-specific hybridoma, ha3p132.3 was co-cultured with DR15 or DR7 expressing human EBV transformed B cell lines CRC103 and CRC021 respectively. B cells were pulsed with either ISLWKGFSFI (denoted here as WISL) or control peptide, DerP1 at a concentration of 20μg/ml (equivalent to 7.5μM). Stimulation of hybridoma was expressed as before as the production of IL-2 in a 24 hour co-culture supernatant. This graph is representative of 3
experiments where DR7 expressing APC pulsed with ISLWKGSFI failed to stimulate ha3p132.3. ISLWKGSFI-pulsed DR15 expressing B cells however strongly stimulated ha3p132.3. No response was seen in cells pulsed with control peptide, DerP1.

3.2.9 Summary of hybridoma screening

<table>
<thead>
<tr>
<th></th>
<th>ISLWKGSFI</th>
<th>P8/AS345</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hybridomas</td>
<td>11</td>
<td>4</td>
</tr>
<tr>
<td>TCR/CD4⁺ve</td>
<td>11</td>
<td>2</td>
</tr>
<tr>
<td>Peptide specific</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Restricted by DR15</td>
<td>7</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 3.1 Summary of characterisation of T cell hybridomas. 11 hybridomas were generated from T cell lines in mice pulsed with ISLWKGSFI and 4 hybridomas from mice pulsed with P8 (AS345). Of the 11 hybridomas generated from mice pulsed with ISLWKGSFI, 9 recognised ISLWKGSFI presented by DR15+/I-A/E⁺ murine splenocytes but only 7 of these recognised peptide expressed on human DR15-expressing EBV transformed B cells and were therefore DR15 restricted. Only 1 of the 4 hybridomas generated from AS345 primed mice recognised AS345 presented by DR15/I-A/E⁺ murine splenocytes. However when tested against DR15-expressing human B cells, this hybridoma did not recognise peptide and was assumed to be restricted to mouse class II and not DR15.
3.2.10 Similarity of fine specificity to patients' T cells

Hybridomas generated against the ISLWKGSFSI peptide were demonstrated to respond to ISLWKGSFSI (dap131-150). I selected one hybridoma (ha3p132.3) to compare with patients' T cells. It was demonstrated to have very similar fine specificity indistinguishable (with 15mer peptides overlapping by 10) from patients' T cells (Figures 3.7). The common sequence of the stimulatory peptides contains only 1 register with high capacity to bind DR15 (Phelps, 1999). Therefore, this hybridoma (and probably the remaining hybridomas) as well as patients' T cells, almost certainly recognise the peptide α3(IV)NC1138-148 with sequence ISLWKGSFSI engaging pockets in the peptide binding groove as shown in Table 3.2.
Figure 3.7 Responses of the T cell hybridoma (ha3p132.3) (panel A) and a patient’s (panels B and C) peripheral blood T cells to 5 overlapping peptides presented by DR15-homozygous EBV transformed B cell lines. Ha3p132.3 and a major population of autoreactive T cells in this representative patient most likely respond to ISLWKGFSF with side chains orientated into pockets in the DR15 peptide-binding groove as shown in Table 3.2 (from Zou et al JASN 2007)
3.3 Discussion

In this chapter I successfully generated 7 DR15 restricted hybridomas specific for the α3(IV)NC1-derived disease associated peptide, dap132-154 (ISLWKGFSFI). The hybridomas were peptide specific and sensitive, maintaining their ability to recognise peptide antigen on fixed APC (although sensitivity was less than in non-fixed APC). This allowed the accurate detection of small amounts of DR15/ISLWKGFSFI complexes on APC surface and in addition can respond to much smaller numbers of peptide pulsed APC. Hybridomas responded in a consistent manner although it became clear at an early stage that 2 of the 8 hybridomas, ha3p132.3 and ha3p132.15, responded consistently well in terms of IL-2 production. They were also easily maintained and for this reason were selected for use in most future experiments.
The major advantage of generating this highly specific detector for one peptide, was the potential to examine the influences of factors thought to be of importance in the α3(IV)NC1 processing pathway, and in particular to focus on the generation or destruction of one particular peptide. This specificity was in some ways limiting as I was restricted to examining only one of many potential peptides. The difficulties I encountered in generating T cell hybridomas specific for AS345 were a disappointment and efforts had to be abandoned eventually due to time constraints. The first 4 hybridomas successfully derived from AS345 immunised mice were specific for peptide but the mice used at this early stage co-expressed mouse class II as well as DR15. These AS345-specific hybridomas were not restricted to presentation on DR15 and could not be used.

A further 3 attempts to generate AS345-specific hybridomas were unsuccessful despite the use mice that were now mouse class II KO. The first attempt related to the loss of the T cell line after a 4th round of re-stimulation. An alternative approach was taken as described in chapter 2 where the cell lines were fused after only 1 round of re-stimulation. This carried the risk of generating a large number of T cell hybridomas, none of which may be peptide-responsive as the AS345-specific T cell lines had not been selected out prior to fusion. This unfortunately proved to be the case. In a final attempt, mice were primed with the intention of retrieving lymph nodes as well as spleen. Lymph nodes offer a greater yield of T lymphocytes if the mouse has responded. Only mice demonstrated to have responded to AS345 in an *in vitro* recall response, were utilised. These lines underwent 2 rounds of re-stimulation before fusion. Once again the hybridomas generated were not specific for AS345. Although the problems encountered in generating these AS345-specific T cell hybridomas were likely related to method initially, later problems related to difficulties achieving an adequate and sustained response to peptide. This may have been due to alterations in the conformation of peptide, AS345 and are discussed further in Chapter 8.
3.4 Appendix

De novo and salvage pathways

De novo pathway

Salvage Pathways

Phosphoribosyl pyrophosphate
+ Uridylate

Thymidine

Hypoxanthine

NUCLEOTIDES

Figure 3.8 The de novo and salvage pathways. The generation of hybridomas was a technique developed originally for the production of monoclonal antibodies by immortalising B cells that produce a specific antibody. It is based on the fact that each B cell produces antibody of single specificity and T cells express a TCR of single specificity. Therefore each monoclonal tumour from a T cell, known as a thymoma, expresses only one TCR. Unfortunately, most thymomas express TCR of unknown specificity as the transformation process giving rise to the tumours affects T cells and B cells randomly. As normal T lymphocytes are unable to grow indefinitely, the alternative approach has been to immortalise T cells. The first and still generally used technique was described by Kohler and Milstein in 1975. The method involved fusion or somatic cell hybridisation between a normal T cell and a T cell myeloma line, and selection of fused cells that express TCR
of the desired specificity derived from the normal T cell line. These fusion-derived immortalised TCR expressing cell lines are known as hybridomas. The technique depends on the development of cultured myeloma lines that would grow in normal culture medium but would be unable to grow in defined "selection" medium as they lack functional gene(s) required for DNA synthesis in this selection medium. Fusion of the normal T cell lines to the defective myeloma fusion partners would provide the necessary gene(s) from the normal cells so that only somatic hybrids would survive in selection media. In addition, genes from the myeloma fusion partner would make such hybrids immortal. Cell lines used as fusion partners can be created by inducing defects in the nucleotide synthesis pathway. Normal cells synthesise purine nucleotides and thymidylate de novo from phosphoribosyl pyrophosphate and uridylate respectively in a number of steps one of which involves the transfer of a methyl or formyl group from activated tetrahydrofolate. Anti-folate drugs such as aminopterin block the reactivation of tetrahydrofolate inhibiting the synthesis of purine and thymidylate. As they are necessary components of DNA, aminopterin blocks DNA synthesis via the de novo pathway. Aminopterin-treated cells can use the salvage pathway where purine is synthesised from exogenously supplied hypoxanthine using the enzyme hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and thymidylate synthesised from thymidine using the enzyme thymidine kinase (TK). Cells can grow normally in the presence of aminopterin if the culture medium is also supplemented with hypoxanthine and thymidine (HAT medium). Cell lines can be made defective in HGPRT and TK by mutagenesis. Such cells cannot use the salvage pathway and therefore die in HAT medium. If normal cells are fused with HGPRT negative or TK negative cells, the normal cells provide the necessary enzyme(s) so that hybrids synthesise DNA and grow in HAT medium. The fused cells are diluted out such that each culture well is expected to contain only one hybridoma cell.
Production of T cell Hybridomas

T cells from DR15 mice

Mixture of fused & unfused cells

Only fused cells (hybridomas) grow

Clonal populations

FUSION

BW5147 Mutant TCR-ve myeloma line

In vitro selection in HAT medium

Limiting dilution to establish clonal populations

Selection for TcR/CD4 expressing T cell hybridomas

**Figure 3.9 Generation of T cell hybridomas.** Advances in the technique described in Figure 3.10 have used thymoma T cell lines that do not express their own TCR. I elected to use the mouse AKR thymoma, BW5147 (kind gift from Professor J. Robinson, Newcastle & Dr S Anderton, Edinburgh). BW5147 has rearranged both of its TCR beta-chain loci, using the same J beta region in each but with different V beta segments. The 2 rearrangements are expressed equally in cytoplasmic RNA but the principle of allelic exclusion ensures that only one rearrangement is in frame and therefore capable of encoding a functional protein. In hybridomas made with BW5147, this protein may combine with the alpha-chain protein derived from the normal cell to form new Ag/MHC specificities.
Chapter 4

Response of ISLWKGFSFI-specific hybridomas to APC pulsed with intact α3(IV)NC1

4.1 Introduction

A number of DR15 restricted hybridomas were successfully generated to recognise ISLWKGFSFI presented on different DR15 expressing EBV transformed B cells as described in Chapter 3. There are many reports of T cells expanded to peptide that do not respond to intact antigen. To examine the influence of processing on the generation of peptides from intact α3(IV)NC1, it was crucial to demonstrate that these hybridomas were also capable of recognising DR15/ISLWKGFSFI expressed on APC pulsed with intact α3(IV)NC1.

The objectives in this chapter were threefold.

1. To ascertain that the hybridomas generated to recognise ISLWKGFSFI peptide were capable of recognising peptide bound to DR15 on the surface of APC pulsed with intact α3(IV)NC1.

2. To determine if DR15/ISLWKGFSFI could be generated from α3(IV)NC1 pulsed into different murine and human DR15 expressing APC

3. To examine the time course of uptake and presentation of α3(IV)NC1 in different human APC.

The first and second objectives were addressed by pulsing intact recombinant α3(IV)NC1 into DR15 expressing APC and co-culturing with the ISLWKGFSFI-specific T cell hybridoma ha3p132.3. In later experiments in this chapter, α3(IV)NC1-pulsed APC were
fixed prior to co-culture with hybridoma. Determination of the optimum time from pulsing with intact α3(IV)NC1 to surface presentation of peptide/DR15 complexes on APC was therefore vital. The third objective addressed this issue. Ultimately, this would allow me to examine the processing influences of factors such as proteases and in particular, cathepsin D on the presentation of ISLWKGSFSL.

The model also provided an ideal opportunity to examine the differences in the processing of α3(IV)NC1 in both human B cells and macrophages and putatively examine whether alternative pathways were involved in processing of α3(IV)NC1.

4.2 Results

4.2.1 ISLWKGSFSL-specific T cell hybridomas respond to DR15 expressing murine splenocytes pulsed with intact α3(IV)NC1

I had generated 7 T cell hybridomas capable of recognising ISLWKGSFSL-peptide and that recognition was not only specific but restricted to presentation on DR15 expressing APC alone. I was now ready to utilise the model to examine what response (if any) ISLWKGSFSL-specific T cell hybridomas made when co-cultured with APC pulsed with recombinant antigen. I elected to utilise mitomycin c treated splenocytes from DR15 transgenic mice as APC for initial antigen pulsing experiments. Methods are described in Chapter 2 and results demonstrated in Figure 4.1 below.

4.2.2 ISLWKGSFSL-specific T cell hybridomas respond to EBV transformed B cells pulsed with intact α3(IV)NC1

Consistent T cell hybridoma response to the antigen pulsed EBV transformed B cell could be obtained using the protocol described in Chapter 2. As with the murine splenocytes, the human B cell line presented both ISLWKGSFSL and intact α3(IV)NC1 to hybridoma ha3p132.3 (Figure 4.2). Presentation of intact α3(IV)NC1 but not ISLWKGSFSL peptide was abrogated by fixation (Figure 4.3) and chloroquine (Figure 4.4), indicating that presentation of intact α3(IV)NC1 requires processing.
Figure 4.1 Representative graph of 1 of 2 experiments determining response of ISLWKGF3FI-specific T cell hybridomas to DR15 splenocytes pulsed with intact α3(IV)NC1. Murine splenocytes retrieved from DR15 transgenic mice were mitomycin c treated then pulsed with either ISLWKGF3FI peptide (denoted in this graph as AS346) at 5 or 20 μg/ml (equates to 1.75 and 7.5μmol) or intact α3(IV)NC1 at 50, 100 or 200 μg/ml (equates to 1.5, 3 and 6μmol). In control wells, neither peptide nor antigen was added. Hybridoma response to ISLWKGF3FI/DR15 expression on splenocytes was read as production of IL-2 in a 24 hour co-culture supernatant. (IL-2 was measured as before using ELISA. All sample wells were in duplicate. Shaded bars represent the mean of 2 samples, error bars represent the SD. Statistical analysis of peptide or antigen response compared to medium is expressed for each sample (unpaired t-test). Both hybridomas ha3p132.3 and ha3p132.15 recognized peptide and intact antigen in a dose responsive manner. Response to cells pulsed with intact α3(IV)NC1 was less than seen with ISLWKGF3FI peptide. 5 other ISLWKGF3FI-specific hybridomas were tested but only 1 of these (ha2p132.2) demonstrated any response to intact antigen.
Figure 4.2 DR15 expressing EBV transformed B cells pulsed with intact α3(IV)NC1 or ISLWKGF5FI-peptide are capable of stimulating ISLWKGF5FI-specific T cell hybridomas. The DR15.15 expressing EBV transformed B cell CRC013 was pulsed with either 20μg/ml (7.5μM), ISLWKGF5FI peptide or 100, 200 or 300 μg/ml (3, 6 or 9 μM) recombinant α3(IV)NC1. In this experiment, cells were not fixed at any stage prior to co-culture with the ISLWKGF5FI-specific T cell hybridoma, ha3p132.3. As before, hybridoma recognition of DR15/ISLWKGF5FI is expressed as production of IL-2 in a 24 hour co-culture supernatant. This is a representative graph demonstrating ha3p132.3 response to DR15/ISLWKGF5FI in EBV transformed B cells pulsed with either peptide or intact antigen. No response was seen to control peptide, Derp1.
Figure 4.3 Processing is required before the epitope recognised by ha3p132.3 is generated by human B cell processing of intact α3(IV)NC1. This graph is representative of one of many experiments where the DR15 expressing EBV transformed B cell, CRC013 was lightly fixed (as described in Chapter 2) before pulsing with either peptide or intact antigen. Cells were washed prior to co-culture with the ISLWKGSFI-specific T cell hybridoma, ha3p132.3. Fixation has no effect on response to cells pulsed with peptide but abrogated response to intact α3(IV)NC1 indicating that processing is clearly required as no response was observed when the APC was lightly fixed before the addition of α3(IV)NC1.
Figure 4.4 Processing is required before the epitope recognised by ha3p132.3 is presented by human B cell incubated with α3(IV)NC1. B cells were pulsed with 9µM intact α3(IV)NC1 (dark shaded bars) or pre-treated with 0.1µM chloroquine for 15 minutes prior to pulsing with intact α3(IV)NC1 (light shaded bars). B cells were incubated for 4 or 18 hours prior to light fixation and assessing DR15/ISLWKGSFI presentation by determining IL-2 concentration in 24 hours co-culture supernatant with ISLWKGSFI-specific T cell hybridoma, ha3p132.3. IL-2 production determined using ELISA. In the presence of chloroquine, presentation of DR15/ISLWKGSFI was reduced at 4 hours and completely abrogated at 18 hours whilst untreated B cells continued to present DR15/ISLWKGSFI.

4.2.3 ISLWKGSFI-specific T cell hybridomas respond to THP-1 cells pulsed with intact α3(IV)NC1

To determine if ISLWKGSFI could be generated from intact antigen when pulsed into different human DR15 expressing APC. I selected the monocyte cell line, THP-1 as it expresses DR1,15 and can be induced to switch from a monocytic to macrophage phenotype by treatment with PMA (Sakamoto, 1999). Class II expression is induced by incubating THP-1 cells with IFNγ (Inagaki, 2002). These features and its relative ease of
maintenance as a cell line, made the THP-1 cell line a suitable alternative human APC in which to study processing of α3(IV)NC1.

Some time was taken optimising the method stimulating the maturation of THP-1 and is described Chapter 2. In brief, THP-1 were pre-treated with 10ng PMA and 100U IFNγ for 24 hours prior to all experiments. Mature cells were adherent and therefore were detached and washed prior to use. Class II expression following culture with IFNγ was assessed as described using a FITC-labelled anti-HLA DR,DP,DQ monoclonal antibody and FACS. Viability was checked as with all other cell lines prior to experiments.

As with the murine splenocytes and human B cells, THP-1 cells presented ISLWKGFSL when pulsed with intact α3(IV)NC1 (Figure 4.5).
Figure 4.5 ISLWKGFsFI-specific T cell hybridomas, ha3p132.3 and ha3p132.15 respond to THP-1 cells pulsed with intact α3(IV)NC1 as well as ISLWKGFsFI peptide. THP-1 cells were treated with PMA and IFNγ as detailed before pulsing with 20 μg/ml ISLWKGFsFI, 100 or 200 μg/ml α3(IV)NC1. In this experiment cells were not fixed at any stage before co-culture with hybridomas ha3p132.3 or ha3p132.15. Both hybridomas responded to THP-1 cells pulsed with intact antigen and this was dose responsive. Response was reduced when compared to roughly equimolar concentrations of peptide (20 μg/ml peptide ~ 7.5μM and 200 μg/ml α3(IV)NC1 ~ 6 μM). Although not demonstrated here, if cells were lightly fixed before adding intact antigen, no response was observed with either hybridoma.
4.2.4 Response to protein antigen α3(IV)NC1 is specific and not due to contamination

As α3(IV)NC1 had been produced in the bacterial vector, pET14b, I was conscious to ensure IL-2 responses could not be due to LPS contamination which is notoriously difficult to exclude from recombinant protein made in E.Coli. To address this concern, 50μg/ml LPS was added to control wells with THP-1 cells co-cultured hybridoma alone. No IL-2 production was observed (Figure 4.6). APC were also pre-treated with polymixin B, an antibiotic with bactericidal action which binds to the lipid A portion of LPS interfering with the permeability of the cytoplasmic membrane. It is also used for the removal of endotoxins from solutions. Results confirmed that polymyxin had no effect on IL-2 production. (Figure 4.7)
Figure 4.6 The presence of LPS in a co-culture of hybridoma ha3p132.3 and EBV transformed B cell, LCL013 but in the absence of either peptide or antigen, fails to generate any significant response.
Figure 4.7 Polymyxin has no effect on the response of hybridoma ha3p132.3 to either EBV transformed B cells (LCL061) or THP-1 cells pulsed with intact antigen. In the presence of polymyxin B recognition of both peptide and α3(IV)NC1 was not significantly affected indicating responses were specific to peptide and not due to LPS contamination.

4.2.5 Time course of uptake and processing α3(IV)NC1 in different DR15 expressing human APC

Current understanding of the class II processing pathway stems from studies using soluble proteins and recombinant antigens expressed in various bacterial species employing various expression vectors. Antigen uptake includes a number of pathways including different cell surface receptors, endocytosis and F-actin microfilament and
microtubule cytoskeleton motility motors. Internalization of antigen is then thought to trigger a number of intracellular events which lead to the production of peptide fragments which are available for binding to MHC class II. Traditionally it was believed that the peptide loading occurred predominantly in the more acidic environment of the late endosome or MHC-II molecule-loading compartment(s) (MHCII) with subsequent binding to newly synthesized class II. Alternative pathways have been investigated where antigen may be targeted to early endosomal compartments and peptide fragments bound to class II MHC recycled from the cell surface through an II-independent recycling MHC-II pathway (reviewed by Robinson, 2002). Recent work therefore is proving this sequence of events to be more complex than previously thought. It appears yet unclear what determines the route a particular antigen may take following uptake and indeed whether the type of antigen presenting cell involved will also influence peptide production.

Having established that the ISLWKGSFVI-specific hybridomas responded to both EBV transformed B cells and THP-1 cells pulsed with intact antigen as well as peptide, I hoped to use this system to examine the time course of uptake and presentation of α3(IV)NC1 in different human APC.

Figure 4.8 shows a schematic diagram of the method employed initially in examining processing of intact α3(IV)NC1 with time in both human B cells and THP-1 cells.

Figure 4.9 is representative of three experiments using this method. Interval times between pulsing with intact antigen or peptide and fixation of APC prior to co-culture varied slightly between experiments. Presentation of DR15/ISLWKGSFVI on both B cells and THP-1 cells pulsed with ISLWKGSFVI-containing peptide occurred at all timepoints providing a positive control. Presentation of DR15/ISLWKGSFVI was not detected until 12 hours in both B cell and THP-1 cells when pulsed with intact α3(IV)NC1. Presentation increased with time thereafter for up to 29 hours.

The presentation of DR15/ISLWKGSFVI was up to 8 fold greater in B cells and up to 10 fold greater in THP-1 cells when pulsed with molar equivalent of ISLWKGSFVI-containing peptide compared to intact α3(IV)NC1. This suggested that natural levels of protease
activity accounted for some degree of generation of ISLWKGFSFI peptide from α3(IV)NC1, but the process must be very inefficient.

Figure 4.8 Schematic diagram demonstrating method used in the first 3 experiments examining processing over time of intact α3(IV)NC1 in EBV transformed B cells and THP-1 cells.
Figure 4.9 Presentation of DR15/ISLWGFSFI by human EBV transformed B cells (A) and THP-1 cells (B) pulsed with intact antigen or ISLWGFSFI peptide for intervals of 4-24 terminated by light fixation. In both graphs APC were pulsed with either 20µg/ml ISLWGFSFI peptide (denoted here as AS346) or 300µg/ml intact α3(IV)NC1. (Cells were pulsed with roughly equimolar concentrations of peptide (7.5µM in 20µg/ml of ISLWGFSFI-containing peptide and 9µM in 300µg/ml of intact α3(IV)NC1)). Cells were fixed at 4, 6, 8, 12, 18, 20 and 24 hours before washing and co-culture with hybridoma ha3p132.3 as described in Figure 4.8 (Error bars represent the SD from the mean of 2 ELISA plate test wells detecting IL-2 levels from the same 24 hour supernatant sample). The presentation of DR15/ISLWGFSFI on both B cells and THP-1 cells.
was determined by level of IL-2 in the 24 hour co-culture supernatant. Expression of HLA DR in both B cells and IFNy/PMA-treated THP-1 cells was similar as determined by median fluorescence of FITC-conjugated mouse anti-human HLA DP DQ DR mAb compared with isotype control.

4.2.6 In human APC, ISLWKGF6SFI peptide is presented by 4 hours of pulsing of intact antigen and presentation increases with time.

The results exhibited variability that possibly related to variation in viability and state of activation of THP-1 cells as well as B cells and hybridomas, despite my considerable efforts. To address this concern I undertook a further set of experiments. The new method is outlined in Figure 4.10 and changes detailed in the legend below.

The most important change ensured that THP-1 cells were stimulated then eventually aliquoted and pulsed with antigen or peptide at the same time. Both B cells and THP-1 cells were co-cultured with hybridoma ha3p132.3 simultaneously and the experiment terminated at the same time. Results were considerably more consistent and are summarised in Figure 4.11.

In contrast to observations in the first set of experiments, presentation of ISLWKGF6SFI peptide was observed as early as 4 hours and in some cases, 2 hours in THP-1 cells pulsed with intact α3(IV)NC1. This was the first time expression of peptide had been detected as early as 2 hours and given that is was observed in more than one experiment, it would seem a true observation. Presentation of DR15/ISLWKGF6SFI was detected at 2 hours in 1 of the 3 experiments in α3(IV)NC1 pulsed EBV transformed B cells.

Presentation of DR15/ISLWKGF6SFI increased with time in both B cells and THP-1 cells pulsed with either ISLWKGF6SFI peptide or α3(IV)NC1. This would seem logical in the case of intact antigen where protein takes time to unfold and digest before peptide is available to bind to class II. In the case of peptide, observations are a little more difficult to explain as it is well known that peptide can bind directly to class II on the surface of APC.
(Lindner, 1996, Aoi, 1997, Harris, 1996) and it would follow that peptide binding should reach an equilibrium of on/off binding and presentation should no longer increase. One possible explanation is the increased synthesis of class II within the cell with time affording an increase in available class II molecules for binding to peptide. In the case of APC pulsed with intact α3(IV)NC1, results clearly demonstrate, as in the first 3 experiments, that both EBV transformed B cells and THP-1 cells are capable of processing and presenting ISLWKGSF1 peptide even in the presence of natural levels of aspartate proteases. Although not shown here, processing was required in these circumstances as APC fixed prior to pulsing with intact antigen were unable to present DR15/ISLWKGSF1 (see figure 4.3).

A difference was demonstrated in the presentation of DR15/ISLWKGSF1 between THP-1 cells and EBV transformed B cells where presentation of DR15/ISLWKGSF1 was optimal at 12 hours in the case of THP-1 cells and 18 hours with EBV transformed B cell. With respect to intact α3(IV)NC1, this may reflect differences in methods of antigen uptake rather than processing. However a similar pattern was seen with peptide which is capable of binding to the surface of APC.
Figure 4.10 Schematic diagram demonstrating method used in experiments discussed in Section 4.3.2 examining time points in processing of intact α3(IV)NC1 in EBV transformed B cells and THP-1 cells. The main changes to method are outlined in red. Minor changes are as follows; The concentration of ISLWKGFSFI peptide used was 10μg/ml (3.75μM) with 100μg/ml (3μM) α3(IV)NC1 ensuring equimolar concentrations of peptide and peptide containing antigen were pulsed into APC. A different B cell line, LCL061 (DR7.15 expressing) was used in these experiments. A different preparation of recombinant ISLWKGFSFI peptide was used in these experiments. Due to a lower protein yield (5mg/ml rather than 16mg/ml) a higher volume of 6M urea/50mM Tris was required to dissolve α3(IV)NC1 (see chapter 2). This relative higher concentration of urea may explain, when protein was tested prior to use, the observation that the greatest response was seen with 100μg/ml rather than 300μg/ml. For this reason, 100μg/ml was selected as the optimum concentration of α3(IV)NC1 to use. Of importance, this still contained a molar equivalent to ISLWKGFSFI-containing peptide (3μM vs 3.75μM).
Figure 4.11 Presentation of DR15/ISLWKFGSFI in human EBV transformed B cells (A) and THP-1 cells (B) pulsed with intact antigen or ISLWKFGSFI peptide. Each graph represents IL-2 production at various time points in response to either ISLWKFGSFI-containing peptide (black) or intact α3(IV)NC1 (red). The continuous line represents the mean of 3 separate experiments. Bars represent the range in IL-2 levels in 24 hours supernatants at each time point. Expression of
HLA DR in both B cells and IFNγ/PMA-treated THP-1 cells was similar as determined by median fluorescence of FITC-conjugated mouse anti-human HLA DP DQ DR mAb compared with isotype control.

4.3 Discussion

Experiments in the first section of this chapter confirmed that at least 2 of the 7 hybridomas generated against ISLWKGFSLFI, were capable of responding to DR15/ISLWKGFSLFI on the surface of APC incubated with intact α3(IV)NC1 as determined by production of IL-2. Both splenocytes from DR15 TG mice and human DR15 expressing APC presented ISLWKGFSLFI to hybridomas ha3p132.3 and ha3p132.15. I confirmed that processing was required before ISLWKGFSLFI was presented, as APC that were lightly fixed prior to incubating with α3(IV)NC1, were unable to stimulate ha3p132.3. Similarly, pre-treatment of human APC with chloroquine abrogated ha3p132.3 response to α3(IV)NC1 pulsed DR15 expressing B cells. Response was specific and not due to LPS contamination.

The second section examined the time taken from incubation with α3(IV)NC1 to presentation of DR15/ISLWKGFSLFI on the surface of APC. Although these experiments could not differentiate time taken for antigen to be internalised from time required for generation of ISLWKGFSLFI and presentation with DR15 on APC surface, it generated some interesting observations despite several initial difficulties in optimising the experiment.

Most noticeable was the difference between B cells and stimulated THP-1 cells in the time required before DR15/ISLWKGFSLFI was presented where presentation of DR15/ISLWKGFSLFI was optimal at 12 hours in the case of THP-1 cells and 18 hours with EBV transformed B cell. Although this may reflect differences in methods of antigen uptake rather than processing, experiments later described in Chapter 6 suggest that variation in processing and, in particular, the level of protease activity within the APC may
account for differences. This is discussed further in Chapter 8. Presentation of DR15/ISLWKGSFI increased with time in both B cells and THP-1 cells pulsed with α3(IV)NC1. This would appear logical given that recombinant antigen is a tightly folded globular structure that requires unfolding and denaturation before further processing can occur.
Chapter 5

Processing of α3(IV)NC1 by Cathepsin D/E

5.1 Introduction

Results from Chapter 4 confirmed that ISLWKGFSLI-specific T cell hybridomas ha3p132.3 and ha3p132.15 responded to APC pulsed with intact antigen. This enabled me to start examining the factors that might influence presentation of ISLWKGFSLI peptide from the Goodpasture autoantigen.

The evidence that processing factors are both critical and influential in the presentation of ISLWKGFSLI has been introduced in Chapter 1. Previous work suggested processing factors diminish presentation of several sites of α3(IV)NC1 peptides including ISLWKGFSLI, but evidently presentation nevertheless occurs. Biochemical analysis of the α3(IV)NC1 peptides bound to HLA-DR15 on the surface of α3(IV)NC1-pulsed APC found that the most abundantly presented α3(IV)NC1 peptides were not those with highest affinity for HLA-DR15 (Phelps 1996, 1998). The results could be interpreted as evidence that processing factors prevent some peptides (including some with higher affinity for DR15) from gaining access to MHC class II molecules within APC. Similar conclusions have been reached in experiments with other antigens (Sercarz, 2002).

Therefore I resolved to examine the effects of the processing milieu within APC on ISLWKGFSLI presentation.

Prior to starting my PhD, the group had used a biochemical approach to examine the processing of α3(IV)NC1 by isolated lysozomes and lysosomal enzymes. The results of these experiments generated crucial information relating to the initial unlocking and sequential processing of α3(IV)NC1, the proteolytic enzymes responsible for this and
their influence on processing of disease associated peptides known to stimulate patients' T cells in Goodpasture's disease.

Goodpasture autoantigen (α3(IV)NC1, ~31kD) incubated with B cell lysosomes was cleaved within a few minutes to form ~9 and ~22kD fragments, then increasing quantities of smaller peptides (Zou, 2007). The processing was completely abrogated by Pepstatin A, a specific inhibitor of Cathepsin D/E, even though lysosomal extracts contain a rich array of proteases. Purified Cathepsin D generated the same major α3(IV)NC1 fragments as entire lysosomes, suggesting Cathepsin D cleavages are required to initiate α3(IV)NC1 processing. The initial unlocking cleavages destroyed 2 major self-epitopes, including ISLWKGFSFI, and subsequent preferred cleavages destroyed all the other T cell epitopes recognised by most patients' autoreactive T cells (Zou, 2007).

The objectives of this chapter were threefold.

1. To examine the effects of pre-digestion with cathepsin D on recombinant α3(IV)NC1 by pulsing human DR15 expressing APC with digestion products and comparing presentation of ISLWKGFSFI with APC pulsed with intact α3(IV)NC1, using the ISLWKGFSFI specific T cell hybridoma, ha3p132.3.

2. To investigate whether aspartate proteases net reduced ISLWKGFSFI presentation by intact B cells.

3. To determine if the balance is for or against the presentation of ISLWKGFSFI peptide in human antigen presenting cells pulsed with intact α3(IV)NC1.

In this chapter I will demonstrate that pre-digestion of α3(IV)NC1 with cathepsin D leads to a significant reduction in presentation of ISLWKGFSFI in B cells when compared to B cell pulsed with intact α3(IV)NC1. Furthermore, in the presence of natural levels of aspartate protease activity, ISLWKGFSFI-presentation by B cells was 2-3 fold greater for B cells incubated with ISLWKGFSFI-containing peptide than molar equivalent quantities of intact α3(IV)NC1. This supports the suggestion that aspartate protease activity in B cells diminishes presentation of the ISLWKGFSFI epitope, as predicted from the earlier in
vitro studies and suggests the balance is more against than for the presentation of ISLWKGF5SI peptide in human antigen presenting cells pulsed with intact α3(IV)NC1

5.2 Results

To determine if cathepsin D influenced the generation of ISLWKGF5SI peptide from intact α3(IV)NC1, I elected initially to adapt the in vitro approach utilised within our lab for biochemical studies. This had the advantage of being an established technique with already optimized methods that had demonstrated the efficient unlocking and subsequent processing of α3(IV)NC1. I determined to pulse human DR15 expressing EBV transformed B cells with these digestion products and compare the presentation of DR15/ISLWKGF5SI with B cells pulsed with intact α3(IV)NC1 assessed by the production of IL-2 by the ISLWKGF5SI-specific T cell hybridoma ha3p132.3.

The hybridoma ha3p132.3 was used in all subsequent experiments because it had the properties of responding to intact α3(IV)NC1 and to overlapping α3(IV)NC1 peptides with fine specificity indistinguishable (with 15mer peptides overlapping by 10 from patients' T cells (Chapter 3). It was also the healthiest of all the hybridomas. It remained clonal throughout all experiments (determined by FACS as described in chapter 2 and 3) and viability was consistently >95% unless the cell line was allowed to become over-confluent. It responded to both the ISLWKGF5SI peptide and intact recombinant α3(IV)NC1 in a consistent and replicable manner above all other hybridomas.

5.2.1 Does pre-digestion of α3(IV)NC1 with Cathepsin D prevent presentation of ISLWKGF5SI peptide?

Figure 5.1 outlines the method employed in these experiments and Figure 5.2 is an example of an SDS page gel comparing intact recombinant α3(IV)NC1 with cathepsin D digested products. Preliminary experiments suggested that pre-digestion of recombinant α3(IV)NC1 with cathepsin D (irrespective of time allowed for digestion) resulted in a reduction in presentation of DR15/ISLWKGF5SI on B cells which had been pulsed with cathepsin D-digested α3(IV)NC1 compared with B cells pulsed with intact α3(IV)NC1
(Figure 5.3). Presentation was however not completely abrogated. Although less profound, a reduction in IL-2 was also seen in the control sample where α3(IV)NC1 had been incubated with DTT in the absence of cathepsin D.

Figure 5.4 summarises 3 separate experiments including the first experiment illustrated in Figure 5.3. Method between these experiments varied only slightly in that later experiments included results from B cells pulsed with cathepsin D pre-treated α3(IV)NC1 which had been digested for as little as 5 minutes only. Results were consistent at all time points in all three experiments and demonstrated a significant reduction in the presentation of DR15/ISLWKGFSLI in B cells pulsed with Cathepsin D digested α3(IV)NC1 compared with untreated α3(IV)NC1. The differences were unlikely to be due to chance (two way ANOVA p=0.01)

In the presence of DTT but no citrate buffer or cathepsin D, production of IL-2 by ha3p132.3 was reduced by approximately 30-50% (not shown here). Thus it appears that DTT does exert some influence on the digestion of α3(IV)NC1 and production of peptide, ISLWKGFSLI. This was irrespective of concentration of DTT used. In these processing experiments, DTT was added as a reducing agent to assist Cathepsin D digestion. Work in other groups examining processing of different protein antigen, such has insulin, have demonstrated that reduction of disulphide bonds alone may be sufficient to allow presentation of antigen derived peptides to class II restricted T cells (Jensen, 1991 & 1993, Collins, 1991). It was in theory possible that DTT itself could be influencing the digestion and processing of α3(IV)NC1 and potential generation of ISLWKGFSLI peptide, however, the reduction in IL-2 production was considerably less than seen in cathepsin D + DTT treated α3(IV)NC1 pulsed B cells (70-80% reduction) suggesting that Cathepsin D was still largely responsible for the reduced presentation of ISLWKGFSLI.
Figure 5.1 Schematic diagram demonstrating method used in experiments examining the influence of cathepsin D digestion of intact α3(IV)NC1 on expression of ISLWKGSFSFI presentation in EBV transformed B cells. Purified cathepsin D was reconstituted (in phosphate buffer-reconstituted in water i.e. 25µg lyophilized from 27.3µl NaPO4 pH 6.5 reconstituted in H2O). The digestion was carried out in acidic and reducing conditions. Recombinant α3(IV)NC1 was dissolved in 6M urea & incubated with purified human liver Cathepsin D (Athens Research & Technology) in the presence of DTT and citrate buffer at 37°C for 30, 60 and 120 minutes. The activity of Cathepsin D and thus digestion was then stopped by raising the pH by removing 2µl from the end volume and adding 2µl 0.5M TrisCl (pH 6.8). EBV transformed B cells were spun, counted and aliquoted at 1x10⁶ cells per well and left on ice before adding digested and undigested samples directly to the wells. Pulsed B cells were incubated for 3 hours before co-cultured with ha3p132.3. 24 hour co-culture supernatant analysed for the concentration of IL-2.
Figure 5.2 Time course of processing of α3(IV)NC1 with purified human cathepsin D SDS PAGE analysis of purified recombinant α3(IV)NC1 (denoted as α3) with (+) or without (-) prior digestion with cathepsin D for different time intervals (15, 30, 60 & 90 minutes). Several darker staining higher mobility bands seen only in combined samples were presumed to be fragments of α3(IV)NC1.
Figure 5.3 Pre-digestion of α3(IV)NC1 with cathepsin D and resultant influence on presentation of DR15/ISLWKGSFI after pulsing EBV transformed B cells with digestion products. CTRL: control Cath D: cathepsin D CB: citrate buffer T: time (minutes) alpha3: α3(IV)NC1. Cathepsin D digestion was carried out as detailed in Figure 5.1. Whole recombinant antigen or digestion products were added to B cells as detailed. Resultant DR15/ISLWKGSFI presentation was assessed by IL-2 levels in a 24 hour co-culture supernatant with DR15/ISLWKGSFI-specific hybridoma, ha3p132.3. This figure shows IL-2 levels in co-cultures from B cells pulsed with digestion products at 30, 60 and 120 minutes (T30, T60 T90). Results demonstrate a reduction of IL-2 levels in all samples pulsed with α3(IV)NC1 pre-treated with cathepsin D when compared with intact α3(IV)NC1.
Figure 5.4 Summary of 3 experiments. Each dot represents presentation of DR15/ISLWKGFSFI on the surface of DR15 expressing EBV transformed B cells as quantified by the level of IL-2 produced by DR15/ISLWKGFSFI-specific T cell hybridomas that had been co-cultured with pulsed B cells. The first series of dots represents every result at different timepoints in all 3 experiments in B cells that were pulsed with α3(IV)NC1 and had been pre-digested with Cathepsin D. The second series represents every result from B cells pulsed with untreated or intact α3(IV)NC. The bar in each column represents the mean level of IL-2 in all experiments. Reduction in DR15/ISLWKGFSFI expression in APC pulsed with α3(IV)NC1 digested with Cathepsin D was consistent at all timepoints in 3 separate experiments and the difference was unlikely to be due to chance (2 way ANOVA p=0.01).

Experiments illustrated in Figure 5.4 provided supportive evidence that Cathepsin D influenced the processing of α3(IV)NC1 and subsequent expression of ISLWKGFSFI peptide on DR15. Conditions in these experiments were clearly non-physiologic however and were established initially to ascertain what influence (if any) Cathepsin D had on the processing of ISLWKGFSFI peptide from α3(IV)NC1. In the final section of this chapter I
wanted to ask whether aspartate protease activity within living B cells and THP-1 cells reduced presentation of ISLWKGFSFI as suggested by the initial studies.

5.2.2 Do natural levels of aspartate protease activity in EBV transformed B cells and THP-1 cells influence the presentation of DR15/ISLWKGFSFI?

In Chapter 4, presentation of the ISLWKGFSFI epitope by EBV transformed B cells incubated with intact α3(IV)NC1 was processing-dependent (inhibited by fixing the APC or pre-treating with chloroquine) and of low efficiency compared to B cells incubated with molar equivalent quantities of α3(IV)NC1 peptide. Although many factors may have contributed to the differences in presentation, including efficiency of antigen uptake and of processing, the observation that ISLWKGFSFI epitope was generated within APC from intact α3(IV)NC1 would allow me to test whether aspartate proteases net reduced ISLWKGFSFI presentation by intact B cells.

Figure 5.5 illustrates results from 3 separate experiments where human DR15 expressing APC (EBV transformed B cells and THP-1 cells) were pulsed with either intact α3(IV)NC1 or equimolar concentration of ISLWKGFSFI-peptide. Thereafter cells were lightly fixed and presentation of ISLWKGFSFI on the surface of APC determined by co-culture with ha3p132.2 as previously described. In the presence of natural levels of aspartate protease activity, ISLWKGFSFI presentation was 2-8 fold greater in EBV transformed B cells and 0.8-10 fold greater in THP-1 cells when incubated with ISLWKGFSFI-containing peptide compared to molar quantities of intact α3(IV)NC1. This would support the hypothesis that aspartate protease activity in B cells diminishes presentation of the ISLWKGFSFI epitope, as predicted from both in vitro studies and initial Cathepsin D digestion studies described earlier in this chapter.
Figure 5.5 Presentation of DR15/ISLWKGFSFI on EBV transformed B cells (A) and THP-1 cells (B) pulsed with either ISLWKGFSFI peptide (light bars) or intact α3(IV)NC1 (dark bars). Each graph demonstrates 3 separate experiments. EBV transformed B cells and THP-1 cells were pulsed with equimolar concentrations of ISLWKGFSFI peptide (3.75μM) and α3(IV)NC1 (3μM) for 12 hours before fixing and washing cells then co-culturing with DR15/ISLWKGFSFI specific hybridoma, ha3p132.3. Production of IL-2 is representative of presentation of DR15/ISLWKGFSFI on the surface of APC after pulsing with either peptide or intact α3(IV)NC1.
5.3 Discussion

Results presented in Chapter 4 demonstrated that DR15 expressing B cells incubated with intact α3(IV)NC1 were able to present the ISLWKGFSl epitope despite its containing 2 peptide bonds known to be cut in early processing by B cell lysosomes in vitro (Zou, 2007). B cells incubated with abundant (300μg/ml) α3(IV)NC1 were able to present ISLWKGFSl, as assessed by IL-2 production by ha3p132.3, and presentation was processing-dependent because it was inhibited by fixation and chloroquine (Figures 4.3 & 4.4, Chapter 4). Therefore processing of α3(IV)NC1 within intact B cells does generate some ISLWKGFSl which is presented on DR15 in the conformation recognised by ha3p132.3.

*In vitro* work by Zou et al had suggested that lysosomes and more specifically, aspartate proteases, cathepsin D/E, were the proteolytic enzymes responsible for the unlocking of α3(IV)NC1 and subsequent destruction of all disease associated peptides including ISLWKGFSl.

The initial experiments in this chapter were set up to re-examine whether the observation that cathepsin D/E destroyed the T cell epitope, ISLWKGFSl *in vitro* had similar consequences in living EBV transformed B cells.

I initially elected to pre-digest recombinant α3(IV)NC1 with Cathepsin D prior to adding to B cells. This approach had some limitations in that the concentration of cathepsin D and subsequent digestion of α3(IV)NC1 was uncertainly physiological. Nonetheless these preliminary experiments were instigated to observe if there was any discernable influence on α3(IV)NC1 processing due to cathepsin D prior to more detailed and physiological work. The concomitant pulsing of equimolar concentrations of α3(IV)NC1 that had not been pre-treated with cathepsin D served as a control for any *in vivo* processing influences on the presentation of ISLWKGFSl. Preliminary results demonstrated a reduction in presentation of ISLWKGFSl in B cells pulsed with α3(IV)NC1 pre-digested with cathepsin D. Results from 3 experiments confirmed this finding to be reproducible.
and statistically significant. Controls confirmed that these effects were largely due to cathepsin D alone.

These initial results utilising an entirely different system, clearly supported the biochemical data demonstrating that pre-digestion of \(\alpha_3(IV)NC1\) with Cathepsin D abrogated presentation of DR15/ISLWKFGFSFI.

The influence of cathepsin D was consistent in a more physiologic setting, where in the presence of natural levels of aspartate protease activity, ISLWKFGFSFI-presentation by living EBV transformed B cells, was 2-3 fold greater for B cells incubated with ISLWKFGFSFI-containing peptide than molar equivalent quantities of intact \(\alpha_3(IV)NC1\). This supports the suggestion that aspartate protease activity in B cells diminishes presentation of the ISLWKFGFSFI epitope, as predicted from the earlier biochemical studies.

Experiments described in chapter 4 had flagged up the quandary that in vitro observations suggested that cathepsin D was required to unlock and process intact \(\alpha_3(IV)NC1\), at the same time destroying self epitopes (including ISLWKFGFSFI peptide). This raised the question, how was it possible that ISLWKFGFSFI peptide was presented in \(\alpha_3(IV)NC1\)-pulsed DR15 expressing APC at all? Is the balance for or against the presentation of ISLWKFGFSFI peptide in human antigen presenting cells pulsed with intact \(\alpha_3(IV)NC1\)?

The final section of this chapter focused on experiments examining the influence of natural levels of aspartate protease activity on presentation of DR15/ISLWKFGFSFI. Results demonstrated that although rapid destruction by endosomal aspartate proteases can account for a reduction in ISLWKFGFSFI presentation in B cells pulsed with intact \(\alpha_3(IV)NC1\) compared with equimolar concentrations of ISLWKFGFSFI, sufficient ISLWKFGFSFI to stimulate T cells can be presented by DR15-expressing B cells under particular conditions, such as culture in high concentrations of \(\alpha_3(IV)NC1\). Cells were pulsed with up to 300\(\mu\)g/ml \(\alpha_3(IV)NC1\) which is likely to reflect a much greater abundance of \(\alpha_3(IV)NC1\) than is naturally available for uptake and processing suggesting that cathepsin D may be 'saturated' (if observations are not an effect of conformation). This
may help explain how many of the stimuli reported to trigger Goodpasture's disease would be expected to increase turnover of basement membrane α3(IV)NC1 (Phelps, 2000) thus flooding the system.
Chapter 6

Inhibition of aspartate protease activity on processing of α3(IV)NC1 in human antigen presenting cells

6.1 Introduction

Results from biochemical studies discussed in the introduction (Zou, 2007) and my own observations in chapter 4, suggested that the aspartate protease, Cathepsin D could play a key role in the unlocking of α3(IV)NC1 allowing further processing. Biochemical results had also demonstrated that Cathepsin D (or an aspartate protease with identical specificities) was crucial in the further processing and subsequent destruction of the major epitopes recognised by patients' T cells with active Goodpasture's disease.

Chapter 5 showed reduced presentation for α3 peptides and confirmed that cathepsin D could be a factor in diminished presentation because pre-incubation lead to reduced presentation, albeit under non-physiologic conditions. In order to test the hypothesis that the cathepsin D levels naturally occurring within human APC might influence presentation of ISLWKGFSFI, I elected to compare presentation by APC either pre-treated with an inhibitor of aspartate protease activity or left untreated.

There are a number of possible approaches available which could be applied to the inhibition of cathepsin D/E activity in vivo. These included the use of specific cathepsin D/E inhibitors such as pepstatin, genetic manipulation using antisense-cathepsin D cDNA, siRNA, or the use of APC generated from cathepsin D-null mice.

I elected initially to use a specific inhibitor of cathepsin D/E, Pepstatin A, for a number of reasons. Firstly, it is a widely used specific inhibitor of aspartate proteases. It can be used without substantial toxic effect in cell culture and was the most straightforward initial
approach available to inhibit Cathepsin D activity. The alternative approaches detailed above are discussed further in the chapter.

The first part of this chapter focuses on the influence of cathepsin D inhibition on $\alpha_3(IV)\mathrm{NC1}$ processing in different antigen presenting cells at varying time points because my earlier work showed the time points selected for analysis of processing had a substantial impact on results (see Chapter 4). The second section addresses describes experiments to determine whether cathepsin D activity within APC is in fact inhibited by pepstatin A.

6.2 Results

6.2.1 Does pre-treatment of THP-1 cells and B cells with an aspartate protease inhibitor influence presentation of DR15/ISLWKFSFI?

In order to investigate the influence of the natural levels of aspartate protease within the processing machinery of human APC, I first examined presentation of DR15/ISLWKFSFI by $\alpha_3(IV)\mathrm{NC1}$-pulsed THP-1 cells that had been either pre-treated with pepstatin A or left untreated. The experimental design is shown in Figure 6.1.

The first experiment compared two ways of limiting the interval during which processing could occur because the duration of action of pepstatin was likely to be less than required for presentation to T cell hybridomas to yield detectable IL-2. The results (Figure 6.2) showed a greater diminuation in IL-2 production when processing was curtailed by light fixation than by washing, compared to APC allowed to continue internalising and processing $\alpha_3(IV)\mathrm{NC1}$ throughout the 24 hours incubation with the T cell hybridomas. Nevertheless it was discernable that pre-treatment with Pepstatin A did increase presentation and that the increase was greater with higher concentrations of pepstatin A.

An essentially similar experiment in which EBV transformed B cells were used in place of THP-1 cells (not shown here) gave inconclusive results with very low levels of IL-2. This was not unexpected as my previous work had shown that presentation of $\alpha_3(IV)\mathrm{NC1}$ by cells was lower level, peaking nearer 12 hours.
Therefore I elected to undertake a set of experiments allowing varying intervals for antigen processing before fixing/washing as well as varying concentrations of pepstatin A pre-treatment. The experimental design is shown in Figure 6.3 and cumulative results of 3 experiments are shown in Figures 6.4 and 6.5.

Demonstrated in figure 6.4, at all four time points, pepstatin A had a significant and consistent effect on presentation of ISLWKGF5FI by α3 pulsed B cells where presentation increased with both 1μM and 10μM pepstatin A. Presentation was not altered by pepstatin A in B cells pulsed with ISLWKGF5FI peptide. Similar experiments employing THP-1 cells as APC shows that an increase in presentation of ISLWKGF5FI only occurred at certain timepoints (4 and 18 hours in presence of 10μM pepstatin A) but presentation of ISLWKGF5FI was either reduced or otherwise unaltered by pepstatin A at other timepoints in THP-1 cells pulsed with recombinant α3(VI)NC1 (Figure 6.5).

The results demonstrated that both THP-1 and B cells presented DR15/ISLWKGF5FI processed from intact α3(VI)NC1, and that the level of presentation increased with longer periods of processing (4-12 hours) suggesting that epitope generation was limiting. Moreover cathepsin D/E activity was shown to be a factor in limiting DR15/ISLWKGF5FI presentation as its inhibition consistently enhanced presentation at least in B cells. (Zou, 2007)
Figure 6.1 Method used in first 3 experiments examining influence of pepstatin A on processing of α3(IV)NC1. Each sample was set up in duplicate and the supernatant from both test wells mixed and aliquoted in duplicate for analysis using ELISA. Cell viability in the THP-1 line was 75%. A control plate of THP-1 cells fixed with FACS fix and washed prior to adding peptide and ha3p132.3 was included to ensure any effects of Pepstatin were due to the specific inhibition of Cathepsin D and its effects on antigen processing.
A: THP 1 cells washed at 5 hrs

- WISL 10µg/ml
- alpha3 300µg/ml

B: THP-1 cells fixed at 5 hours

- WISL 10µg/ml
- alpha3 300µg/ml

C: THP-1 cells: No wash/fix

- WISL 10µg/ml
- alpha3 300µg/ml
Figure 6.2 THP-1 cells washed (A), fixed (B), neither washed nor fixed (C) after 5 hours pulsing with ISLWKGSFSl or α3(IV)NC1 in the presence of Pepstatin A. THP-1 cells were pre-treated with PMA and INFγ as described in Figure 6.1. The were pre-treated with 0-100μM pepstatin A (indicated on x axis) for 30 minutes before pulsing with either 10μg/ml (3.75μM) ISLWKGSFSl peptide (denoted as WSL and indicated as black diamonds) or 300μg/ml (9μM) α3(IV)NC1 (indicated as black squares). Cells were incubated for 5 hours before lightly fixing then washing (A), washing (B) or leaving without fixing or washing (C). A number of issues arose from this first experiment which were addressed in future experiments demonstrated in Figure 6.4

1. **Cell viability** In previous experiments, viability of THP-1 cells was ~70-80%. A new cell line was thawed and split regularly so cells were not allowed to become over-confluent. Cell viability was >95% for THP-1 cells in all subsequent experiments. I also elected to use a different EBV transformed B cell line, LCL061( DR15.7). Cell viability was >95%.

2. **Antigen and peptide** A different preparation of recombinant α3(IV)NC1 was used in the next set of experiments. Prior to use, the antigen had been tested against ha3p132.3 (see experimental methods in chapter 2). Testing antigen demonstrated that in this case 100μg/ml gave the best response compared to 200 & 300μg/ml. One possible explanation was that the protein yield from this preparation was smaller (approximately 6mg/ml rather than the previous 16mg/ml). As samples were dissolved in 6M urea, higher concentrations of α3(IV)NC1 such as 300μg/ml used in assays contained relatively higher concentrations of urea which could in turn have a toxic effects on cells). In subsequent experiments, I ensured that approximately equimolar concentrations of peptide were pulsed into cells (3.75μM ISLWKGSFSl and 3μM α3(IV)NC1).

3. **Method** Experimental details are highlighted in figure 6.3. There were a number of key differences: At the end of each time point, APC were fixed and washed as previously described. Before the third wash, the plate was left at 4°C until all time points had been completed. It was at this stage that all plates underwent a third and final wash before adding Ha3p132.3.
Figure 6.3 Method used examining influence of pepstatin A on processing of α3(IV)NC1
Figure 6.4 Pepstatin A enhances presentation of ISLWKGSFI by B cells. DR15 expressing B cells were pulsed with equimolar concentrations of ISLWKGSFI or α3(IV)NC1 with or without pepstatin A (1 or 10µM). Cells were incubated for 4 (A), 6 (B), 12 (C), and 18 (D) hours before fixing then incubating with ha3p132.3. Influence of pepstatin was assessed by comparing IL-2 concentration in co-cultures with or without pepstatin and expressed as % change in IL-2 concentration (y axis). Each graph represents 3 experiments (denoted as • • •). At all 4 time points, pepstatin A had a significant effect on presentation of DR15/ISLWKGSFI in B cells pulsed with α3 but no demonstrable influence on B cells pulsed with peptide. Mean of 3 experiments (bar) confirmed the effect of Pepstatin A was consistent and significant (unpaired t-test).
Figure 6.5 Pepstatin A and presentation of ISLWKGFSFI by THP-1 cells. Change in presentation of ISLWKGFSFI to T cell hybridomas effected by 2 concentrations of pepstatin A, expressed as % change in IL-2 concentration measured by ELISA. DR15 expressing THP-1 cells were pulsed with equimolar concentrations of ISLWKGFSFI peptide or whole recombinant antigen, α3(IV)NC1. Cells were incubated for 4 (A), 6 (B), 12 (C), and 18 (D) hours before fixing, washing and incubating with DR15/ISLWKGFSFI the specific hybridoma, ha3p132.3. Each graph represents 3 separate experiments (denoted as •••). The mean of all 3 experiments is represented by a bar. Pepstatin A had no clear or consistent effect on presentation of ISLWKGFSFI by THP-1 cells pulsed with either peptide or antigen.
6.2.2 Levels of constitutive aspartate protease activity in B cells & THP-1 cells.

One explanation for the much more variable but generally weaker effect of pepstatin A on THP-1 cells as compared to B cells was the potential presence of higher concentrations of cathepsin D within THP-1 cells. To examine this possibility, I measured cathepsin D activity in both THP-1 cells and B cells.

There are now numerous assays available to examine the activity of cathepsin D. The principle in the majority of these kits relies on the use of a substrate which contains a specific cathepsin D (and or E) cleavage site. Substrates are either labelled in some way such as biotinylation or contain a fluorescent moiety and a quenching group. When cleaved the fluorescence increases and can be detected at specific wavelengths due to a reduction in quenching following the separation of the fluorescent and quenching moieties. Pepstatin A is in fact commonly used in these assays as a control in determining cathepsin D activity.

I chose the Calbiochem InnoZyme™ Fluorogenic Cathepsin D Immunocapture Activity Assay Kit (see chapter 2). This is a selective fluorogenic assay for the quantitative in vitro determination of cathepsin D activity.

In preliminary cathepsin D assays, I detected cathepsin D activity in the lysates of as few as 0.5 x10⁶ APC, (not shown here), measuring cathepsin D concentrations of up to 1.56ng/ml (minimal detectable concentration of assay of 0.2ng/ml).

The assay clearly showed much higher aspartate protease activity (in the order of ten-fold increase) in THP-1 lysates compared to B cell lysates (Figure 6.6) but further interpretation was frustrating. Preliminary work demonstrated a trend where cell lysates from APC pre-treated with pepstatin A demonstrated less cathepsin D activity than those untreated, as would be expected, but subsequent experiments showed no effect of pepstatin A. I experienced considerable difficulties in establishing this assay. This was initially due to a faulty assay where the controls failed to develop. Time constraints also limited my ability to pursue this further. It was relevant however that the assay
recommended the use of $1 \times 10^6$-$1 \times 10^7$ cells when analysing lysates. The majority of my experiments were attempting to detect cathepsin D activity from lysates of $0.5 \times 10^5$-$1 \times 10^5$ cells.

Figure 6.6 Cathepsin D/E activity in lysates of THP-1 and B cells. Graph representative of 4 experiments where $1 \times 10^5$ B cells (LCL061) or PMA/IFNγ treated THP-1 cells were pulsed with 100μg/ml α3(IV)NC1 and incubated with 0, 1, 5, 10 and 50μM pepstatin A. Assays were set up as in processing experiments in 96 well plates and incubated at 37°C 5% CO2 for 24 hours. Cells were then collected and lysed as described in methods (Chapter 2). Protein concentration was determined using a BCA assay as described before adding samples and standards to the 96 well assay plate, adding substrate solution and assay buffer as described and measuring fluorescence of the unquenched peptide on a fluorescent plate reader.
6.2.3 Probing Cathepsin D activity by substrate modification.

As an independent further test of the influence of aspartate protease activity on ISLWKGF5F1 presentation, I utilised analogues of ISLWKGF5F1 with reduced susceptibility to cathepsin D.

ISLWKGF5F1 is most scissile to cathepsin D at the LW peptide bond. Since cathepsin D has a strong preference for L over its isomer, I, at the P position around substrate peptide bonds, I reasoned an analogue peptide with substitution of leucine for isoleucine (ISLWKGF5F ISLWKGF5F) should allow much more resistant cleavage by cathepsin D but very likely to retain recognition by the T cell hybridomas I had made (given time constraints and availability of suitable IA/E/α3KO mice, it was not possible to generate T cell hybridomas specific for this mutated peptide). Figure 6.7 demonstrates that only 1 (ha3p132.6) of 8 DR15/ISLWKGF5F1 specific T cell hybridomas was also responsive to the altered peptide.

This allowed me to observe the influence of pepstatin A on the presentation of modified peptide, which in theory if resistant to cleavage by cathepsin D, should not be influenced by Pepstatin A. Contrary to prediction, pepstatin A enhanced presentation of both peptide (Figure 6.8) as well as intact antigen suggesting that the modified peptide presentation was enhanced by protection from aspartate protease despite its expected greater resistance to at least cathepsin D.

To investigate the effect of the mutation on Cathepsin D susceptibility directly, I next incubated modified and unmodified peptide with Cathepsin D in vitro and determined the extent of digestion by MALDI-tof mass spectrometry. The results showed that whilst resistant at the IW peptide bond, as predicted, the altered peptide was still very scissile to cathepsin D on account of its containing other Cathepsin D scissile peptide bonds (Figure 6.9 & 6.10).
Figure 6.7 Response (IL-2 production) of T cell hybridoma to WISL & WISI peptide analogues
A. Only one hybridoma out of 7, ha3p132.6, was capable of recognising modified peptide WISI. B. Response to ISLWKGSFI (denoted as AS346), modified antigen, WISI and intact α3(IV)NC1 using the T cell hybridoma, ha3p132.3. Response to ISLWKGSFI peptide and α3(IV)NC1 was clearly demonstrated but no response was seen to modified antigen, WISI. C. Response to ISLWKGSFI, modified antigen, WISI and α3(IV)NC1 in the T cell hybridoma, ha3p132.6. Response to modified peptide, WISI was observed although response to ISLWKGSFI and whole antigen was weaker in this particular experiment compared with ha3p132.3 (note relative concentration of IL-2 was around 10 fold less). This representative one of 3 similar experiments.

Figure 6. 8 Influence of pepstatin A on presentation of WISL (denoted as AS346), modified peptide, WISI or intact α3(IV)NC1.
6.2.4 Susceptibility of modified peptide, WISI, to cathepsin D cleavage

As presentation of both ISLWKGFSFI and, unexpectedly, modified peptide were increased in the presence of pepstatin A, it was postulated that modified peptide was susceptible to cleavage by Cathepsin D. To address this, both modified peptide, ISIWKGFSFI and control peptide, ISLWKGFSFI were digested with cathepsin D as previously described (Zou, 2007) and the digested products analysed and identified using MALDI-tof mass spectrometry. (For method detail, see Chapter 2)

Table 6.1 represents the digestion products of non-modified peptide (ISLWKGFSFI) and modified peptide (ISIWKGFSFI) after treatment with cathepsin D as determined using MALDI-tof mass spectrometry.

The known molecular weight of ISLWKGFSFI is 2599.29. The strongest peak was identified at 2599.1 pre-digestion. On exposure to Cathepsin D for 30 minutes, a number of new peaks were identified; 771 and 969 were the strongest (see below). Letters in red represent the sequence of the identified digestion. These results confirmed previous work, where digestion with cathepsin D cleaved between WISL/WKG FSF/IMF.

The digestion products from cathepsin D-treated modified peptide, ISIWKGFSFI (where leucine has been replaced by isoleucine) demonstrates the strongest peak at 1856 pre-digestion. The known molecular weight of ISIWKGFSFI is 1855.93. On exposure to Cathepsin D for 30 minutes, a number of new peaks were identified. 1464.2 and 1855.93 were the strongest (see below). Letters in red represent the sequence of the identified digestion. Therefore the modified peptide, ISIWKGFSFI, was not significantly cleaved at WIS/WKG, compared to the natural peptide which was cleaved at WISL/WKG to release fragments. This would suggest that, as predicted, ISIWKGFSFI in this critical area was less susceptible to cleavage by cathepsin D. However, the modified peptide was extensively cleaved at other sites. This could explain the results observed with modified peptide in processing experiments where both ISLWKGFSFI and modified peptide were influenced by inhibition with pepstatin A.
<table>
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<th>Peak</th>
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<td>1856</td>
<td>SPHGWI SLWKGFSFIMFTSAGSR</td>
</tr>
<tr>
<td>771</td>
<td>WKGFSF</td>
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<td>969</td>
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A Cathepsin D treated ISLWKGFSFI digestion products

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<th>Peak</th>
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<tr>
<td>1855.93</td>
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B Cathepsin D treated ISIWKGFSFI digestion products

Table 6.1 Cathepsin D digestion products

6.3 Discussion

The results presented in Chapter 4 showed that presentation of ISLWKGFSFI from intact antigen was inefficient compared with peptide, and confirmed previous biochemical data that aspartate proteases within the APC cleaved within ISLWKGFSFI, as might account for less efficient processing.

Initial work discussed in this chapter demonstrated that the level of protease activity within human EBV transformed B cells was sufficient to substantially diminish presentation of ISLWKGFSFI. Pre-digestion of α3(IV)NC1 with cathepsin D prior to
pulsing into APC suggested that it may be the enzyme responsible but this needed to be demonstrated. The next step in identifying cathepsin D/E as the critical enzymes would be to observe the differences in presentation of peptide under conditions of reduced activity or concentration of the enzyme. In theory, a reduction in enzyme availability within APC would conversely lead to an increase in peptide expression.

The methods by which cathepsin D activity can be reduced or effectively abolished are numerous. Some microbial protease inhibitors are known to inhibit the intracellular proteases of living cells. They also have the advantage of being site specific and have a small molecular weight so that they can easily penetrate the cell membrane (Puri, 1988).

Cathepsin D is a representative of the acid or carboxyl-dependent classes of endopeptidases which also include pepsin, chymosin and renin. The enzymes can be inhibited by a variety of reagents which covalently react with carboxyl groups, e.g. (Lundblad and Stein, 1969, Takahashi, 1972, Tang, 1971). However few effective reversible inhibitors are known. Initial study of cathepsin D relied heavily on the use of inhibitory antisera. This had certain important disadvantages including the high molecular weight of the antibody molecules and the complex chemical nature of the inhibition. A naturally occurring tight binding inhibitor of carboxyl-proteinases, pepstatin, was first described by Morishima in 1970. Pepstatin is obtained from filtrates of cultures of streptomyces (Morishima, 1972). It is a potent but reversible inhibitor of aspartic proteases. It forms a 1:1 complex with proteases such as Cathepsin D, pepsin, renin, bovine chymotrypsin (Umazawa, 1976, Marciniszyn, 1977) and protease B (Takahashi, 1976). As an inhibitor it is highly selective.

Initial interest in the inhibiting effects of pepstatin on cathepsin D was as a means of determining the concentration of cathepsin D and thus its activity in a variety of biological and pathological processes. The nature of the interaction between cathepsin D and pepstatin A have therefore been intensively studied. Cathepsin D has a tight binding to pepstatin. Gel-chromatographic experiments have shown that binding of pepstatin and its derivatives is strongly pH dependant.
Results from preliminary experiments gave inconclusive results with respect to the influence of pepstatin A on presentation of DR15/ISLWKGFSLFI in EBV transformed B cells. Results with THP-1 cells were more encouraging where pre-treatment with Pepstatin A did increase presentation DR15/ISLWKGFSLFI and that the increase was greater with higher concentrations of pepstatin A.

Both B cells and THP-1 cells pulsed with ISLWKGFSLFI-containing peptide then left without washing or fixing demonstrated at least a 2-3 fold increase in presentation of ISLWKGFSLFI in the presence of natural levels of B cell and THP-1 cell aspartate protease activity than in cells pulsed with molar equivalent quantities of intact α3(IV)NC1. Partial inhibition of aspartate protease activity with pepstatin A increased ISLWKGFSLFI-presentation on intact α3(IV)NC1 in a dose dependant manner although this was mainly observed in the THP-1 cells which had been neither fixed nor washed.

I therefore undertook a second set of experiments using a modified protocol that allowed varying intervals for antigen processing before fixing/washing as well as varying concentrations of pepstatin A pre-treatment. This set of 3 experiments generated several observations:

- Both B cells and THP-1 cells when pulsed with intact α3(IV)NC1 presented DR15/ISLWKGFSLFI in the presence of natural levels of aspartate proteases and this expression increased with time.

- Partial inhibition of the aspartate protease, Cathepsin D by the specific inhibitor, Pepstatin A resulted in an increase in presentation of DR15/ISLWKGFSLFI in B cells pulsed with intact α3(IV)NC1 but no influence on expression was observed in APC pulsed with ISLWKGFSLFI-containing peptide. These findings were consistent in 3 experiments and significant (unpaired t-test)

- Presentation of DR15/ISLWKGFSLFI on THP-1 cells was extremely variable, where in some cases expression of DR15/ISLWKGFSLFI fell. For this reason it was not possible to determine any clear influence of Pepstatin A on α3(IV)NC1 processing in THP-1 cells.
One putative explanation for this last observation could be the increased presence of cathepsin D in activated THP-1 cells in comparison with B cells. If enzyme levels were significantly higher in THP-1 cells, it is possible that Pepstatin A was ‘saturated’ and of insufficient concentration to abrogate the effects of Cathepsin D. It is difficult to quantify ‘physiologic’ levels of enzyme activity and in turn measure the extent of cathepsin D inhibition, especially in THP-1 cells. My attempts to measure Cathepsin D activity and inhibition were initially thwarted by a faulty assay where the controls consistently failed to develop. Time constraints also limited my ability to optimise the assay. Nonetheless, one interesting observation was apparent from later experiments. The assay clearly showed much higher aspartate protease activity (in the order of ten-fold increase) in THP-1 lysates compared to B cell lysates (Figure 6.6) supporting the idea of increased aspartate protease activity within THP-1 cells.

It is perhaps relevant to note that the assay recommended the use of $1 \times 10^6$ to $1 \times 10^7$ cells when analysing lysates. The majority of my experiments were attempting to detect cathepsin D activity from lysates of $0.5 \times 10^6$ to $1 \times 10^6$ cells. Attempts latterly to use cell numbers closer to those recommended still failed to detect any significant change on cathepsin D activity in the presence of increasing concentration of pepstatin in both B and THP-1 cells.

Further attempts to confirm that cathepsin D was responsible for abrogating expression of DR15/ISLWKGFSFI by modifying ISLWKGFSFI peptide such that leucine was exchanged for the cathepsin D resistant, isoleucine, proved disappointing. Having fortuitously demonstrated that one of the ISLWKGFSFI-specific T cell hybridomas was also capable of recognising ISIWKGFSFI in addition to α3(IV)NC1, results demonstrated that expression of either peptide increased in a dose responsive manner with pepstatin. If the mutated peptide was indeed resistant to cathepsin D cleavage this would suggest that either cathepsin D was not the proteolytic enzyme responsible for cleavage (as we would have expected pepstatin A to have no effect on the mutated peptide). Alternatively the single amino acid substitution may not have resulted in resistance to cathepsin D. This second explanation was supported by data analysing digestion products of both original and modified peptide. Although isoleucine did prevent cleavage at the crucial scissile
bond, cleavage was demonstrated to occur at 2 different sites. Work with overlapping peptides (15mers overlapping by 10) has identified the sequence most likely to be recognised by patient's T cells as well as the hybridomas to be ISLWKGFSL. As this sequence also contains the two sites cleaved by cathepsin D in the digestion of modified peptide this could explain where in the presence of pepstatin A, the presentation of modified peptide is augmented.

Although time precluded further attempts to confirm Cathepsin D as the enzyme responsible for reduced expression of DR15/ISLWKGFSL, alternative approaches may have included the use of a cathepsin D knock out mouse or the utilisation of a cathepsin D specific probe to substantiate observations. An alternative approach could have included the use of other inhibitors such as the AEP inhibitor, MV026630 (AEP is required for the cleavage of pro-cathepsin D into the proteolytically active Cathepsin D and its inhibition in turn would abrogate cathepsin D activity). Alternative approaches are discussed further in Chapter 8.
Chapter 7

Dominant protection

7.1 Introduction

As detailed in Chapter 1, Goodpasture's disease has very striking associations with alleles inherited at the MHC class II loci. A strong positive disease association is described with greater than 80% of patients carrying DRB1*1501 (one of the alleles contributing to DR specificity). An even more impressive but contrasting association is that seen with DR7 alleles which appear to protect from disease even in the presence of DRB1*1501 (Phelps, 1999). The phenomenon has been termed dominant protection and similar HLA associations have been described in other autoimmune diseases and animal models of autoimmunity (Phelps, 1999).

The exact mechanism accounting for the association of MHC with autoimmunity is not known but it seems likely and indeed there is evidence to support that expression of class II molecules themselves can account for the associations (Deng, 1993). We know that MHC molecules can influence immune response by their peptide binding preferences and subsequent display of peptide-MHC complexes to T cells. It seems likely that peptide binding preferences would also influence immune response to self proteins.

Evidence supporting the direct influence of class II molecule expression and susceptibility to autoimmune disease has been demonstrated in mice transgenic for HLA class II alleles (Taneja, 1998). How MHC differences can in turn influence susceptibility is controversial. The mechanisms involved are likely to vary in different autoimmune diseases and in turn models of disease.

Postulated mechanisms by which HLA may protect from disease relates to influencing presentation of self antigen which in turn can lead to a) deletion of auto reactive T cells or
the inhibition of their selection b) induction of anergy of autoreactive T cells c) diversion of the phenotype of autoreactive T cells to that of a regulatory phenotype d) "capture" of the determinants recognised by autoreactive T cells and e) positive selection of regulatory T cells.

Work by Phelps et al (Phelps, 2000) examining the peptide binding preferences of HLA-DR molecules with divergent associations with susceptibility to Goodpasture's disease demonstrated that DRB1*0701 confers strong dominant protection from disease (OR 0.3) such that those individuals inheriting B1*1501 with *0701 have no higher risk than the general population. Using an inhibition assay they demonstrated that DR7 molecules have greater affinity for α3(IV)NC1 peptides than DR15. They suspected dominant protection could arise by DR7 capturing peptides in turn preventing their display bound to DR15. Indeed their binding data suggested that the major α3(IV)NC1 peptides presented bound to DR15 by DR15 homozygous APC would bind preferentially to DR7 in DR7,15 heterozygote APC.

T cell hybridomas specific for DR15/ISLWKGFSFI afforded an opportunity to examine this hypothesis by comparing presentation of ISLWKGFSF by antigen presenting cells with and without the co-expression of DR7.

7.2 Results

7.2.1. DR7,15 expressing APC demonstrate a trend for generating more abundant DR15/ISLWKGFSFI complex than DR15/15 homozygous APC

Initial experiments compared ISLWKGFSFI presentation by four different EBV-transformed B cells which were either DR15 homozygotes or DR15,7 heterozygotes pulsed with ISLWKGFSFI peptide or intact α3(IV)NC1. Two lines were homozygous for DR15 (LCL013 & LCL036) and the second two heterozygous for DR15 and DR7 (LCL039 & LCL041) (see table 7.1).

Surprisingly, when pulsed with intact α3 there was a trend for DR7-expressing APC to generate more abundant DR15/ISLWKGFSFI complex than DR15/15 APC (Figure 7.1),
contrary to what would be expected if DR7 was capturing ISLWKGSFI peptide, preventing presentation by DR15. Variability differed between cell lines and could putatively account for by variation in DR15/ISLWKGSFI between the B cell lines. To address the issue of viability, a second experiment was performed (Figure 7.2) including a third DR15,7 expressing B cell line, LCL047. As a control, a non-DR7 expressing heterozygote was included, LCL106 (DR15, 11). Viability of B cell lines did not account for differences observed in presentation of DR15/ISLWKGSFI where the B cells presenting DR15/ISLWKGSFI in the greatest abundance had the poorest viability.
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</tr>
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</table>

Table 7.1
HLA DR expression of EBV transformed B cells
Figure 7.1 DR15,7 heterozygous EBV transformed B cells demonstrate a trend for presenting DR15/ISLWKGFSFI in greater abundance than DR15,15 homozygous cells. Each graph is representative of 1 experiment. APC were pulsed as previously described (chapter 2) with either 20μg/ml (7.5μM) ISLWKGFSFI-containing peptide or 300μg/ml (9μM) recombinant α3(IV)NC1 except in the third experiment where concentration of peptide and α3(IV)NC1 was 3.75μM and 6μM respectively. APC were pulsed for 4 hours at 37°C 5% CO2 before adding the DR15/ISLWKGFSFI-specific T cell hybridoma ha3p132.3 in a ratio of 2:1. Co-cultures were incubated for 24 hours and supernatant analysed for production of IL-2 as previously described. Light bars represent IL-2 levels in a 24 hour co-culture in APC pulsed with ISLWKGFSFI-containing peptide and dark bars with intact α3(IV)NC1. CRC013 and 036 are DR15 homozygous and LCL039 and 041 DR15,7 heterozygous EBV transformed B cells. Standard error bars represent the SD of the mean of duplicate samples used to analyse IL-2 levels in an ELISA assay.
Figure 7.2 DR15,7 heterozygous EBV transformed B cells demonstrate a trend for presenting DR15/ISLWKGFSFI in greater abundance than DR15,15 homozygous cells. B cells were pulsed with either ISLWKGFSFI peptide (3.75µM) or α3(IV)NC1 (9µm). Experimental set up was as described in Figure 7.1. There was a trend for increased presentation of DR15/ISLWKGFSFI in the DR15,7 expressing heterozygotes when pulsed with intact α3(IV)NC1. Non-DR7 expressing heterozygotes (LCL106) failed to stimulate hα3p132.3. The viability of APC did not appear to influence DR15/ISLWKGFSFI expression as the 2 highest stimulators of hα3p132.3 (LCL047 and LCL061) had a viability of around 50%. It was unusual however that the DR15 homozygote, CRC013 failed to express DR15/ISLWKGFSFI when pulsed with either ISLWKGFSFI-containing peptide or intact α3(IV)NC1 despite a viability of >95%.

7.2.2 The expression of HLA DR does not correlate with the increased abundance of DR15/ISLWKGFSFI seen in DR15,7 heterozygous EBV transformed B cells

To assess if expression of HLA DR as determined by flow cytometry corresponded with any variation in presentation of DR15/ISLWKGFSFI in α3(IV)NC1-pulsed B cells, I repeated the above experiments using a number of EBV transformed B cells from healthy
volunteers with different HLA DR expression (a kind donation from Mrs Gwen Wilkie, Dick Vet School, Edinburgh). These cells were homozygous for DR15,15 or heterozygous for DR7,15 (see Table 7.1). APC were pulsed with intact α3(IV)NC1 and the presentation of DR15/ISLWKGSFI complex determined as above. Cell lines were also stained for expression of class II using a FITC-labelled monoclonal antibody to HLA DR,DP,DQ (described in Chapter 2).

Results shown in Figure 7.3 demonstrate that there was no correlation between expression of DR and presentation of DR15/ISLWKGSFI on DR15,15 and DR15,7 B cells and is unlikely to explain the trend for greater abundance of DR15/ISLWKGSFI in DR15,7 heterozygous B cells when pulsed with intact α3(IV)NC1.
Figure 7.3 Scatter plot comparing expression of HLA DR on EBV transformed B cells with presentation of DR15/ISLWKGFSFI as determined by production of IL-2 assessed using ELISA. In this experiment, the expression of HLA DR DP DQ on 4 different DR15 homozygous and 4 different DR15,7 heterozygous EBV transformed B cells was determined using flow cytometry (demonstrated on x axis as median fluorescence) and was compared with presentation of DR15/ISLWKGFSFI as assessed by production of IL-2 in a 24 hour co-culture (demonstrated on y axis as IL-2) when B cells were pulsed with intact α3(IV)NC1. DR15,7 heterozygous EBV transformed B cells again presented DR15/ISLWKGFSFI as well as DR15,15 homozygotes. There was a trend for presenting DR15/ISLWKGFSFI in greater abundance in the DR15,7 expressing B cells pulsed with intact α3(IV)NC1. Expression of HLADR is expressed as the median fluorescence using a mouse anti-human FITC labelled monoclonal antibody as described in chapter 2. All samples were gated using a FITC conjugated mouse IgG2a to exclude any non-specific binding. There was no clear correlation with the abundance of DR15/ISLWKGFSFI expressed on B cells as determined by IL-2 production and level of expression of HLA DR using flow cytometry.
7.2.3 The DR7-associated enhancement of DR15/ISLWKGF/SFI presentation is consistent and significant.

The experiments shown in Figures 7.2 and 7.3 demonstrated that viability and expression of HLA DR did not appear to explain differences in presentation of DR15/ISLWKGF/SFI between DR15,15 and DR15,7 expressing B cells. The results of preliminary experiments could, however, be accounted for by the variability between the B cell lines other than that of their HLA expression. This was because they were derived at different times from different individuals and inevitably exhibited somewhat different growth characteristics in culture. To address these sources variability, a further experiment using larger numbers of DR15,15 and DR15,7 expressing EBV transformed B cell lines among which one could hope other sources of variability would average out. To ensure that any observed increase in abundance of DR15/ISLWKGF/SFI was specific to the DR7,15 and not a feature of all DR15 heterozygotes, a number of non-DR7 heterozygotic DR15 expressing EBV transformed B cells were included in the processing experiments.

Figure 7.4 demonstrates that the DR7-associated enhancement of DR15/ISLWKGF/SFI presentation observed in DR15/7 heterozygotes compared with DR15 homozygotes (and DR15/non-DR7 heterozygotes) was consistent across 8 lines in 4 separate experiments. The difference was unlikely to be due to chance (Mann-Whitney Rank Sum Test P=0.005).

It was of interest that non-DR7 expressing DR15 heterozygotes were also capable of expressing DR15/ISLWKGF/SFI in a similar abundance to DR15, 15 homozygotes.
Figure 7.3 Scatter plot comparing expression of HLA DR on EBV transformed B cells with presentation of DR15/ISLWKGFSFI as determined by production of IL-2 assessed using ELISA. In this experiment, the expression of HLA DR DP DQ on 4 different DR15 homozygous and 4 different DR15,7 heterozygous EBV transformed B cells was determined using flow cytometry (demonstrated on x axis as median fluorescence) and was compared with presentation of DR15/ISLWKGFSFI as assessed by production of IL-2 in a 24 hour co-culture (demonstrated on y axis as IL-2) when B cells were pulsed with intact α3(IV)NC1. DR15,7 heterozygous EBV transformed B cells again presented DR15/ISLWKGFSFI as well as DR15,15 homozygotes. There was a trend for presenting DR15/ISLWKGFSFI in greater abundance in the DR15,7 expressing B cells pulsed with intact α3(IV)NC1. Expression of HLADR is expressed as the median fluorescence using a mouse anti-human FITC labelled monoclonal antibody as described in chapter 2. All samples were gated using a FITC conjugated mouse IgG2a to exclude any non-specific binding. There was no clear correlation with the abundance of DR15/ISLWKGFSFI expressed on B cells as determined by IL-2 production and level of expression of HLA DR using flow cytometry.
7.2.4 Can competitive inhibition with peptides of high affinity to DR7 help confirm that co-expression of DR7 accounts for the increased abundance of DR15/ISLWKGFSFI presentation on EBV transformed B cells?

One possible mechanism for the difference in DR15/ISLWKGFSFI expression was the direct influence of DR7 on peptide binding to DR15. I determined to inhibit ISLWKGFSFI uptake by DR7 and observe the effects (if any) on expression of DR15/ISLWKGFSFI. As an initial approach I elected to inhibit ISLWKGFSFI binding to DR7 by pulsing B cells and THP-1 cells with DR7-high affinity peptides. Both peptides P16 and P21 are reported to bind to DR7 with much greater affinity than DR15 so might be expected to increase presentation on DR15 by competitive displacement for DR7 if present on the peptide binding compartment at a suitable concentration.

Figure 7.5 demonstrates that all 3 DR15,7 expressing cell lines both P16 and P21 reduced presentation of DR15/ISLWKGFSFI when APC were pulsed with ISLWKGFSFI containing peptide and α3(IV)NC1. Reduction in presentation fell with increasing concentration of inhibitory peptide.

As a similar reduction in DR15/ISLWKGFSFI presentation was observed in cells pulsed with both peptide and antigen, this raised the possibility of a non-specific effect of the inhibitor on DR15/ISLWKGFSFI presentation. As cell were not fixed at any time point it was not possible to determine whether any effects observed were due to interference with DR7 binding or a non-specific or even toxic effect of the inhibitory peptide.

One way to control for this was to examine the effects of inhibitory peptide on DR15/ISLWKGFSFI presentation in DR15,15 homozygotes. P16 did not affect presentation of DR15/ISLWKGFSFI in DR15 homozygotes pulsed with ISLWKGFSFI peptide or antigen however the level of presentation of DR15/ISLWKGFSFI (including controls without inhibitory peptide) on these APC was low compared with DR15,7 heterozygotes (shown in Figure 7.6 which represents one of two similar experiments). It was not possible to conclude with certainty that there was a true difference in the effect of P16 on DR15 homozygous or DR15,7 heterozygous cell lines. Observations remained
consistent with DR15,7 heterozygous cell lines, however, where in the presence of P16, presentation of DR15/ISLWKGF\text{SF}I was reduced when pulsed with intact \( \alpha3(\mathrm{IV})\mathrm{NC}1 \). Levels of IL-2 were approximately 10 fold lower in comparison with B cells pulsed with ISLWKGF\text{SF}I-containing peptide.

Due to time constraints, despite intriguing results, it was not possible to conclude with certainty from these experiments with inhibitory peptide, whether any observed reduction in presentation of DR15/ISLWKGF\text{SF}I in DR15,7 B cells when pulsed with \( \alpha3(\mathrm{IV})\mathrm{NC}1 \) had been as a result of blocking the DR7 peptide binding site or a non-specific and potential toxic effect of inhibitory peptide on any cell line irrespective of DR7 expression.
Figure 7.5 Competitive inhibition of DR7 binding by two peptides (P16 at top, P21 at bottom) with high affinity for DR7 compared to DR15. Both inhibitory peptides were made up to a stock concentration of 10mg/ml in DMSO and dissolved further to 0.5mg/ml in sterile PBS. Inhibitory peptides, P16 or 21 were added at a concentration of 10, 20 or 40μg/ml. Cells were then left for 60 minutes on ice. ISLWKGSFSFI peptide was then added to APC at a concentration of 20μg/ml (7.5μM) and α3(IV)NC1 was pulsed at 200μg/ml (6μM). APC were incubated for a further 2.5 hours at 37°C before adding ha3p132.3 and incubating at 37°C for 24 hours. 3 different DR15,7 expressing B cell lines (LCL041, LCL047 & LCL061) were used. Graph demonstrates the
presentation of DR15/ISLWKGFSSFI on respective B cells pulsed with either ISLWKGFSSFI containing peptide (AS346) or α3(IV)NC1 (alpha3).
7.3 Discussion

In this chapter I demonstrated that contrary to initial expectations, DR15/ISLWKFGSFI presentation was enhanced in DR7,15 compared with DR15,15 expressing EBV transformed B cells.

Despite the difficulties encountered confirming the influence of co-expression of DR7 on expression of DR15/ISLWKFGSFI, this chapter produced some intriguing results.

Class II molecules are capable of binding unfolding proteins and peptides of long lengths in addition to the previously acknowledged smaller peptide fragments. On unfolding, the most available high affinity binding regions then bind preferentially to class II MHC. These bound protein sequences would then be protected from proteolysis by MHC and go on to become immunodominant T cell determinants (Deng, 1993). For self antigens, T cells with specificity for those dominantly displayed determinants will be subject to strong tolerance induction. This process whereby determinants within an unfolding protein antigen compete for binding to the same class II molecule has been termed determinant capture and has since been extended as a means by which protection could be provided by the expression of specific class II molecules in autoimmune diseases such as DM and MS.

Deng et al published seminal work in where they compared the immunogenicity of hen egg lysozyme (HEL) in a NOD mouse (NOD x BALB/c) F1 and Eβ transgenic NOD. Response to the subdominant A^NOD- restricted determinant epitope disappeared on introduction of Eβ molecule and was restored when scission of HEL separated this
determinant from its adjoining competitively dominant Eα-restricted determinant. This suggested that Eα bound and protected its dominant determinant if exposed on a long peptide while neighbouring determinants were lost during proteolysis (Deng et al 1993).

There is a dominant negative association observed with DR7 and susceptibility to Goodpasture’s disease. With the knowledge that, ISLWKGSFI, a key epitope recognised by patients’ T cells with disease has one of the strongest affinities for DR7 with only intermediate affinity for the disease associated DR15 (Phelps, 1996), the generation of ISLWKGFSFI-specific T cell hybridomas provided an ideal opportunity to examine whether determinant capture of ISLWKGFSFI peptide could account for the dominant protective effect associated with co-expression of DR7 with DR15. In theory this immunodominant peptide would bind preferentially to DR7 in turn preventing binding to DR15 leading to a decrease in response by the DR15/ISLWKGFSFI specific T cell hybridomas.

Results illustrated in this chapter suggested that DR7 did indeed influence expression of DR15/ISLWKGFSFI on the surface of alpha3 pulsed APC. However observations were contrary to expectations where I found that DR15/ISLWKGFSFI presentation was considerably enhanced in DR15/7 compared to DR15/15 expressing EBV transformed B cells. Therefore DR7 co-expression does not reduce ISLWKGFSFI presentation on DR15, and determinant capture is unlikely to account for dominant protection.

One alternative hypothesis is that as a result of determinant capture, DR7 could afford protection by enhancing presentation of DR15/ISLWKGFSFI in health leading to deletion or regulation of DR15/ISLWKGFSFI specific T cells during establishment of central tolerance. This in fact seems more logical given previous arguments that T cells recognising self peptides escape tolerance as a result of reduced presentation of the same self peptides during central tolerance.

There remains the question how does co-expression of DR7 with DR15 enhance binding of ISLWKGFSFI to DR15 when binding data suggests it would bind preferentially to DR7?
Competitive capture is one potential explanation where different determinants of an unfolded antigen compete for binding to a class II molecule. This has been demonstrated in an animal model of MS where the hierarchy of 3 overlapping determinants within a region of the Golli-MBP complex was characterised. All were demonstrated to compete for binding to I-Au, but due to competitive capture by flanking determinants, one lower affinity determinant was not expressed (Mavarakis et al 2008). If other DR7 high affinity determinants were competing for DR7 binding, this could in turn “free” ISLWKGFSFI peptide to bind to the lower affinity DR15.
Chapter 8

Discussion

8.1 Introduction

In this study of α3(IV)NC1 processing and presentation I set out to examine whether the destructive processing of the major disease associated peptides influenced presentation by APC so as to potentially account for the existence of T cells in the peripheral repertoire with reactivity towards these self peptides.

In the course of my research I successfully generated T cell hybridomas from a humanised mouse transgenic for human HLA DR15 which were specific for one of the major disease associated α3(IV)NC1 peptides, dap131-150. These T cell hybridomas were used as peptide detectors to study the processing of α3(IV)NC1 by living human and mouse APC. The peptide, dap131-150, was demonstrated to be susceptible to destruction by a protease in the endosome of APC, cathepsin D. The response of the T cell hybridomas specific for the same major disease associated peptide when incubated with intact α3(IV)NC1-pulsed APC were significantly enhanced by the cathepsin D/E inhibitor, Pepstatin A. Taken together this work shows that naturally occurring Cathepsin D activity within human B cells or APC, significantly diminishes presentation of at least one α3(IV)NC1-derived peptide known to be recognised by patients' T cells. The results suggest that destructive processing could be a factor in the escape from central-tolerance of major auto reactive T cells destroying them in the course of antigen processing supporting the hypothesis suggested above.

In this chapter I hope to highlight a number of strengths and limitations of this work and discuss some of the controversies raised before discussing the implications of my results in the context of the immunopathogenesis of Goodpasture's disease and potentially to autoimmunity in general.
8.2 Generation and utility of T cell hybridomas

Since the early reports of hybridoma production, a number of technical advances have been introduced to facilitate and improve the production of both B and T cell hybridoma clones therefore maximising the chance of generating peptide specific hybridomas.

Wood et al were the first to describe a mouse T cell hybridoma capable of specifically responding to human PBMC. The mouse used to generate the hybridomas was an HLA-DR4 transgenic mouse (Woods, 1994) but since this time little further work has been carried out to further characterise and utilise this technique. Indeed, until recently, few groups had attempted to evaluate the use of HLA transgenic mice to construct HLA class I and II restricted T cell hybridomas capable of recognising peptide MHC complexes on human APC.

Canaday et al described methods for systematic production of T cell hybridomas related to human HLA alleles and their application to study human APC function involving processing of Influenza A, Mycobacterium TB and tetanus toxin (Canaday, 2003). More recently Von Delwig et al and Boots et al have utilised T cell hybridomas generated from mice TG for human HLA DR1 and DR4 respectively in the study of processing of different epitopes in the study of collagen-induced arthritis (Von Delwig, 2006, Boots, 2007). Chou et al have also generated a number of humanised myelin peptide specific T cell hybridomas from mice TG for human DRB1*1501 (Chou, 2004). To our knowledge, this is the first description of T cell hybridoma production restricted to human HLA and specific for disease associated peptides to a known human renal auto antigen.

I generated 7 DR15 restricted hybridomas capable of recognising the α3(IV)NC1 derived disease associated peptide, ISLWKFGSFI. The hybridomas were peptide specific, and recognition was restricted to presentation of ISLWKFGSFI on DR15 expressing APC. Canaday et al demonstrated hybridoma responses on exposure to 1x10^4 macrophages and 2x10^3 dendrite cells. In my hands, the smallest number of EBV transformed B cells and THP-1 cells used was 2.5x10^4. Hybridomas responded in a consistent manner although it became clear at an early stage that 2 of the 7 hybridomas, ha3p132.3 and
ha3p132.15 responded better in terms of IL-2 production. In addition, the viability of these cells was good. They consistently expressed both TCR and CD4 and the T cell populations remained clonal. For this reason these lines were selected for use in most of the future experiments.

One of the biggest setbacks in the course of my research was the difficulty in generating hybridomas that were specific to other disease associated peptides, in particular, the other major disease associated peptide, dap71-90. The peptide, AS345 (SPFLFCNVNDVCNFASRND) was used to prime mice and again used with in vitro recall responses. The difficulties encountered in generating AS345 specific hybridomas were unfortunately numerous. Problems initially related to the use of transgenic mice which co-expressed mouse class II with human HLA DR15. Although 4 T cell hybridomas were generated that were specific for AS345, none of these were restricted to presentation by DR15. Mouse class II KO/DR15+ transgenic mice were subsequently generated and used for all further immunisations. However, difficulties were then encountered maintaining healthy cell lines prior to fusion. Initial attempts resulted in the loss of T cell lines after the fourth round of re-stimulation despite demonstrating in vitro recall response on retrieval of splenocytes from immunised mice. Subsequent attempts adopted an alternative approach where fusion was attempted after only 1 round of re-stimulation. This carried the risk that peptide specific T cell clones would not be selected out and indeed, despite the production of greater than 40 healthy T cell clones, none were specific for AS345. A final attempt established T cell lines using lymph nodes from primed mice (which contain around 80% T lymphocytes as opposed to 10% in splenocytes). Despite once again demonstrating in vitro recall responses to AS345, none of the subsequent hybridomas generated recognised this peptide.

Although the reasons for failure to generate DR15 restricted T cell hybridomas against this particular peptide are not clear, it was apparent in later experiments that part of the problem was an inability to maintain a sustained response to peptide prior to fusion. One explanation is the presence of 2 cysteine amino acids in AS345 which form a disulphide bond within the peptide. This in turn alters the conformation such that AS345 forms a
loop-like structure. It was possible that peptide used to immunise mice may have been reduced thus altering the conformation and immunogenicity of the peptide.

Although results with other disease associated peptides were perhaps disappointing, I had generated a number of T cell hybridomas against the disease associated peptide, ISLWKGFSDFI. These hybridomas were a powerful tool with which to examine aspects of $\alpha_3(IV)$NC1 processing. The particular advantage of T cell hybridomas over mass spectrometry is their specificity. Although mass spectrometry has the advantage of exquisite sensitivity, it generates a complex array of peptides in which the identification of specific peptides of interest can be difficult (particularly if peptides are expressed at low levels). Hybridomas, in contrast, can be used as "peptide detectors" to recognise specific peptide/HLA complexes presented on the surface of antigen presenting cells with high sensitivity. This targeting of specific disease associated peptides such as ISLWKGFSDFI, allows the influence of processing factors on individual peptides to be investigated.

8.3 Intracellular processing of intact $\alpha_3(IV)$NC1 is necessary before T cell hybridomas respond to DR15/ISLWKGFSDFI complexes.

Most exogenous antigens require intracellular processing before presented by class II on the surface of APC. There is evidence however demonstrating that cell surface MHC class II can be directly loaded with native protein if epitopes are located in structurally flexible and accessible regions (Sette, 1989). In addition, MHC class II molecules on the surface of fixed APC have been shown to directly bind and present extra cellular epitopes and fragmented proteins (Lindner, 1996, Aoi, 1997, Harris, 1996) and even denatured intact protein (Jensen 1991 & 1993, Carrisco-Marin 1998). The data show that this is not the case with $\alpha_3(IV)$NC1.

For the approach proposed to investigate the influences of endosomal proteolytic enzymes within the MHC class II processing pathway, it was crucial to demonstrate intracellular processing within APC was necessary before T cell hybridomas were capable of recognising DR15/ISLWKGFSDFI complexes on the surface of APC pulsed with
intact α3(IV)NC1. Different DR15 expressing APC including the human monocytic cell line THP-1 and several DR15 expressing EBV transformed B cell lines, were shown capable of stimulating 5 of the 7 DR15/ISLWKGFSFI specific hybridomas in a dose responsive manner when pulsed with intact α3(IV)NC1, but in every case processing was confirmed necessary by the inability of pre-fixed DR15 expressing APC to stimulate hybridomas. In contrast, pre-fixed APC pulsed with peptide did induce a response, implying that ISLWKGFSFI is almost certainly capable of binding DR15 directly on the surface of fixed APC. Response to antigen was specific and not due to LPS contamination.

The demonstration that processing of α3(IV)NC1 was required before the peptide specific T cell hybridomas could respond to DR15/P14 complexes on the surface of APC ensured that I could study putative factors thought important in the generation or destruction of the disease associated peptide, ISLWKGFSFI.

8.4 Influence of aspartate protease activity on processing of α3(IV)NC1 in human antigen presenting cells

Biochemical studies had strongly implicated cathepsin D as the enzyme responsible for initial unlocking and processing of α3(IV)NC1, it was logical that this should be the first proteolytic enzyme on which to focus.

As a first attempt to examine the influences of cathepsin D specifically on processing of α3(IV)NC1, I elected to use an in vitro approach by pre-incubating recombinant α3(IV)NC1 with cathepsin D prior to pulsing the digested protein into APC. This had the advantage of focusing on cathepsin D specifically which had been identified in biochemical studies as a putative enzyme in the destructive processing of ISLWKGFSFI/dap131-150 (Zou, 2007).

Results from this set of experiments demonstrated that there was indeed a fall in IL-2 production by the T cell hybridoma, ha3p132.3 in cells were co-cultured with APC pulsed with cathepsin D-digested α3(IV)NC1 compared with intact α3(IV)NC1. This approach
had some limitations in that the concentration of cathepsin D and subsequent digestion of α3(IV)NC1 was non-physiological. The results however confirmed biochemical data showing a reduction in presentation of ISLWKGSFI peptide following digestion of intact α3(IV)NC1 with cathepsin D.

To examine the effect of natural levels of cathepsin D on presentation of ISLWKGSFI peptide, the presentation of DR15/ISLWKGSFI in DR15 expressing APC pulsed with abundant intact α3(IV)NC1 or ISLWKGSFI peptide was compared using hybridoma, ha3132.3. In the presence of natural levels of B cell aspartate protease activity ISLWKGSFI-presentation by B cells was greater for B cells incubated with ISLWKGSFI-containing peptide than molar equivalent quantities of intact α3(IV)NC1. This supports the suggestion that aspartate protease activity in B cells diminishes presentation of the ISLWKGSFI epitope, as predicted from the earlier in vitro studies and preliminary work with cathepsin D pre-digestion experiments. It was difficult to control for these experiments, however, due to the uncertainty of knowing the exact concentration of antigen compared to peptide used each the assay. This was due to variation in quantifying stock concentration of protein antigen. Nevertheless, it was a consistent finding in experiments that despite incubation with Cathepsin D for up to 120 minutes or comparing the presentation of peptide in B cells pulsed with either ISLWKGSFI-containing peptide or molar equivalent quantities of intact α3(IV)NC1 in the presence of natural levels of aspartate proteases, B cells were still capable of presenting ISLWKGSFI albeit at a significantly reduced level in comparison with intact α3(IV)NC1.

The data suggested that although rapid destruction by endosomal aspartate proteases can account for a reduction in ISLWKGSFI presentation, sufficient ISLWKGSFI to stimulate T cells can be presented by DR15-expressing B cells under particular conditions, such as culture in high concentrations of α3(IV)NC1. This may help explain how many of the stimuli reported increase the turnover of basement membrane α3(IV)NC1 (thus flooding the system) are associated with Goodpasture’s disease (Phelps, 2000).
8.5 Inhibition of cathepsin D and influence on processing of α3(IV)NC1

Crucial to further studies was the confirmation that cathepsin D was the responsible proteolytic enzyme for the destruction and resultant reduction in surface presentation of ISLWKGFSFI peptide.

There are now a number of methods by which cathepsin D activity can be reduced or effectively abolished. Microbial protease inhibitors are known to inhibit the intracellular proteases of living cells in a non-cytotoxic manner. They also have the advantage of being site specific and have a small molecular weight so that they can easily penetrate the cell membrane (Puri, 1988). For these reasons I elected to use the specific aspartate protease inhibitor, Pepstatin A to inhibit Cathepsin D activity.

Experiments involving the aspartate protease inhibitor, pepstatin A, demonstrated partial inhibition of aspartate protease activity where pepstatin A increased ISLWKGFSFI-presentation on intact α3(IV)NC1 in a dose dependent manner. In cells pulsed with α3(IV)NC1 that were neither fixed nor washed prior to co-culturing with ha3p132.3, a tenfold increase in IL-2 production in the supernatant was observed. One explanation for this could be increasing expression of surface DR15/P14 complexes with time. Alternatively, the proteolysis of IL-2 has been attributed to a large extent Cathepsin D/E. Ohnishi et al demonstrated a ten fold increase in the half life of IL-2 in mice injected with the cathepsin D inhibitor, pepstatin A (Ohnishi, 1990). In vitro work has also supported a role of aspartate proteases on IL-2 production where pepstatin A significantly inhibited IL-2 production in ova specific T cell hybridomas (Mizuochi, 1994, Rodriguez, 1992). This may well be of relevance in the experiments where cells were neither washed nor fixed prior to co-culture with the T cell hybridoma ha3p132.3. To address this concern, cells were lightly fixed then washed after incubation with peptide and antigen, ensuring no pepstatin remained prior to co-culture. An increase in IL-2 in the 24 hour co-culture supernatant was seen as before, suggesting this was a true observation and not a direct effect of pepstatin on IL-2 production.
DR15 expressing EBV transformed B cells, in the presence of pepstatin A demonstrated a clear increase in expression of DR15/ISLWKGFSFI after pulsing with intact recombinant α3(IV)NC1. Results were more difficult to interpret in the case of THP-1 cells where it was not possible to identify even a trend in the level of expression of DR15/ISLWKGFSFI in cells pulsed with either ISLWKGFSFI-containing peptide or intact α3(IV)NC1 in the presence of pepstatin A.

8.6 Influence of different antigen presenting cells on processing of intact α3(IV)NC1.

The role of different professional antigen presenting cells such as dendritic cells, B cells and macrophages in the initiation and maintenance of autoimmune responses is well documented. They have been demonstrated to play a pivotal role in the pathogenesis of autoimmune diseases where they present self-epitopes to auto reactive T cells in animal models of autoimmunity such as RA and MS (Bayry, 2004, Holmdahl, 1991, Panayi, 2005, Dittel, 1999) but the differences in processing and presentation of auto antigen are less clear.

B cells are certainly effective APC and antigen-specific B cells may be important in the initiation of autoimmunity (Roth, 1996, Panayi, 2005). Human EBV transformed B cells from healthy volunteers were initially decided upon as APC as they provided a range of B cells homozygous for DR15, the class II molecules strongly associated with disease susceptibility. Previous work within the lab had utilised EBV transformed B cells in the study of α3(IV)NC1 processing using biochemical approaches and methods had therefore already been well established by which B cells could be effectively pulsed with recombinant antigen resulting in efficient uptake and subsequent processing.

Both quantitative and qualitative differences have been documented between antigen-specific and non-specific B cells. Clearly antigen-specific B cells would have been more desirable (Simitsek, 1995, Watts, 1993) as would α3(IV)NC1-specific B cells from patients with active and untreated Goodpasture's disease. It should also be borne in mind that EBV transformation may alter aspects of the phenotype of B cells including the level of
surface class II expression and possibly the efficiency of antigen presentation (De Campos-Lima, 1993). It is not known however whether antigen processing is qualitatively different in transformed B cells.

It was for this reason, as well as to investigate whether any differences in processing could be observed between alternative DR15 expressing cell lines, that I elected to include a different APC. THP-1 cells are a DR15,1 expressing monocytic cell line that can be differentiated into a macrophage phenotype on exposure to PMA and induced to express MHC class II by IFNγ (Sakamoto, 1999). It is also interesting to note that IFNγ has been described as capable of inducing apoptosis in THP-1 cells (Inagaki, 2002) which may explain the poorer cell viability at time points of 18-24 hours and reduced response to both peptide ISLWKGFSFI and α3(IV)NC1 in general. (discussed later)

Marked variation in the uptake, processing, presentation and dissemination of antigen between APC has been described. Differences in protease content is a clear example where macrophages have been shown to contain high levels of lysosomal proteases which rapidly degrade proteins whereas DC and B cells were protease poor, retaining antigen for longer with a more limited capacity for degradation. This in turn however favoured antigen presentation (Delamarre, 2005). Whether a similar explanation could account for differences in the presentation of ISLWKGFSFI over time between EBV transformed B cells and THP-1 cells would have been a further avenue of interest had time allowed. Unfortunately, preliminary studies using the cathepsin D assay to determine the level of aspartate protease activity within each cell line proved problematic as discussed below.

8.7 Influence of antigen conformation on α3(IV)NC1 processing

The observation from processing experiments in both B cells and THP-1 cells demonstrated that if APC were incubated with abundant α3(IV)NC1, ISLWKGFSFI was still presented as assessed by IL-2 production by ISLWKGFSFI specific hybridoma, ha3p132.3. If rapid destruction by the endosomal protease, cathepsin D, is postulated to cleave and destroy ISLWKGFSFI, how can this peptide ever be presented to ha3p132.3?
One explanation lies in the knowledge that APC were incubated with high concentrations of $\alpha$3(IV)NC1, suggesting that in the presence of abundant antigen, the system may be 'flooded' allowing sufficient ISLWKGFSFI to stimulate ha3p132.3. An alternative explanation is variability in the conformation of recombinant antigen and its influence on processing and presentation of ISLWKGFSFI peptide.

Although $\alpha$3(IV)NC1 was made with the human sequence, it differed from native $\alpha$3(IV)NC1 in a number of structural respects. Quaternary structure is quite different where most native $\alpha$3(IV)NC1 is complexed with other type IV collagen chains into hexamers that are stable to significant protease and denaturant attack. The structure of recombinant protein is monomeric. Structure has been shown to influence the presentation of other complex proteins such as hCG (Rouas, 1993) haemoglobin (Atassi, 1989) In addition, patients with Goodpasture's disease have antibodies to epitopes within $\alpha$3(IV)NC1 which may in turn protect or enhance presentation of certain peptides as shown in other antigens such as tetanus toxin (Simitsek, 1995).

Recombinant protein was subjected to a refolding protocol that promoted disulfide bond formation allowing some degree of folding and disulfide bond formation, but it is likely that several conformations of recombinant $\alpha$3(IV)NC1 may have resulted. This may account for the incomplete effects of cathepsin D pre-digestion of $\alpha$3(IV)NC1 on abrogating ISLWKGFSFI presentation. This may also explain the presentation of ISLWKGFSFI peptide on APC pulsed with recombinant $\alpha$3(IV)NC1 in the presence of natural levels of aspartate proteases.

Just as the conformation of protein antigen may influence the processing of antigen, there is evidence to suggest that the processing of antigen by alternative pathways may influence the T cell repertoire.

There is a substantial body of evidence supporting different populations of T cells (type A and type B) that recognise alternative conformational isomers (conformers) of the same MHC-peptide complexes (Viner, 1995, 1996, Peterson, 1999, Pu, 2002).
Studies of HEL and its presentation by I-AkCD4+ T cells primed by immunisation by the dominant peptide processed from HEL segregate into two subsets; type A T cells which recognise a MHC-peptide complex formed in the APC with exogenous peptide that recapitulates that of complexes formed by intracellular processing of native protein. There is a second subset (type B) that recognise the complex formed by peptide derived from exogenous protein but not the complex from identical peptide derived from that of intracellular native protein. (Viner, 1995 & 1996, Peterson, 1999, Pu, 2002). The importance of this phenomenon was demonstrated with the observation that type B T cells that escape thymic negative selection can peripheralise in conditions where type A T cells to the same epitope undergo complete thymic central deletion. This was demonstrated by Peterson et al with type B T cells reactive to the 48-62 HEL epitope in mice transgenic for HEL expression (Peterson, 1999) and Lovitch et al with type B T cells recognising an abundant autologous epitope in wild-type B10.BR mice (Lovitch 2003). This latter finding suggested that self-reactive type B T cells are present in the naïve repertoire where their activation can result in autoimmune pathology.

Pu et al demonstrated that type A and type B conformers are generated in distinct vesicles with type A conformers forming in late endosomes/lysosomes and type B conformer restricted to early endosomes and the cell surface and in addition this restriction is mediated through the action of DM (Pu, 2002).

As type B reactivity is not explained by failure of APC to generate peptide during processing, post-translational modification of the peptide or peptide binding in an alternative register, it has been hypothesized that formation of MHC-peptide complexes in distinct intracellular compartments may underlie the generation of the two conformers.

8.8 Confirmation of Cathepsin D as the proteolytic enzyme responsible for reduced expression of DR15/ISLWKGFSFI

Results from chapter 5 where pre-digestion of α3(IV)NC1 with purified human cathepsin D reduced presentation of ISLWKGFSFI peptide and chapter 6 where inhibition of cathepsin D increased presentation of DR15/ISLWKGFSFI strongly support a role for
cathepsin D in α3(IV)NC1 processing. Proteolytic enzymes have been implicated in the destruction of epitopes in other studies (Rodriguez, 1995) and interestingly, cathepsin D has been implicated in the destructive processing of myoglobin (Watts, 2003, Moss, 2005). Redundancy amongst proteases is well documented and this could prevent detection of important protease involvement in single-protease knockout experiments. Phelps et al have demonstrated that Cathepsins D and E have similar activity in processing of α3(IV)NC1 (Zou, 2008).

Difficulties with both the cathepsin D assay and the use of cathepsin D resistant modified antigen (discussed in chapter 6) meant that it was not possible to identify cathepsin D conclusively as the proteolytic enzyme responsible for the reduced surface expression of DR15/ISLWKGFSFI.

However, biochemical data from our lab along with results from pre-digestion experiments with cathepsin D and inhibition experiments using the cathepsin D/E specific inhibitor provided strong evidence supporting the hypothesis that cathepsin D or a proteolytic enzyme with similar substrate specificities was responsible for the destructive processing of the disease associated peptide ISLWKGFSFI.

8.9 Alternative approaches to confirm the role of cathepsin D in α3(IV)NC1 processing

Time constraints unfortunately prevented me from selecting an alternative approach to confirm or refute the role of cathepsin D in the processing of α3(IV)NC1. Discussed below are a number of possible approaches used to abrogate the effect of cathepsin D that have been employed in other areas of research.

1. Site directed mutagenesis

The use of site directed mutagenesis to alter or delete an amino acid thought critical in the cleavage and subsequent processing of a protein or peptide has been used for some years now to confirm the identity of putative enzymes in processing (Abboud-Jarrous, 2005, Dunn, 2000). The alternative approach of mutagenesis of cathepsin D itself has
also been used. Glondu et al used this approach to assess the mitogenic role of cathepsin D in the proliferation of cancer cells. Mutagenesis in this respect abrogated the proteolytic activity of cathepsin D although it maintained its ability to stimulate cells (Glondu, 2001).

2. siRNA

RNA interference has become in recent years a powerful tool for understanding gene function. The use of siRNA has been applied to investigate the role of cathepsin D in programmed cell death (Gui, 2006) as well as antigen processing. Singh et al used siRNA to knock out cathepsin D in macrophages thus demonstrating a crucial role in the processing and presentation of an immunodominant epitope Ag85B from mycobacterium tuberculosis. (Singh, 2006). This technique would be an attractive alternative approach to the use of aspartate protease inhibitors. This could be of particular importance in delineating the specificity of cathepsin D in processing of α3(IV)NC1 as available inhibitors such as pepstatin A are still capable of inhibiting other aspartate proteases such as renin and pepsin and cathepsin E.

3. Cathepsin D Knockout

As the study of lysosomal proteases got underway in the 70s and 80s it was noted that genetic defects in lysosomal proteases had not as yet been described. This suggested that the defect of one protease may be compensated for by another i.e. a degree of redundancy was present or that a deficiency may in fact be lethal early on in embryonic life (Dean, 1975).

Saftig et al described a cathepsin D -/- mouse generated by gene targeting. These mice were found to develop normally in the first 2 weeks but stopped thriving in the third week due to mucosal atrophy and subsequent necrosis resulting in death by day 26+/-. There was also loss of lymphoid populations with the destruction of the thymus and spleen where the ratio of cell proliferation to elimination was altered to favour elimination with T and B cell populations particularly affected (Saftig, 1995).
Obtaining APC from cathepsin D -/- mice would have been an attractive alternative approach to examine the influence of cathepsin D on α3(IV)NC1 processing. However in practice, this would not have been possible due to the restricted lifespan of cathepsin D -/- mice and the resultant inability to cross breed with mouse class II KO DR15 mice.

8.10 Cathepsin D and apoptosis

In the last few years several reports have implicated Cathepsin D in apoptosis (Turk, 2000, 2001, 2002). The role of cathepsin B in apoptosis was described recently by Guicciardi (2000) and Stoka (2001). Schestkowa et al induced apoptosis in Hela cells and human fibroblasts by microinjecting either mature cathepsin D or its inactive precursor pro cathepsin D into the cytosol. The results confirmed that its effects were independent of its catalytic activity (Schestkowa, 2007). The molecular mechanisms of cell death promotion from lysosomal proteases is not yet clearly understood. A pro-apoptotic member of the Bcl-2 family, Bid appears to be central in cathepsin (including cathepsin D) -mediated apoptosis however. Bid is a linker between extrinsic (via death receptors) and intrinsic (mitochondrial) pathways (Li, 1998, Luo, 1998). How Bid is activated by proteases is not clear but Cathepsin D is linked to the Bid signaling pathway and may act as a downstream target (Heinrich, 2004). The role of cathepsins in mediating apoptosis is likely to be complicated and involving many mechanisms. Understanding of these mechanisms is however improving and we know that different cell types can favour different routes and/or mechanisms of cell death. Whether the role of cathepsin D in mediating apoptosis in either the EBV transformed B cells or THP-1 cells has any influence of the results from the processing experiments detailed above is still purely speculative.

8.11 Implications of observations and immunopathogenesis of disease

Although a considerable amount of work has been published examining the influence of proteases including aspartate proteases on the processing of protein antigens, little has been published examining the processing of autoantigen.
This work has demonstrated that presentation of a key epitope in Goodpasture's disease is susceptible to destruction by the aspartate protease, cathepsin D. In EBV transformed B cells the level of aspartate protease activity was sufficient to diminish presentation of peptide despite this peptide having high affinity for HLA DR15 molecules. In addition, presentation was significantly enhanced in the presence of the protease inhibitor, Pepstatin A.

The potential importance of destructive processing in autoimmunity was first demonstrated by Manoury et al where they demonstrated the influence of AEP on the presentation of a key epitope from myelin basic protein, the target of autoimmunity in multiple sclerosis (Manoury, 2002).

This work supports the hypothesis that destructive processing can prevent or reduce presentation of self peptides to potentially auto reactive T cells and in doing so, shape autoimmunity to α3(IV)NC1 and establish self-tolerance. As suggested in animal models of autoimmunity (Moudgil, 2000), cathepsin D cleavage of α3(IV)NC1 and AEP cleavage of MBP may be human examples of antigen processing that generates "cryptic epitopes" that is to say epitopes with high class II binding affinity but low constitutive presentation. Self-proteins as they are constitutively presented such that T cells that recognise epitopes from self-proteins that are not usually presented or cryptic epitopes may be less subject to control (Elson, 1995, Gammon, 1989). This has implications in turn for their ability to develop a pathogenic autoimmune response in certain conditions.

Recent work published by Zou et al has demonstrated that healthy individuals as well as patients have auto reactive T cells specific for epitopes in the Goodpasture autoantigen (Zou, 2008). Of interest, the 6 peptides which evoked a response in >50% of the healthy individuals was not caused by selectivity of HLA class II but rather was a result of susceptibility to Cathepsin D digestion where there was a high correlation between susceptibility to cathepsin D digestion and the capacity to stimulate primary T cell responses.

There remains the key question however, that if destructive processing does account for the presence of auto reactive T cells in the peripheral repertoire by suppressing
constitutive presentation of self-epitopes, how are these epitopes presented in the disease state to drive the autoimmune response?

A number of possible explanations exist. Antigen processing may be modulated by for example bound antibodies (Antoniou, 2002, Simitsek, 1995) or by the actions of aberrant APC (Hall, 2005). Non-autoimmune inflammation may alter accessibility extra cellular modifications of APC or indeed their handling by APC or responsive T cells. Of note in Goodpasture’s disease there are associations with disease onset and certain inflammatory events such as renal vasculitis, renal trauma and membranous nephropathy (Phelps, 1996). In addition to creating an inflammatory response, many of these conditions may result in an increase turnover of glomerular basement membrane and in theory an increase in production of α3(IV)NC1. The data from my own work demonstrate that destruction by aspartate proteases can account for a deficit if ISLWKFGFSFI but importantly it also demonstrated that sufficient ISLWKFGFSFI to stimulate T cells can be presented by both DR15-bearing B cells and macrophages under certain circumstances such as culture high concentrations of α3(IV)NC1 and that partial inhibition of aspartate protease activity is sufficient to substantially increased its presentation.

Therefore the expression of ISLWKFGFSFI is likely to be low level rather than absent and the level of presentation a balance which may be influenced by positive factors such as abundance of α3(IV)NC1 (perhaps determined by basement membrane turnover) and negative factors such as activity of endosomal proteases.

Although the transformation of this work into a therapeutic approach is clearly still some years away, this work adds to that of others demonstrating that individual enzymes do make clear and non-redundant contributions to the processing of key epitopes in certain autoimmune diseases. I hope this in turn would help encourage efforts to manipulate potential vaccine candidates in order that processing is re-tuned to processing machinery.
8.12 Future work

The work generated from this thesis has firstly provided a powerful tool in which to dissect the influences of processing on the generation or destruction of α3(IV)NC1 peptides recognised by patients' T cells. It was perhaps a disappointment that I managed to generate hybridomas that were specific and HLA DR15-restricted for only one of a number of α3(IV)NC1 potential peptides of interest. This particular disease associated peptide was however one of two major self-epitopes recognised by patients' T cells and therefore of key interest in this work. The demonstration that cathepsin D activity could significantly diminish presentation of this α3(IV)NC1 peptide has helped to provide strong evidence supporting the hypothesis that destructive processing may account for the survival of self reactive T cells in the mature peripheral repertoire.

This work has also resulted in an opportunity to go on to study many other aspects of the processing in autoimmune disease.

Firstly, are other disease associated peptides in Goodpasture's disease and other autoimmune diseases influenced similarly by aspartate proteases? Biochemical approaches in our lab have shown this to be the case. Generation of T cell hybridomas specific for other disease associated peptides as well as naturally processed α3(IV)NC1 peptides would help elucidate if the destructive propensity of aspartate proteases on these peptides also occurred in live DR15 expressing human APC.

Secondly, for epitopes such as ISLWKGFISFI whose processing influences presentation, it is likely that disease initiation depends on a change in presentation. These methods described may enable the study of other factors postulated to influence aspects of uptake and processing of α3(IV)NC1. Of particular interest would be the influence of conformation and steric effects on α3(IV)NC1 and subsequent processing. Although bacterial recombinant techniques have advanced considerably since their first description, the protein yielded is unfolded and its conformation less accurate. The generation of native α3(IV)NC1 would be the ideal although yield can often be low. Alternative production techniques such as the use of insect cell lines to generate
α3(IV)NC1 could also be considered. Other conformational influences would also be of interest such as the importance of disulphide linkages and the degree of unfolding of α3(IV)NC1. The steric effects of α3(IV)NC1 bound to patients’ antibodies or in complex with other chains have been shown in other studies to influence the processing and resultant expression of disease associated peptides.

Finally, preliminary work examining the influence of DR7 co-expression on the processing and expression of DA peptides such as ISLWKGFSFI generated intriguing results. Future work determining if DR7 was responsible for increased presentation of ISLWKGFSFI peptide involving either more specific DR7-blocking peptides or further attempts at DR7 transfection into DR7- cell lines would be desirable. If this was demonstrated clearly, study of the influences of DR7,15 co-expression on other disease associated peptides would be fascinating. In a similar vein, given the association of other HLA class II molecules with disease susceptibility and protection, it would be relatively easy to perform similar processing experiments with a wide range of DR15/non-DR7 heterozygous human APC. Examination of the co-expression of a different class II molecule and potential influence on presentation of various DA peptides would be fascinating—particularly if level of DA peptide expression on certain HLA DR15/non-DR7 combinations correlated with genetic studies.
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Publications

Results from this study have been presented at a number of national and international meetings including: The Renal Association, London 2004 and Brighton 2007; The Scottish Society of Experimental Medicine, Edinburgh 2005; The European Renal Association, Glasgow 2006; The Scottish Renal Association, Aberdeen 2006 and The American Society of Nephrology, San Diego 2006.

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