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Functional consequences of single nucleotide polymorphisms in ERAAP

By

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ABSTRACT

Optimal peptide loading of MHC class I molecules is essential for antigen presentation to $CD8^+$ Cytotoxic T lymphocytes. Endoplasmic Reticulum Aminopeptidase associated with Antigen Processing (ERAAP) is responsible for N-terminal trimming of peptides to the optimal length for stable loading and presentation on MHC class I. In humans, ERAAP is also known to play a role in pro-inflammatory cytokine receptor cleavage as well as regulating blood pressure and angiogenesis. Recently, a single nucleotide polymorphism (SNP) linkage analysis study has identified ERAAP as being associated with increased risk of the autoimmune inflammatory disorder, ankylosing spondylitis (AS). A HapMap comparison of AS positive patients against normal controls revealed susceptible and protective ERAAP alleles.

In this study, SNP mutation of ERAAP is shown to alter the ability to trim peptides and facilitate IL-6R cleavage from the cell surface. Transfection of $ERAAP^{-/-}$ cells with individual SNP mutant hERAAP revealed a hierarchy of reduced function. Trimming function was further reduced when selected double SNP mutants were generated. Significantly, a mutant hERAAP, incorporating all the SNPs identified in the linkage analysis, completely abrogated its trimming function. The consistent reduction in activity of K528R and R725Q SNPs highlight these amino acids as important for ERAAP trimming function. Analysis of ERAAP alleles and haplotypes from AS patients identified novel polymorphic combinations which demonstrated a defective trimming activity in comparison to those identified in control samples. This has important implications on the role of these SNPs within ERAAP and the susceptibility of AS.

Although the mechanism for the effect of SNP mutation on ERAAP function is unclear, it appears that they cause a dramatic effect on trimming of N-terminally extended peptides.

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DECLARATION OF AUTHORSHIP

I, Emma Reeves, declare that the thesis entitled

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Definitions and abbreviations

ACE	Angiotensin converting enzyme
ADAM	Metalloprotease disintegrin
AITD	Autoimmune thyroid disease
A-LAP	Adipocyte derived Leucine Aminopeptidase
Allele	Individual ERAAP molecules
APCs	Antigen presenting cells
APM	Antigen processing machinery
ARTS1	Aminopeptidase Regulator of TNFRSF1a Shedding 1
AS	Ankylosing Spondylitis
ATF6	Activating transcription factor-6
ATP	Adenosine triphosphate
β_2m	β2-microglobulin
BH	Bleomycin hydrolase
BiP	Immunoglobulin binding protein
CDRs	Complementary determining regions
CLIP	Class II associated invariant chain peptide
CNX	Calnexin
CPRG	Chlorophenol red-beta-D-galactopyranoside
CRT	Calreticulin
CTL	Cytotoxic T lymphocytes
DCs	Dendritic cells
DRiPs	Defective ribosomal proteins
DTT	Dithiothreitol
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked-immuno-sorbent assay
ER	Endoplasmic Reticulum
ERAAP	ER aminopeptidase associate with antigen processing
ERAAP$^{-/-}$	ERAAP deficient fibroblasts
ERAD	ER associated degradation
ERAP1	ER aminopeptidase 1
ERAP2	ER aminopeptidase 2
ES	ER signal sequence peptide
Haplotype	Two ERAAP alleles

HC	Heavy chain
hERAAP	Human ERAAP
hERAAPtr	hERAAP truncated splice variant
HLA	Human Leukocyte antigens
HLA-B27	Human Leukocyte Antigen – B27
HPV	Human papillomavirus
hsp70	Heat shock protein 70
hsp90	Heat shock protein 90
IAA	Iodoacetamide
IFN-γ	Interferon- γ
Ig	Immunoglobulins
Ii	Invariant chain peptide
IL-1RII	Type II IL-1 receptor
IL-6	Interleukin-6
IL-6R	Interleukin-6 receptor
IP	Co-immunoprecipitation
IRE1	Inositol-requiring 1 homologue
JAK	Janus Kinases
KIRs	Killer immunoglobulin-like receptors
LAP	Leucine aminopeptidase
LILR	Leukocyte immunoglobulin-like receptors
L-RAP	Leukocyte derived arginine aminopeptidase
MAPKs	Mitogen-activated protein kinases
MHC	Major histocompatibility complex
MIIC	MHC II containing compartment
MS	Multiple sclerosis
Mutant	hERAAP containing six SNPs
nsSNPs	Nonsynonomous polymorphisms
NUCB2	Nucleobindin 2
PA28	Proteasome activator subunit
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
PDK1	Phosphatidylinositol-dependent kinase 1
PI3K	Phosphatidylinositol-3-kinase
PILSAP	Puromycin-insensitive leucyl-specific aminopeptidase

PKC	Protein kinase C
PLC	Peptide loading complex
PMSF	Phenylmethyl sulfonyl fluoride
P-RAP	Placental leucine aminopeptidase
PSA	Puromycin sensitive aminopeptidase
RA	Rheumatoid Arthritis
RBMX	RNA-binding motif gene, X chromosome
RP-HPLC	Reverse-Phase High Performance Liquid Chromatography
S6K	p70 s6 kinase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHL8	SIINFEHL
siRNA	Small interfering RNA oligonucleotide
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphisms
SpA	Spondyloarthropathies
STAT	Signal transducers and activators of transcription
TACE / ADAM 17	TNFα converting enzyme
TAP	Transporter associated with Antigen Processing
Tapasin	TAP associated glycoprotein
T_H2	T helper cells
TNF	Tumour necrosis factors
TOP	Thimet oligopeptidase ()
TPPII	Tripeptidyl peptidase II
TRiC	TCP-1 ring complex
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome system
VEGF	Vascular Endothelial Growth Factor
WTCCC	Wellcome Trust Case Control Consortium
X5-SHL8	AIVMK-SIINFEHL
X6-SHL8	LEQLEK-SHL8

Chapter 1: Introduction

1.1 The Immune system

The human immune system has developed over millions of years and is a vital part of the body's defense mechanism to protect from potentially harmful invading pathogens. The immune response has evolved in order to deal with advancing pathogenic mechanisms able to survive destruction by the immune system. Innate immunity is present from birth and is the first line of defense against infecting pathogens, consisting of both barrier mechanisms and chemical components. This initial response acts quickly but with little specificity. Adaptive immunity is a defense mechanism built up over time with exposure to different antigens and acquires the ability to recognise previous foreign pathogens through immunological memory, eliminating them more efficiently. This type of immunity is antigen-specific and is mediated by B and T lymphocytes with antigen receptors present on the cell surface. The genes for these receptors undergo somatic recombination, which enables a wide diversity of receptors for specific antigens.

1.2. B-Lymphocytes

The B cell produces immunoglobulins (Ig) which are able to recognise pathogens that have not undergone any form of processing. These Ig molecules consist of two light chains linked to two heavy chains by disulphide bonds. The earliest B cell precursor is the pre-pro B cell which differentiates into pro-B cells. V(D)J immunoglobulin gene rearrangement does not occur in the earliest B cell precursor, but once differentiated into the pro-B cell, this recombination can begin (Allman et al., 1999). Pro-B cells later mature into pre-B cells in the presence of stromal cells within the bone marrow. Pre-B cells proliferate and differentiate into immature B cells expressing IgM and are transported to secondary lymphoid organs such as the spleen and lymph nodes. Within the periphery, these cells further mature into mature B cells which are dependent on the specificity of the B cell receptor expressed (Levine et al., 2000). Activation of cells through the interaction of membrane bound Ig with specific antigens results in clonal expansion. Activation of B cells through different stimuli such as T cell mediated activation, causes class switching by which the variable heavy chain region of Ig can associate with the constant region of any isotype of Ig, varying the biological effect through change in isotype expression (Stavnezer et al., 2008). These cells will go on to differentiate into ether memory B cells or plasma cells that produce and secrete Ig in the form of antibody.

1.3. T-Lymphocytes

T cells express receptors that recognise antigens processed and presented on the cell surface through the major histocompatibility complex (MHC) molecule. Recognition of the peptide:MHC complex is aided by a co-receptor, either $CD4^+$ in the case of T helper cells, or $CD8^+$ on cytotoxic T lymphocytes (CTL). These two types of T cells recognise antigen presented on MHC class II (MHC II) and MHC class I (MHC I) molecules respectively and it is the latter that is of interest within this study. $CD8^+$ CTL are an important component of the adaptive immune response to invading viral, bacterial and protozoan pathogens (Townsend and Bodmer, 1989). These pathogens are able to infect the cell, however their proteins are processed within the cytosol to generate peptide antigens which are presented on the cell surface in complex with the MHC I molecules. Circulating $CD8^+$ CTL will recognise this antigen as 'foreign' through the T cell receptor expressed on its cell surface and go on to elicit an immune response by causing clonal expansion of $CD8^+$ CTL. The CTL then release interleukins and cytotoxins like perforin to induce apoptosis of the cell containing 'foreign' peptide to eliminate infected or transformed cells (Townsend & Bodmer, 1989).

1.4. Recognition of peptide:MHC I complex

The T cell receptor of $CD8^+$ CTL is able to recognise peptide:MHC I complexes on the surface of cells. The receptor consists of two chains, α and β chain, which both contain transmembrane regions, anchoring the receptor to the cell surface (figure 1.1). Both chains consist of constant (C) domains and variable (V) domains and in these V domains of both the α and β chains, there are complementary determining regions (CDRs). These are hypervariable loops that are responsible for the recognition of the peptide:MHC I complex. Both α and β chains have 3 CDRs, CDR1, CDR2 and CDR3 and it is CDR3α and CDR3β loops that are the most variable and capable of contacting the MHCI and peptide antigen (Katayama et al., 1995). CDR1 and 2 are able to contact the MHC I but have little interaction with antigenic peptide. Binding of the TCR to the MHC I is aided by the co-receptor CD8 molecule, which is able to pro-long the interaction of the T cell with the antigen presenting cell (Hennecke and Wiley, 2001).

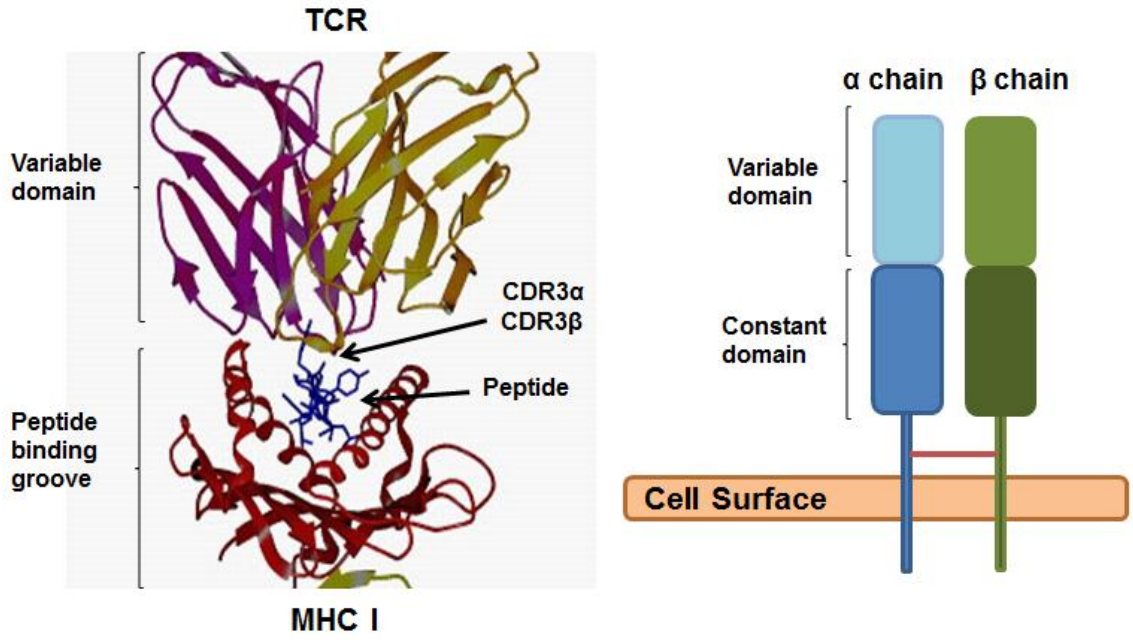

Figure 1.1. Structure of the T cell receptor

TCR consists of an α and β chain both containing a constant and variable domain. The CDR3α and CRD3β are responsible for the contact between peptide:MHC I and the TCR. Sourced from http://nfs.unipv.it/nfs/minf/dispense/immunology/mhcstr.html (pdb accession: 1ao7).

1.5. Antigen Processing and Presentation

Processing of peptide antigen for loading on to the MHC molecule is essential for immunosurveillance by T lymphocytes. The generation of peptide antigen for presentation on MHC molecules occurs by three different pathways. Firstly, the endogenous pathway processes proteins derived within the cell into smaller fragments which are expressed at the cell surface following binding to MHC I. Secondly, the exogenous pathway processes peptide antigens through endocytosis of circulating peptide antigens and are subsequently directed to lysosomes resulting in loading on to MHC II molecules. The final pathway is termed cross presentation. This process involves the processing and presentation of exogenous antigen on MHC I molecules. MHC molecules are highly polymorphic and encoded by the MHC gene region located on chromosome 6 in humans, spanning 3.6Mb, and chromosome 17 in mice. The MHC region is divided into three regions, MHC I, II and III regions. MHC I encodes the Human Leukocyte antigens HLA-A, -B and -C in humans and histocompatibility 2 –D (H2-D) H2-K and H2-L in mice. MHC II encodes HLA-DP, -DQ, -DR and –DO in

humans and H2-A and H2-E in mice. MHC III encodes other immune system components such as factors involved in the complement system.

1.5.1. Endogenous antigen presentation pathway

Endogenous peptide antigens are presented through the expression of the MHC I and are present on the surface of all nucleated cells within the body. These MHC I have the capacity to present an array of peptide antigens generated within cells to circulating $CD8^+$ CTL. These CTLs are able to monitor cells for the presentation of 'foreign' peptides from invading pathogens or malignancies within those cells (Townsend & Bodmer. 1989). The MHC I has three components; the heavy chain (α-chain, HC), an invariant β2-microglobulin (β_2m) subunit and the peptide antigen (figure 1.2). The HC can be one of three subsets, human leukocyte antigen (HLA) -A, -B or –C in humans, corresponding to the positioning within the MHC gene region of chromosome 6 (Ortmann et al., 1997). This HC is constructed of three domains; α1, α2 and α3. The α1 and α2 domains fold to form the peptide binding cleft, which is known to be highly polymorphic to increase the diversity of peptide antigens presented. This is able to increase the T cell repertoire to provide optimal immune responses to non-self peptides (Parham et al., 1988; Zernich et al., 2004). The α3 domain of the HC contains a transmembrane region that is essential for anchoring MHC I to the plasma membrane at the cell surface (Madden, 1995). The invariant β_2m is a member of the immunoglobulin family of proteins and is not encoded by the MHC gene region. This protein associates with the HC and lies laterally to the α3 subunit. The final component of MHC I is peptide antigen. To generate stable surface expression of MHC I, optimal peptide loading is required. Antigenic peptides for MHC I are specific in length, usually 8-9 amino acids, which are able to bind and stabilise the MHC I complex (Elliott et al., 1991b; Falk et al., 1991). Binding of peptide antigen occurs through a network of hydrogen bonds between the N- and C-termini and the peptide binding groove, with studies showing conformational changes occur in this region upon binding of peptide (Elliott et al., 1991a). At one end of the peptide binding groove is a deep pocket, in which specific residues interact with the preferred hydrophobic C-terminal of the peptide and its associated side chains, stabilising the binding (Elliott, 1997). Therefore optimal N- and C-termini for stable binding are essential.

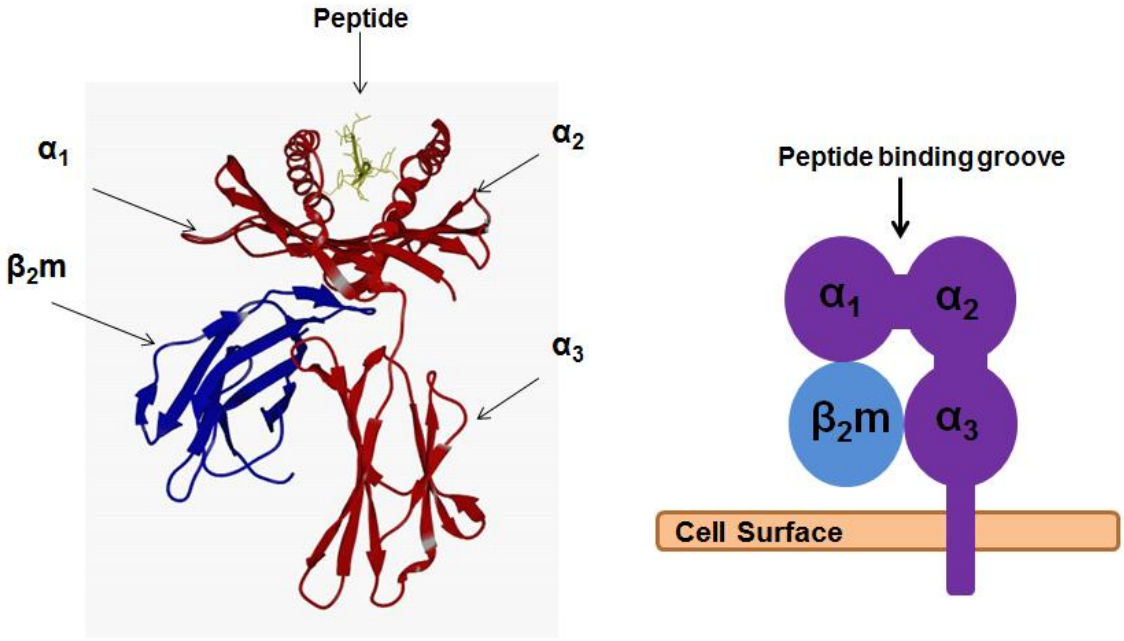

Figure 1.2. Structure of the MHC I

MHC I consists of β_2m, HC and peptide. The α domains of heavy chain fold to form the peptide binding groove between α1 and α2 where optimal peptide binds. Sourced from http://nfs.unipv.it/nfs/minf/dispense/immunology/mhcstr.html (pdb accession: 1mhc).

MHC I folding from an immature state to the mature complex takes place within the lumen of the endoplasmic reticulum (ER). Proteins folded within the ER, such as MHC I, are destined for secretory pathways. The folding of MHC I occurs in association with the peptide loading complex (PLC), which consists of four main components; ERp57, calreticulin, tapasin and TAP (Hirano et al., 1995; Sadasivan et al., 1996). Through interactions with the PLC, MHC I molecule can be stably folded in the presence of optimal peptides, exits through the trans-golgi and is presented at the cell surface to circulating $CD8^+$ T cells. As this antigen processing pathway is a major focus of this study, components of this pathway will be discussed in more detail below.

1.5.2. Exogenous antigen presentation pathway

Dendritic cells (DCs), monocytes/macrophages, B cells and thymic epithelium are known as professional antigen presenting cells (APCs) and express MHC II molecules at the cell surface. These specific cells have the ability to internalise exogenous antigens by endocytosis into lysosomal and endosomal vesicles. They will then be processed and loaded onto MHC II for presentation at the cell surface to circulating

$CD4^+$ T helper cells (T_H2) which can initiate the appropriate immune response. This pathway consists of compartments that increase in acidity, the early endosome (pH6-6.5), the late endosome (pH5-6) and the lysosome (pH4) which aids the processing and stable loading of antigenic peptide onto the MHC II.

The MHC II molecule is synthesised and folded in the ER and consists of two homologous membrane spanning proteins, approximately 30kDa in size, that associate non-covalently to form a heterodimeric complex. These α (light) and β (heavy) chains contain two domain regions, α1, α2 and β1, β2. The MHC II is tethered to the membrane through the membrane spanning domains of α2 and β2. The α1 and β1 domain, situated furthest from the membrane, define the peptide binding groove and is formed of 2α helices above a β-pleated sheet (figure 1.3). Antigenic peptides of 15-20 amino acids are the optimal length for peptide binding (Rudensky et al., 1991). The peptide binding groove is the site with most variability within the MHC II in order to stably bind and present a vast range of antigenic peptides. In combination with this, the two ends of the peptide binding groove are in an open conformation and have smaller residues, such as glycine and valine, to allow longer length peptides to bind. The open ended nature of the peptide binding groove provides potential problems with binding of longer and less specific peptides rather than the optimal peptides required. However these potential problems are overcome by the binding of class II associated invariant chain peptide, CLIP (Gautam et al., 1995).

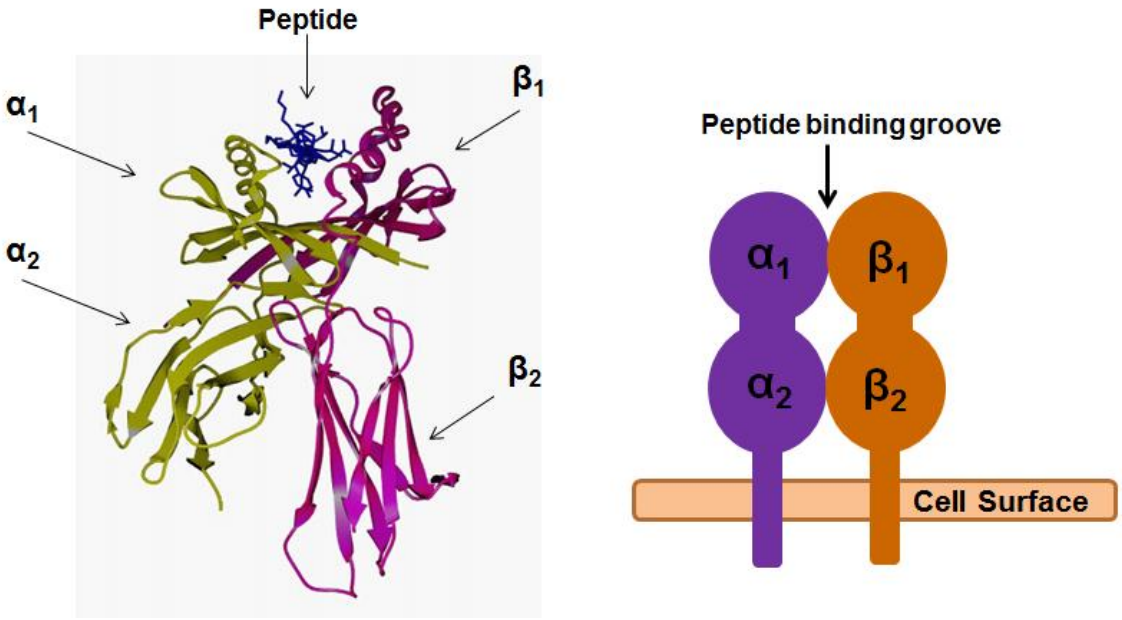

Figure 1.3. Structure of the MHCII

MHC II consists α (light) and β (heavy) chains and peptide. The α and β chains each contain two domains with the α1 and β1 domains form the peptide binding groove. Sourced from http://nfs.unipv.it/nfs/minf/dispense/immunology/mhcstr.html (pdb accession: 1dlh).

Folding and association of the MHC II α and β chains occurs in the ER, however antigenic peptides are internalised into lysosmal vesicles and stable loading of peptide occurs in MIIC compartments (known as MHC II containing compartment (Watts, 2004). In the ER, a preassembled protein trimer, known as the Invariant chain (Ii), is able to associate with the class II αβ chain and a small part of this Ii, known as CLIP, can occupy the peptide binding groove blocking both endogenous and premature peptides from binding whilst in the ER (Roche and Cresswell, 1990; Stumptner and Benaroch, 1997). The Ii also contains a cytosolic di-leucine-targeting motif which guides the MHC II either directly from the trans-golgi network, or indirectly by internalization, into the endocytic pathway where antigen loading can occur in the MIIC compartments (Neefjes et al., 1990). In preparation for antigenic peptide loading, the Ii is degraded by several late endosomal proteases such as cathepsin S and L to leave the small CLIP fragment still occupying the peptide binding groove. These cathepsins are responsible for this degradation as cathepsin S and L deficient mice were shown to have reduced Ii degradation activity and antigen presentation (Nakagawa et al., 1998; Shi et al., 1999). Proteolytic cleavage, low pH and the presence of a non-classical

MHC II (HLA-DM) found in endosomal compartments is required to catalyse the exchange of CLIP for exogenous peptide antigen in sub-compartments of the MIIC (Sloan et al., 1995). Upon stable binding of exogenously derived peptide onto the MHC II, it can be expressed at the cell surface to $CD4^+$ T cells.

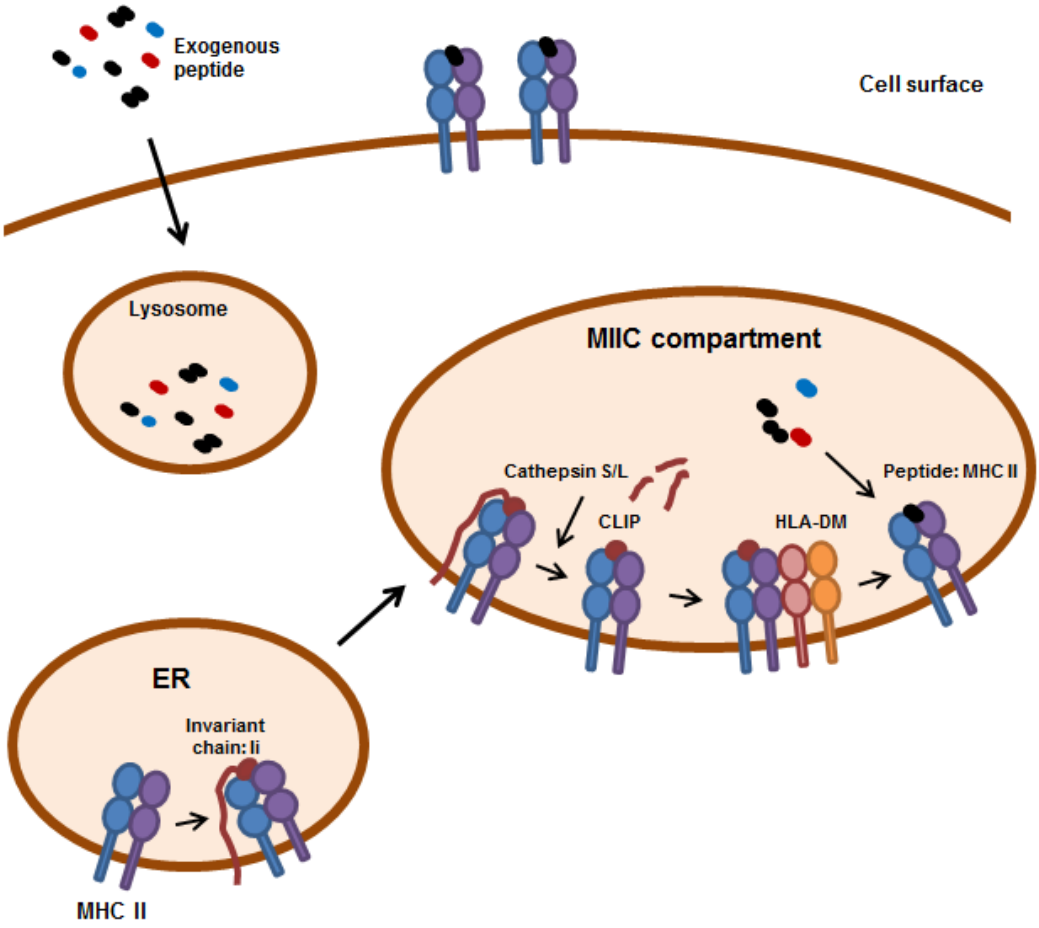

Figure 1.4. The exogenous processing pathway

Endogenous peptides are endocytosed or phagocytosed into endosomes. Immature αβ class II heavy chain associates with Invariant chain (Ii), which is degraded leaving a small fragment bound in the peptide binding grove (CLIP). HLA-DM aids the dissociation of CLIP and association of the αβ chain with peptide antigen for expression at the cell surface.

1.5.3. Cross-presentation of exogenous antigens

Exogenous peptide antigen can be internalised and processed but loaded and expressed at the cell surface on MHC I, a process termed cross-presentation. This form of antigen presentation occurs in DCs and macrophages that express both MHC I and II and have the capacity to internalise exogenous antigens and induce $CD8^+$ T cell

response to pathogens that have not directly infected the cells. There are two defined pathways of cross-presentation, Transporter associated with Antigen Processing (TAP) independent and TAP-dependent. In the TAP-dependent pathway, exogenous antigens are internalised by endocytosis or phagocytosis. The antigens are then transported into the cytosol via transporters such as Sec61 and are degraded by the proteasome (Di Pucchio et al., 2008). The proteasome products are transported via TAP into the ER and loaded on to MHC I (Rodriguez et al., 1999). The TAP-independent pathway requires the internalisation of antigens as above, but these antigens are degraded by endosomal proteases. MHC I have been found within the endosomes and it is thought that MHC I trafficking into these compartments allow loading and expression of MHC I, though the mechanism of trafficking is unknown (Rock and Shen, 2005). A third pathway has recently been suggested in which the phagosomes may fuse with the ER to promote cross presentation (Guermonprez and Amigorena, 2005; Guermonprez et al., 2003). However this pathway still remains elusive.

1.6. Origin and generation of antigenic peptides destined for MHC class I presentation

Proteolytic systems are present within the cytosol of all cells to maintain a normal homeostatic cellular environment in response to protein production and folding. The proteasome, a multicatalytic enzyme residing within the cytosol is responsible for the turnover of the majority of cellular proteins. The proteasome undertakes selective protein degradation to maintain cellular protein concentration and prevent aggregation of misfolded or incorrectly folded proteins (Hughes et al., 1997). Selective protein degradation is important for many biological processes such as transcription factor activation, cell proliferation and differentiation, regulation of gene expression, responses to oxidative stress and for the generation of antigens for MHC I presentation (Goldberg et al., 2002; Pagano et al., 1995; Wang and Maldonado, 2006).

1.6.1. Generation of peptide antigens by the proteasome

The generation of antigenic peptide, the final component of MHC I, arises from the degradation of intracellular derived proteins into smaller peptide fragments of precise length. The proteasome is responsible for degradation of polyubiquitinated proteins targeted for degradation through the ubiquitin-proteasome system (UPS, (Goldberg et al., 2002). The 26S proteasome is the central proteolytic enzyme in this system, made from a 20S multiple subunit core and a 19S cap at either end of the core. The 20S

proteasome, responsible for the catalytic activity, is comprised of four stacked rings, each containing seven subunits (Groll et al., 1997). The two outer rings contain seven α subunits (α1-α7) and the two inner rings contain seven β subunits (β1- β7). Due to the N-terminal threonine residues on the subunits β1, β2 and β5, the β rings are responsible for the 6 sites of proteolytic activity of the 20S proteasome (figure 1.6). These three β subunits were shown to confer differing activity in hydrolysing fluorogenic substrates, referring to them as β1 caspase-like, β2 trypsin-like and β5 chymotrypsin-like (Groll et al., 1997; Heinemeyer et al., 1997). These sites are located on the interior of the ring, therefore the target peptide must enter the central core before degradation begins. The outer rings, made from the α subunits function as a 'gate' to maintain the protein entry into the core. The two 19S caps act as regulatory ATPases that can recognise and bind peptide targets with a polubiquitin chain. It is the S6' subunit within the 19S cap which is responsible for the recognition and binding of target proteins. Binding of ATP promotes the association of the 19S cap with the 20S proteasome, in turn activating the α subunits of the 20S core which act as 'gates' (Lam et al., 2002; Liu et al., 2006). Once ubiquitinated targets are bound, they are unfolded and translocated through the gated pore into the 20S core where they are hydrolysed into smaller peptides by threonine-dependent nucleophilic attack (Groll et al., 1997; Lam et al., 2002; Liu et al., 2006).

1.6.2. Ubiquitin-proteasome system

Ubiquitin-proteasome system (UPS) is a process whereby the target protein must firstly be ubiquitinated, a process involving three enzymes, E1, E2 and E3, which mediate this process and add at least four successive ubiquitin molecules to the protein (figure 1.5). After the ubiquitin chain has been added to the protein, it can be recognised and degraded by the 26S proteasome complex.

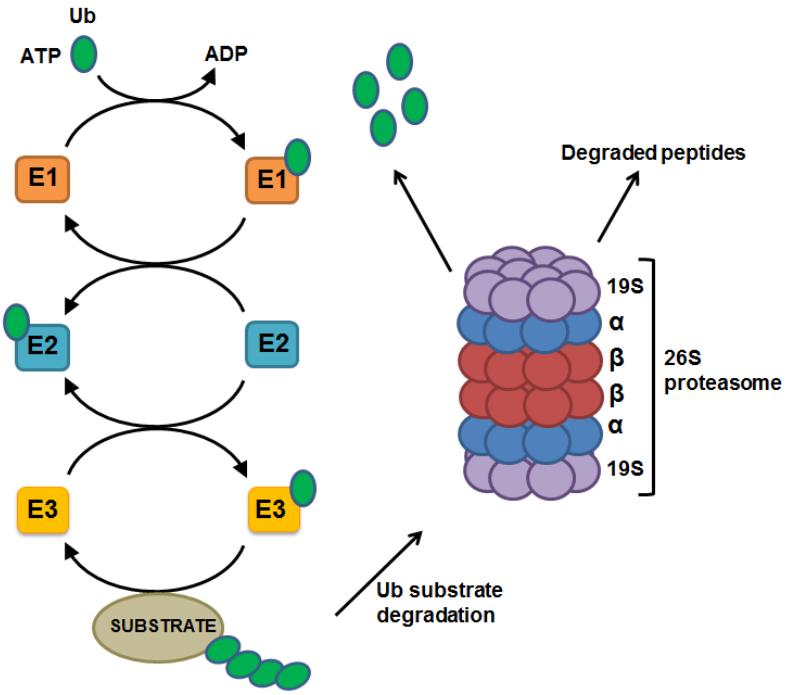

Figure 1.5. The ubiquitin-proteasome system

The substrate is targeted for proteasomal degradation by the addition of ubiquitin in an ATP dependent manner. Three enzymes, E1, E2 and E3 are involved in the transfer of ubiquitin to protein substrates, which requires the addition of a polyubiquitin chain for recognition and subsequent degradation.

Ubiquitin is a 76 amino acid protein which is attached to the protein substrate requiring degradation. Initially, the first enzyme involved in ubiquitination, E1, is activated in an ATP dependent manner and catalyses the activation of ubiquitin. One E1 enzyme is capable of activating many ubiquitin molecules required for modifications. Upon activation, the ubiquitin molecule is transferred to a cysteine residue of one of many ubiquitin conjugating enzymes, E2. This E2 enzyme is responsible for the transfer of ubiquitin to the ubiquitin ligase enzymes, E3 which can in some instances be substrate specific. Each E2 acts with either one or several E3's and it is the E3 that allows conjugation of ubiquitin to its target through a bond between the C-terminal glycine of ubiquitin and an amine group of a lysine on the target protein (Eletr et al., 2005) As there are many different E3 enzymes available there can be a wide range of targets for the ubiquitin proteasomal degradation pathway within a cell. Additional ubiquitin is added to the initial molecule on the lysine48 residue of the previous ubiquitin, generating a polyubiquitin chain which acts as a proteasome recognition signal for the targeted degradation of protein. A target protein is required to have at least four

ubiquitin monomers in the form of a chain before being recognised by the proteasome (Lam et al., 2002). After a protein has been ubiquitinated, it is recognized by the 19S regulatory particle in an ATP-dependent binding step and can enter the interior of the 20S particle to come in contact with the proteolytic active sites. Deubiquitination is coordinated with substrate translocation and proteolysis (Zhu et al., 2005).

1.6.3. Role of the immunoproteasome in the generation of antigenic peptides

The proteasome can preferentially cleave after hydrophobic and basic residues, creating the optimal C-terminal anchor for binding to the peptide binding groove of MHC I. Inhibiting the proteasome with specific inhibitors reduces the antigenic peptide supply and also almost completely abolishes MHC I presentation at the cell surface (Craiu et al., 1997; Schwarz et al., 2000). However, class I heavy chains are still synthesised but a stable peptide:MHC I complex formation does not occur due to lack of antigenic peptide, providing evidence that the proteasome plays a major role in the generation of antigenic peptides (Rock et al., 1994).

Like other antigen processing machinery, IFN-γ stimulation up regulates the expression of the proteasome. However in this instance, IFN-γ stimulation induces a change in the specific catalytic subunits of the 20S proteasome. Alternative subunits LMP2, LMP7, encoded within the MHC II gene region of chromosome 6, along with MECL1 are incorporated into the 20S proteasome in place of β1, β2 and β5 active subunits (Glynne et al., 1991; Kelly et al., 1991; Ortiz-Navarrete et al., 1991). This 20S proteasome with alternative active subunits is termed the immunoproteasome for its role in the generation of antigenic peptides (Wang and Maldonado, 2006).

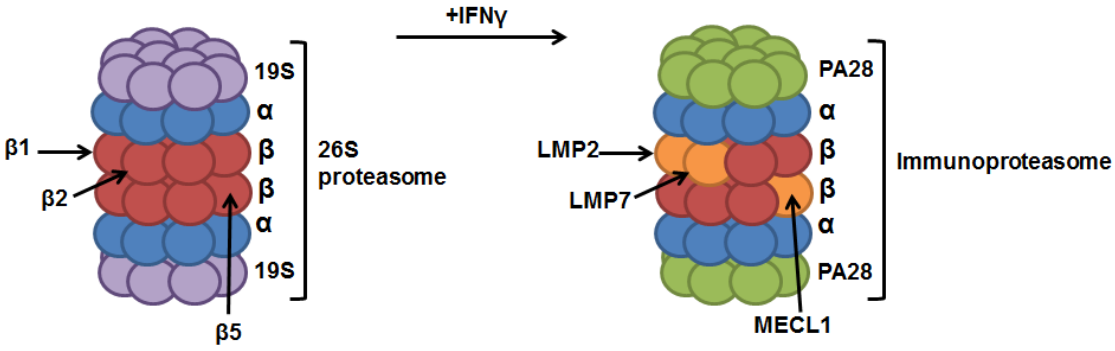

Figure 1.6 Structure of the proteasome and immunoproteasome

The proteasome consists of 20S subunit and 19S regulatory cap. The 20S subunit is formed of 2 outer α rings, containing 7 subunits, and 2 inner β rings also containing 7 subunits. Upon IFN-γ stimulation, the LMP2, LMP7 and MECL1 are up regulated and replace β1, β2 and β5 catalytic subunits.

The cleavage specificity of the immunoproteasome can influence both the C-terminal and N-terminal regions of antigenic peptides. In comparison to the proteasome, the immunoproteasome was not shown to generate an increase of SIINFEKL, an immunodominant epitope derived from ovalbumin, but had 4-6 times greater rate of producing N-terminally extended precursors of SIINFEKL. The alteration of the active site subunits in response to IFN-γ causes a change in cleavage site preference and an increase in cleavage rate (Boes et al., 1994). The increase in chymotryptic-like activity and decrease in the caspase like activity, and preferential cleavage at sites following hydrophobic, basic or asparagine residues could account for the increase in N-terminally extended peptides (Cascio et al., 2001; Driscoll et al., 1993; Ehring et al., 1996; Groettrup et al., 2010). The deletion of LMP2 or LMP7 genes in mice suppresses the presentation of certain antigenic peptides (Fehling et al., 1994). In addition to this, recent studies have shown that mice lacking all three alternative subunits (LMP2, LMP7 and MECL1) demonstrated both an impaired presentation of antigenic peptide epitopes and approximately 50% reduction in surface MHC I expression compared to wild type mice (Groettrup et al., 2010; Kincaid et al., 2012). Also, each alternative subunit has distinct functions in the elimination of pathogens, with mice lacking the LMP7 subunit unable to clear *Toxoplasma gondii* infection (Groettrup et al., 2010). The impaired presentation and alteration of viral clearance in the absence of the immunoproteasome further reinforces the importance of this alternative proteolytic enzyme within the generation of antigenic peptides for presentation on MHC I (Groettrup et al., 2010; Kincaid et al., 2012). IFN-γ stimulation also induces an

alternative regulatory subunit, proteasome activator subunit, PA28 (or 11S REG). PA28 is a ring-shaped multimeric complex and like the 19S cap it can bind both ends of the 20S core but does not share any homology with 19S regulatory unit (Dubiel et al., 1992; Ma et al., 1992). PA28 is thought to influence the uptake and cleavage of shorter peptides, playing an important role in the generation of antigenic peptides (Ma et al., 1992; Stohwasser et al., 2000). Although both normal and immunoproteasomes have the ability to generate MHC I epitopes, the immunoproteasome has a greater influence over the quantity of peptides available, therefore producing a greater number of epitopes that can be detected by pathogen specific $CD8^+$ T cell response (Chen et al., 2001). The immunoproteasome serves to preferentially cleave polypeptides and proteins after hydrophobic amino acids, which is conducive for MHC I loading. However, the production of peptides with an optimal length of 8-11 amino acids is in the small minority as shown by studies that indicate a large proportion of immunoproteasome products are either too short or too long to fit within the peptide binding groove and stabilise the MHC I molecule (Goldberg et al., 2002)

Antigenic peptides are often generated with the correct hydrophobic C-terminus but with an extended N-terminus and therefore require processing before stable loading on to an MHC I molecule can be achieved (Cascio et al., 2001). N-terminally extended peptides with a hydrophobic or basic C-terminal region are optimal peptides for translocation through the TAP into the ER, the site of MHC I biogenesis. Thus, two proteolytic steps must exist for the generation of antigenic peptides; proteasomal cleavage within the cytosol, generating the correct C-terminal region for MHC I binding and cleavage by proteases in the ER to generate the optimal N terminal region for stable MHC I complex formation (Craiu et al., 1997).

1.6.4. Source of antigenic peptides

The source of proteins for degradation comes from both properly folded and fully functional proteins involved in cellular processes and from misfolded or truncated proteins, termed DRiPs (defective ribosomal proteins) which arise from the defective protein synthesis and are rapidly degraded (Qian et al., 2006; Yewdell et al., 1996). As the proteasome aids the regulation of normal cellular processes it acts to control the amount of cellular protein present but also stops the accumulation of DRiPs. However, newly translated polypeptides are an important but not exclusive source of MHC I epitopes. Seifert et al, showed that under pro-inflammatory conditions, IFNs trigger a strong accumulation of oxidant damaged poly-ubiquitinated proteins and the

immunoproteasome is required to eliminate the aggresome like induced structures formed by an increase in accumulation of DRiPs. This implies DRiPs are a major source of antigenic peptide and the immunoproteasome acts to expand the antigen pool for presentation (Seifert et al., 2010).

Peptide precursors generated by the proteasome are not free floating within the cytosol of the cell but are bound to higher molecular weight proteins, i.e. chaperones. TRiC (TCP-1 ring complex) is a group II chaperonin that can transport intermediate peptide precursors generated by the proteasome throughout the cytosol. This chaperone has a protective role over a specific subset of peptides and acts to defend them from degradation on their route to the ER (Kunisawa and Shastri, 2003). In addition hsp90 (heat shock protein 90) also demonstrates a role in chaperoning proteins within the cytosol of the cell. Hsp90α inhibition saw a reduction in the level of correct MHC I folding most likely due to reduced loading of MHC I with stable antigen (Callahan et al., 2008). The hsp90α is known to associate with N-terminally extended peptides, and therefore along with TRiC, plays an important part in chaperoning these intermediate peptides along the course of the antigen processing pathway within the cytosol of the cell (Callahan et al., 2008; Kunisawa and Shastri, 2003). These chaperones direct the antigenic peptides generated within the cytosol of the cell to the TAP within the ER membrane that translocates these peptides into the lumen of the ER.

1.6.5. Non-proteasomal degradation of antigenic peptides

Various proteasome inhibitors have shown the key role of the proteasome in the generation of antigenic peptides for MHC I presentation (Rock et al., 1994). However, many MHC I molecules are still expressed even when high concentrations of proteasome inhibitors have been administered, suggesting a role for other cytosolic proteases in the generation of antigenic peptides (Rock et al., 1994; Vinitsky et al., 1997).

Tripeptidyl peptidase II (TPPII) is a rod-shaped cytosolic subtilisin-like peptidase that is larger than the 26S proteasome, exerting both exopeptidase and endopeptidase activity to generate antigenic peptides by cleaving the N-terminus of peptides longer than 15 amino acids in length (Geier et al., 1999; Reits et al., 2004). TPPII exhibits enhanced activity in proteasome-inhibited cells, potentially substituting for proteasomal activity (Geier et al., 1999). Reduced TPPII activity halts the processing of certain

virally derived epitopes, however the presentation of these epitopes are not altered with reduced proteasomal activity (Seifert et al., 2003).

Leucine aminopeptidase (LAP) was identified by Beninga et al as an IFN-γ inducible cytosolic protease (Beninga et al., 1998). Upon IFN-γ stimulation, LAP was able to trim LEQ-SIINFEKL to the final SIINFEKL optimal epitope in HeLa cytosolic extracts However, over-expression of LAP correlated with a decrease in MHC I expression having potential to destroy MHC I peptide ligands (Beninga et al., 1998; Reits et al., 2003; Towne et al., 2005). Conversely, LAP deficient cells generated normal MHC I responses and trimming was not reduced under both normal and increased IFN-γ conditions, indicating that although LAP can trim peptide precursors, it may not appear to be responsible for the generation of MHC I peptide ligands (Towne et al., 2005).

Thimet oligopeptidase (TOP) is a metalloendopeptidase cleaving peptides of between 9-17 amino acids. It is thought that this protease is primarily responsible for the degradation of a variety of antigenic peptides (Saric et al., 2001). Overexpression of TOP led to a decrease in antigen presentation and when TOP expression was reduced, MHC I antigen presentation increased (Kessler et al., 2011; York et al., 2003). These results suggest TOP acts primarily to inhibit antigenic peptide generation, through the destruction of a large proportion of antigenic peptides. However, in contrast to this, a small subset of antigenic peptides may be generated by the activity of TOP. It was revealed that by cleaving the C-terminal region of peptides, TOP was required for the generation of tumour specific and immunodominant CTL epitopes from both tumour associated PRAME and Epstein-Barr virus proteins (Kessler et al., 2011). In addition to this, nardilysin, another cytosolic endopeptidase, was shown to process peptides at both the N and C-terminal regions prior to processing activity by TOP in order to general the final peptide antigen (Kessler et al., 2011).

Bleomycin hydrolase (BH) is a cysteine protease of the papain family present within the cytosol. It is broadly expressed in human tissues, with up regulation seen in bleomycin-resistant tumours and cell lines (Ferrando et al., 1996). BH influences some antigenic peptide presentation, but its role when other aminopeptidases are present was shown to be largely redundant in the generation of MHC I ligands (Towne et al., 2007).

In 2010, Parmentier et al demonstrated the involvement of Insulin degrading enzyme (IDE) activity in the generation of antigenic peptides (Parmentier et al., 2010). Initially

identified for its ability to degrade insulin, IDE is a cytosolic metallopeptidase with ubiquitous expression. Although the cleavage specificity of IDE is currently poorly defined, it was shown to act independently of the proteasome to produce the MAGE-$A3_{168–176}$ peptide, from the MAGE-A3 protein, presented on HLA-A1 (Parmentier et al., 2010).

Puromycin sensitive aminopeptidase (PSA) is a 100kD cytosolic protease that was shown to cleave the extended N terminal residues from a VSV nuclear protein epitope (Stoltze et al., 2000). Reduction in PSA expression reduced the CTL mediated recognition of antigen presenting cells. Also, PSA has preference for hydrophobic or basic resides at position 1 and cannot cleave before or after proline or glycine residues (Levy et al., 2002). PSA was shown to cleave peptides that are not successfully cleaved by TOP, suggesting a role for PSA in the generation of peptide antigen. However, this enzyme is not up-regulated in response to IFN-γ stimulation (Saric et al., 2001). Although there are a number of proteases present within the cytosol, it is apparent that the proteasome/immunoproteasome provides the major source of antigenic peptides for MHC I loading.

1.7. MHC I folding and assembly in the Endoplasmic Reticulum

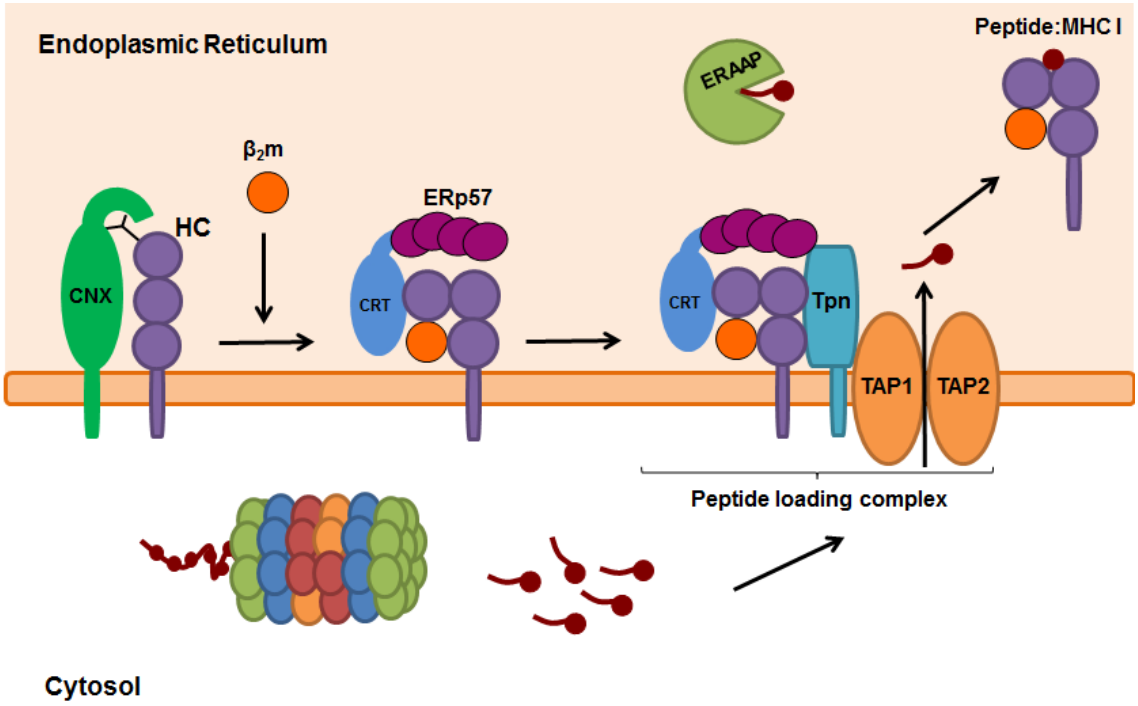

Figure 1.7. Assembly of the MHC I in the endoplasmic reticulum

Immature heavy chain associates with calnexin (CNX) before the recruitment of β_2m and ERp57 and subsequent association with Calreticulin (CRT) aids HC folding. The peptide loading complex; CRT, ERp57, Tapasin (Tpn) and TAP associate with the heavy chain and β2-microglobulin to load peptides generated within the cytosol that have undergone processing to the required length, forming the complete MHC I.

The endoplasmic reticulum facilitates the folding of many proteins destined for the secretory pathway. Within the ER, there are a number of protein chaperones that aid the appropriate folding of newly synthesised proteins through disulphide bond formation. These chaperones act as quality control machinery preventing the accumulation of unfolded polypeptides and in certain circumstances can initiate protein degradation. Such chaperones consist of immunoglobulin binding protein (BiP), GRP94, calreticulin (CRT) and calnexin (CNX). During the early stages of folding, immature MHC heavy chain can associate with the chaperone BiP, a member of the heat shock protein 70 family (Hsp70). Immature glycoproteins are initially synthesised with a core N-linked glycan to which 3 glucose residues are constitutively added. During the folding process, the first glucose residue is removed by glucosidase I and

further trimming is undertaken by glucosidase II to generate a monoglucosylated core glycan which is recognised by CNX and CRT. The chaperone activity of CRT and CNX is therefore dependent on the interaction with glycans of the newly synthesised proteins.

1.7.1. Calnexin and Calreticulin

CNX is a 65kDa transmembrane protein, including a single transmembrane helix and a cytoplasmic tail region containing an ER retention motif. CRT, the soluble homologue of CNX, is also resident in the lumen of the ER through the presence of retention and KDEL based retrieval motifs. Both proteins belong to the lectin-like family of chaperones resident within the ER. These chaperones are able to recognise and transiently bind to newly synthesised monoglucosylated N-linked glycans and encourage their folding (Leach et al., 2002; Leach and Williams, 2004). CNX and CRT have a lectin like globular domain which aids recruitment of glycans and can aid the sequestering of polypeptides to divert them away from degradation. CNX can also act in the targeting of terminally misfolded proteins for degradation. Interactions of CNX and CRT with glycans have a fast off-rate so the protein is able to bind and un-bind, cycling through the glycoprotein quality control pathway. If glucosidase II removes the third glucose, dissociation from CNX and CRT occurs. In this instance, the protein may follow one of three pathways. Firstly, if the protein is properly folded, it exits the ER. Secondly, if folding is incomplete, UDP-Glc:glycoprotein glucosyltransferase can re-add the glucose, allowing re-binding to CNX and CRT. Zhang et al has shown that UGT1 reglucosylated MHC I molecules bind to suboptimal peptides, suggesting a role for UGT1 in sensing optimal peptide loading (Zhang et al., 2011). Thirdly, ER associated degradation (ERAD) occurs in proteins that have resided in the ER in an immaturely folded state, eliminating the unfolded/misfolded proteins. As CNX is resident at the ER membrane, it is able to recruit and bind immature MHC I heavy chain (Danilczyk et al., 2000). The CNX and calreticulin chaperones recruit the thiol-dependent oxido-reductase ERp57, which has the ability to aid disulphide bond formation that can facilitate accurate folding of the immature heavy chain (Hirano et al., 1995).

1.7.2. ERp57

When two cysteine residues are within close proximity, a disulfide bond can form, and many proteins within the ER destined for the secretory pathway contain disulfide bonds that stabilise their folded conformation. The formation of disulfide bonds are dictated by the local redox conditions and results from the deprotonation of one cysteine thiol and

donation of two electrons to an acceptor such as oxygen (Sevier and Kaiser, 2002). The environment of the ER is relatively oxidizing which can partially enable spontaneous disulfide bond formation in folding proteins (Hwang et al., 1992). ERp57, a 57kDa member of the protein disulphide isomerase (PDI) family, is a glycoprotein specific oxidoreductase within the ER that facilitates the formation of disulphide bridges in folding glycoproteins. In vitro studies have shown ERp57 to exert reductase, oxidase and isomerase activity. The structure of ERp57 contains four thiolredoxin-like domains, a b b' a' with two redox-active motifs, CXXC (C = cysteine and X = any amino acid) conferring catalytic activity (a, a') and are separated by two redox inactive domains (b,b'). ERp57 also contains a QEDL motif at the C-terminal region, acting as an ER retention signal. The b' domain of PDI family members mediates interactions with substrates, and recognition is likely to involve exposed hydrophobic regions within proteins (Klappa et al., 1998; Pirneskoski et al., 2004). Members of the PDI family either act directly by binding with non-glycosylated proteins or indirectly through chaperones that assist glycoprotein folding. ERp57 is recruited by CNX and CRT to aid folding of nascent protein chains. Binding of CRT and CNX to ERp57 occurs through b' domain interactions of ERp57 with the P-domains of CRT and CNX and is further enhanced and stabilised by additional contacts between the b domain of ERp57 and the P domain (Frickel et al., 2002; Pollock et al., 2004; Russell et al., 2004).
After initial interactions of the nascent HC with CNX and CRT the recruitment of β_2m initiates the dissociation of CNX but remain associated with CRT due to it being monoglucosylated (Danilczyk et al., 2000; Farmery et al., 2000). ERp57 is able to facilitate disulfide bond formation within the membrane-proximal Ig-like α3 domain of HC (Zhang et al., 2006b) . A second disulfide bond between cys101 and cys164 residues within the α2 domain forms, with β_2m enhancing the stability of this bond (Warburton et al., 1994). The presence of CNX or CRT increases ERp57 activity towards glycosylated substrates, such as nascent MHC I heavy chains, and subsequently a disruption of CNX or CRT interactions with ERp57 prevents substrate interactions with ERp57. Zhang et al also found that reduction in ERp57 expression results in impaired HC oxidative folding but provided evidence that CNX is dispensable for the folding and assembly of MHC I HC (Sadasivan et al., 1995; Zhang et al., 2006b). In addition, reduction in β_2m results in the degradation of the majority of HC and low expression of misfolded MHC I (Warburton et al., 1994). In mouse B cells lacking ERp57, Garbi et al found that the level of H-2K^b surface expression was reduced by 50%. The recruitment of MHC I into complexes with TAP and tapasin as part of the PLC could occur, but dissociate more rapidly in the absence of ERp57.

Therefore ERp57 may not be essential for MHC I interacting with TAP and tapasin, but it acts to stabilise the PLC complex (Garbi et al., 2006).

1.7.3. Protein Disulphide Isomerase

PDI is a 55kDa protein member of the PDI family that shares 33% homology with ERp57. The structure of PDI is similar to that of ERp57, containing two redox active domains and two redox-inactive domains. PDI has the capacity to interact with nascent or incorrectly folded proteins through two peptide binding sites, one in the b' and one in the C terminal region, 57 residues after the active a' domain. PDI functions to facilitate disulphide bond formation and facilitate early folding of immature proteins. The role of PDI within the PLC remains controversial and there are many contradictions within the literature. Two independent groups have identified PDI in association with TAP (Park et al., 2006; Santos et al., 2007). Park et al found PDI interacting with TAP and plays a role in regulating the oxidation of the α2 disulphide bond in the peptide binding groove, aiding optimal peptide selection and stabilising the MHC I into a peptide receptive state (Park et al., 2006). However, alongside this, several other groups have failed to detect PDI associating with TAP (Kienast et al., 2007; Peaper et al., 2005; Rufer et al., 2007).

1.7.4. Tapasin and Transporter associated with Antigen Processing

Transporter associated with Antigen Processing (TAP) belongs to the large family of ABC transporters. This family of transporters utilise ATP to translocate substrates across membranes (Deverson et al., 1990). TAP consists of two ATP-hydrolysing subunits, TAP1 and TAP2 which forms a heterodimer, with both subunits containing transmembrane domains. Both subunits are required for antigen translocation and successful incorporation into the PLC for peptide loading (Androlewicz et al., 1994; Kelly et al., 1992). This ER membrane spanning protein contains a hydrophobic membrane spanning domain and a hydrophilic cytoplasmic nucleotide binding domain, which are common to all ABC transporter family members. The peptide binding to TAP is an ATP independent process; however translocation from the cytosol into the ER requires ATP hydrolysis (Neefjes et al., 1993). The nucleotide binding domains, located within the cytoplasm, are involved in ATP binding and hydrolysis. TAP has been shown to transport peptides between 8-21 amino acids in length into the ER in vitro and are likely to transport 8-15 amino acid peptides in vivo (Schumacher et al., 1994). Therefore, N-terminally extended peptides are preferred and can be transported into the ER through TAP, where they are processed to the appropriate length before loading on to MHC I (Cascio et al., 2001; Goldberg et al., 2002). TAP is required to

associate with MHC I through associations with the PLC in order to supply peptides for MHC I binding (Grandea et al., 1995; Ortmann et al., 1994). The function of TAP can be inhibited by viral proteins. ICP47 is a protein encoded from the Herpes simplex virus and can bind to TAP, inhibiting the peptide translocation, subsequently reducing peptide supply to nascent MHC I (Fruh et al., 1995). Inhibition of peptide transport through down regulation of TAP expression is correlated with a loss of MHC I expression in malignant cervical carcinoma (Cromme et al., 1994).

TAP associated glycoprotein (Tapasin) is a 48kDa glycoprotein, and along with TAP, plays an important role in generating the stable peptide:MHC I complex. Tapasin interacts with TAP through N-linked glycans and the N-terminal helices of TAP1 and TAP2 domains (Koch et al., 2006). Although the presence of tapasin is independent of TAP, this association is essential for loading of TAP dependent peptides (Sadasivan et al., 1996; Tan et al., 2002). Tapasin serves as a bridge between MHC I, TAP and calreticulin and is essential for ERp57 to associate with the TAP-tapasin complex by formation of a disulphide bond (Hughes and Cresswell, 1998; Sadasivan et al., 1996). Tapasin may also act as a quality control of peptides that bind to MHC I and retain immature MHC I in the ER. Low affinity peptide pre-loading may be required for associations with tapasin, subsequently dissociating from the complex, allowing optimal peptides to bind through TAP associated peptide loading (Paulsson et al., 2001). Tapasin can recruit ERp57 into the PLC to form a semi-stable disulphide linked heterodimer through the Cys95 residue on tapasin and the Cys57 residue within the N-terminal thioredoxin active site region of ERp57 (Dick et al., 2002). All tapasin within the PLC are stably disulphide linked to ERp57 demonstrating that this association affects the stability of the PLC. Dong et al also found that this dimer is further stabilised by non-covalent interactions between the a' domain and tapasin (Dong et al., 2009). Tapasin deficiency has severe effects on most MHC I alleles (Barber et al., 2001), however a subset of MHC I alleles are unaffected by this deficiency, being termed tapasin independent. In majority of cases, tapasin deficiency results in few MHC I expressed at the cell surface with a decreased stability and ability to present peptide antigens (Williams et al., 2002) The loading of these high affinity peptides may then cause the dissociation of MHC I complex from tapasin and the PLC to exit via the trans-golgi to be expressed at the cell surface (Diedrich et al., 2001).

Tapasin can influence peptide repertoire, as tapasin competent cells have a greater presentation of more stable peptides with a longer half-life compared to peptides with

shorter half-lives, indicating the MHC I-tapasin interaction is essential (Howarth et al., 2004). Correlating with this, the HLA-B8 allele is able to form in the absence of tapasin but is much less stable than those expressed in the presence of tapasin, indicating the connection between stability and conformational state of MHC I after peptide binding. Some studies, however, have indicated a subset of MHC I alleles that are independent of tapasin for loading of peptide and optimal cell surface expression (Williams et al., 2002). Tapasin is involved in editing the repertoire of peptides on MHC I, acting to replace sub-optimal peptides with a fast off-rate with higher affinity optimal peptides. This editing activity of tapasin is most optimal when occurring as part of the fully formed PLC and increases both the loading rate and the stability of peptide:MHC I at the cell surface. Amino acid residues at positions 114 and 116 of the MHC I F pocket region are thought to be the major determining factors of MHC class I dependence upon tapasin (Park et al., 2003; Williams et al., 2002). As tapasin shows little variation in expression or polymorphisms, it is the highly polymorphic nature of the peptide binding groove that alters the dependence on such molecules for optimal peptide loading (Park et al., 2003).

1.8. Endoplasmic Reticulum Aminopeptidase associated with Antigen Processing

The immunoproteasome is responsible for the generation of majority of the antigenic peptides displayed by MHC I. The proteasome generates the C-terminal of the antigenic peptide appropriate for MHC I binding. However, TAP, the transporter responsible for translocation of antigenic peptides from the cytosol to the ER, can transport peptides with N-terminal extensions (optimum 12-13 amino acids), with peptides up to 40 amino acids in length being transported (Schumacher et al., 1994). TAP cannot transport peptides with a proline residue at position 1-3, however some MHC I have been shown to optimally bind peptides with proline at p2. As the peptides are often N-terminally extended, ER proteolysis and processing of the peptides to generate the optimal N-terminal region for MHC I binding are essential. It was identified in 1995 that proteolysis of peptides is not restricted to the cytoplasm and trimming of N-terminal residues to generate the optimal peptide length for binding can occur in the ER (Elliott et al., 1995). A number of studies have indicated the presence of proteolytic activity in the ER to result in the stably loaded MHC I molecules for presentation at the cell surface.

1.8.1. Identification of ERAAP in the generation of peptide antigens

The processing of N-terminally extended peptides in the ER requires the activity of an aminopeptidase. In 2002, two independent groups identified an aminopeptidase responsible for N-terminal peptide trimming; ER aminopeptidase associate with antigen processing (ERAAP) in mice and ER aminopeptidase (ERAP1) in humans (Saric et al., 2002; Serwold et al., 2002). Mouse ERAAP was identified from solubilisation and fractionation of microsomes derived from mouse liver and spleens at a similar time to the identification of human ERAAP through isolation from HeLa S cells (Saric et al., 2002; Serwold et al., 2002). The involvement of ERAAP in antigen processing was first indicated when recombinant protein was incubated with peptides that had N-terminal extensions. In the presence of human ERAAP the N-terminally extended peptides disappeared, correlating with increased ERAAP activity. However, when human ERAAP was incubated with shorter 8mer peptides, ERAAP activity ceased (Saric et al., 2002). In mouse cells, ERAAP expression was knocked down using RNA interference and as a consequence, MHC I expression at the cell surface was reduced. These ERAAP knockdown cells were unable to generate the 8mer SIINFEHL (SHL8) peptide from the N-terminally extended (X7-SHL8), 15 amino acid precursor. Conversely, the 8mer SHL8, which did not require trimming, was unaffected (Serwold et al., 2002). These data provided the evidence that ERAAP was associated with N-terminal peptide trimming and thus the peptide supply for antigen presentation (Saric et al., 2002; Serwold et al., 2002).

York et al showed that limiting ERAAP expression increased the number of overall peptides available for MHCI binding (York et al., 2002). Elimination of ERAAP expression results in a reduction in peptide generation in the form of N-terminal trimming. Many N-terminally extended peptides are generated by the proteasome and undergo final trimming on N-terminal extensions by ERAAP within the ER (Hammer et al., 2006; York et al., 2002). ERAAP proteolytic activity is controlled by the length of the substrate, with activity ceasing when the peptide reaches 8-9 amino acids in length, the optimal length for high affinity MHC I binding (Saric et al., 2002; York et al., 2002). ERAAP cannot be compensated by another aminopeptidase within the ER, showing ERAAP to be an exclusive aminopeptidase responsible for generation of MHC I ligands (Hammer et al., 2006). Expression of ERAAP is greatest in tissues with high expression of MHC I molecules and further indications of ERAAP activity in antigen processing was noted when the protein was up-regulated by IFN-γ. Upon treatment

with IFN-γ, ERAAP up regulated processing activity, however, in the absence of IFN-γ, there was limited presentation and can even be seen to destroy some peptide epitopes (Saric et al., 2002; Saveanu et al., 2002; Serwold et al., 2002; York et al., 2002).The localisation of ERAAP has varied in different studies. Initially, ERAAP was shown to be secreted into culture medium when over-expressed in COS-7 cells therefore being reported as a secretory protein (Hattori et al., 1999). It was then termed a cytosolic protein due to its function in promoting angiogenesis, but also was shown to localise at the plasma membrane due to its involvement in facilitation of cytokine receptors (Cui et al., 2002; Miyashita et al., 2002). However since then ERAAP has been shown to localise as a soluble monomeric protein within the ER, and although does not contain an ER retention sequence or KDEL motif, it localises with proteins containing such motifs (Kanaseki et al., 2006; Saric et al., 2002; Serwold et al., 2002). ERAAP is EndoH sensitive, confirming its presence in early secretory compartments and studies further confirmed its localisation to the ER (Serwold et al., 2002). It is plausible, since ERAAP has been shown to interact with Nucleobindin 2 (NUCB2) and PDK1, that depending on its differing functions, the aminopeptidase can alter its cellular localisation through altering its binding partners (Adamik et al., 2008; Islam et al., 2006; Yamazaki et al., 2004).

1.8.2. ERAAP substrate specificity

The generation of ERAAP deficient mice further shows the importance of the role of ERAAP in generation of peptide supply. In such mice, the levels of classical H-2K^b and H-2D^b were reduced. Also, the non-classical MHC Ib molecule Q-a2 shows a reduction in expression compared to wild type cells. When treated with IFN-γ, the reduction was still apparent but less pronounced than steady-state. A reduction was also seen in CD8 CTL responses in ERAAP deficient cells (Yan et al., 2006). In conjunction with this, Firat et al also generated ERAAP deficient mice and equally saw a decrease in H-2K^b and H-2D^b expression, analysed by flow cytometry. As expected, MHC II levels were unaffected. Upon IFN-γ treatment, similar to the recent study by Yan et al, they did not see a significant difference in MHC I levels from fibroblasts compared to WT, however immature-DCs still displayed a reduction in MHCI expression compared to WT. ERAAP has also been shown to be required for efficient cross-presentation (Firat et al., 2007; Yan et al., 2006).

In ERAAP deficient mice (ERAAP KO), each of the five mouse MHC I molecules (H-2K^k, K^b, D^b, D^d, L^d) were reduced in surface expression compared to wild type.

However H-2L^{d} expression significantly decreased by approximately 70% compared to 20% for the other 4 MHC molecules. The expression of MHCII molecules was the same in wild type and ERAAP deficient cells. The H-2L^{d} molecule, most affected by ERAAP expression, presents peptides with an X-pro-Xn sequence. ERAAP fails to cleave X-pro bonds, a characteristic of ER resident aminopeptidases, showing that the presence of proline blocked antigen processing in the ER, however has been shown to cleave lysine, leucine, asparagine and tyrosine residues (Kanaseki et al., 2006; Saric et al., 2002; Serwold et al., 2001; Serwold et al., 2002). The expression of ERAAP is essential for MHC I expression in both steady state and IFN-γ induced cells. However, ERAAP does not affect the peptide acquisition or MHCI trafficking but does affect the MHC I stability by optimising peptide repertoire rather than limiting peptide:MHC I assembly. In the absence of ERAAP, only a small fraction of peptides remain unchanged by the difference of ERAAP expression with a subset of peptides absent in ERAAP KO mice (Hammer et al., 2006). ERAAP KO mice are unable to generate immunodominant eptiopes. Immunisation of MHC matched wild type mice with ERAAP deficient splenocytes generated strong CD8^{+} T cell and B cell responses, proving ERAAP deficient cells to be immunogenic in wild type mice. The absence of ERAAP sees depletion of a number of peptide ligands for MHCI, but also sees the generation of a large volume of new peptides displayed at the cell surface. The peptide:MHC I molecules presented at the cell surface of ERAAP deficient cells are structurally distinct, due to the retention of their N-terminal extension (Hammer et al., 2007). ERAAP is shown to serve as a susceptibility factor for infectious pathogens. In the case of Toxoplasma gondii, ERAAP deficiency impairs the generation of a CD8^{+} response to this pathogen, proving ERAAP KO mice are susceptible to infection, most likely due to lack of presentation of the pathogenic peptide:MHC I complexes (Blanchard et al., 2008). Trimming activity of N-terminally extended peptides within the ER of ERAAP deficient fibroblasts is reduced, with ERAAP deficiency altering the magnitude and hierarchy of T cell responses to viral epitopes (York et al., 2006). The absence of ERAAP can result in the increased presentation of a subset of peptides, indicating that ERAAP destroys these peptides under normal conditions. However in the absence of ERAAP these peptides are not destroyed and subsequently presented on MHC I (Hammer et al., 2006). The lack of ERAAP expression disrupts the presentation of antigens and affects the ability to induce CD8^{+} T cell responses. Endogenous peptide antigens appear to fall into 3 categories, ERAAP dependent, ERAAP independent and ERAAP sensitive. Whereas those that are dependent on ERAAP are reduced in its absence and are unable to induce a CTL response, the presentation of others are

increased and therefore increases T cell stimulation, indicating ERAAP has the ability to destroy sub-optimal peptides. The final subset of peptides appear to have little variation in presentation in the presence and absence of ERAAP (Hammer et al., 2007; York et al., 2002). This was shown when ERAAP deficient mice lacked the expression of some subsets of peptide:MHC I complexes that were presented in wild type mice. Those peptide:MHC I complexes that were expressed in ERAAP deficient mice were highly unstable and unique. When wild type mice were immunised with ERAAP deficient splenocytes, a major $CD8^+$ T cell and B cell response was elicited (Hammer et al., 2007). Therefore, the absence of ERAAP alters the peptide repertoire for presentation and indicates a role of ERAAP in generating optimal peptides for loading rather than just a role in the supply of peptides (Hammer et al., 2006; Hammer et al., 2007).

ERAAP synergises with MHC I molecules to regulate the quality of processed peptides presented at the cell surface. Absence of ERAAP changes the repertoire of peptides presented. The length of a large proportion of antigenic peptides is dependent upon ERAAP trimming activity and proteolysis by ERAAP controls the length of MHC I ligands and influences the epitope specificity of the $CD8^+$ T cell response, showing ERAAP is an essential regulator of the optimal peptide repertoire presented on MHC I at the cell surface (Blanchard et al., 2010).

1.8.3. ERAAP mechanism of action

The mechanism of ERAAP to trim N-terminally extended precursors to the final optimal peptide remains unclear. Based on the ability to trim peptides in vitro, it has been suggested that ERAAP works alone or in concert with ERAP2 to generate the final peptide MHC I ligand for optimal binding (Saveanu et al., 2005; York et al., 2002). However other studies have shown that the presence of the appropriate MHC I molecule is essential for optimal peptide generation (Kanaseki et al., 2006). Thus two mechanisms of action have been proposed. Firstly, Chang et al have proposed the molecular ruler mechanism, in which ERAAP acts as a template for the generation of peptides of 8-9 amino acids in length (Chang et al., 2005). In this proposed model, ERAAP is thought to bind substrates with higher affinity due to their hydrophobic C-terminal at the hydrophobic pocket, and the active site with the N-terminal extension and trim the precursor to optimal length. Evidence to support this was generated by using recombinant ERAAP and a panel of synthetic peptides. ERAAP was shown to have substrate length preference similar to TAP, preferring peptides of 9-16 residues.

Hydrolytic activity is reduced with peptides less than 8 amino acids, and trimming is also affected by both N- and C- terminal residues, with its ability to monitor the C terminal amino acid, favouring a hydrophobic C-terminal region. To also support this, York et al showed that in the absence of MHC I, ERAAP could trim peptide precursors to 8-9 amino acids, where trimming activity of ERAAP would cease (Chang et al., 2005; York et al., 2002). Once the precursors are trimmed, they can then bind with high affinity to the appropriate MHCI molecule for presentation.

The analysis of ERAAP deficient mice also revealed substrate preferences for ERAAP, but does not support the molecular ruler mechanism (Hammer et al., 2006; Yan et al., 2006). In vivo, it was shown that ERAAP could generate 8-9 amino acid substrates that were presented by differing MHC I molecules, however acts to degrade other peptides of similar size. The second mechanism of ERAAP activity proposes that the appropriate MHC I for the precursor substrate is required to act as a template for ERAAP peptide hydrolysis. MHC I can bind N-terminally extended peptides which can be trimmed by ERAAP, acting as the template for ERAAP activity. The evidence that MHC I acts as a template for ERAAP trimming activity comes from observations that optimal peptides for MHC I are only generated in the presence of the correct MHC I molecule for the peptide. This was demonstrated by using the QL9 peptide specific for H-2L^{d} and an N-terminally extended version (X6-QL9) requiring trimming by ERAAP. To generate the QL9-H-2L^{d} complex, both H-2L^{d} and ERAAP presence was required and in the absence of H-2L^{d}, the precursor was degraded and could no longer be recognised by QL9 specific hybridoma, therefore eliminating the precursor (Kanaseki et al., 2006). This supports the original model proposed by Falk et al in which the MHC I itself acts to define the peptides presented and in the absence of the correct MHCI, the peptide is degraded and eliminated (Falk et al., 1990; Kanaseki et al., 2006).

1.8.4. ERAAP Structure

ERAAP is a member of the zinc M1 family of metalloproteases and resides within the lumen of the ER localising with proteins known to contain the KDEL ER retention motif (Kanaseki et al., 2006; Saric et al., 2002). The human gene for ERAAP is located on chromosome 5q15 and the transcribed protein consists of 941 amino acids, with a molecular weight of 106kDa. In comparison to this, murine ERAAP is located on chromosome 13 and consists of 930 amino acids, sharing 86% homology with human ERAAP (Cui et al., 2002; Hammer et al., 2007; Saric et al., 2002; Serwold et al., 2002). With regards to genomic organisation, ERAAP has a close relationship to the other M1

family member, P-LAP/IRAP (Hattori et al., 2001). Like all M1 metalloprotease family members, ERAAP has an extracellular active site which spans 375 amino acids (figure 1.8). Within this active site are two motifs common to the M1 metalloprotease family, a zinc metalloprotease catalytic motif HEXXH$(X)_{18}$E and also a 5 amino acid GAMEN motif encoded within exon 6 of the protein (Cui et al., 2002; Hattori et al., 2001; Kanaseki et al., 2006). The GAMEN motif determines the enzymatic function of ERAAP. When glutamic acid (E) is mutated to alanine (A), giving rise to GAMAN, antigen presentation is impaired. However an intact GAMEN motif had normal antigen presentation. The glutamic acid is proposed to bind to the final amine group at the N-terminus of peptides (Kanaseki et al., 2006). Another important residue in the activity of ERAAP was identified by Goto et al also showing the glutamine residue at position 181 is important for both the enzymatic activity of ERAAP but also its substrate specificity (Goto et al. 2008).

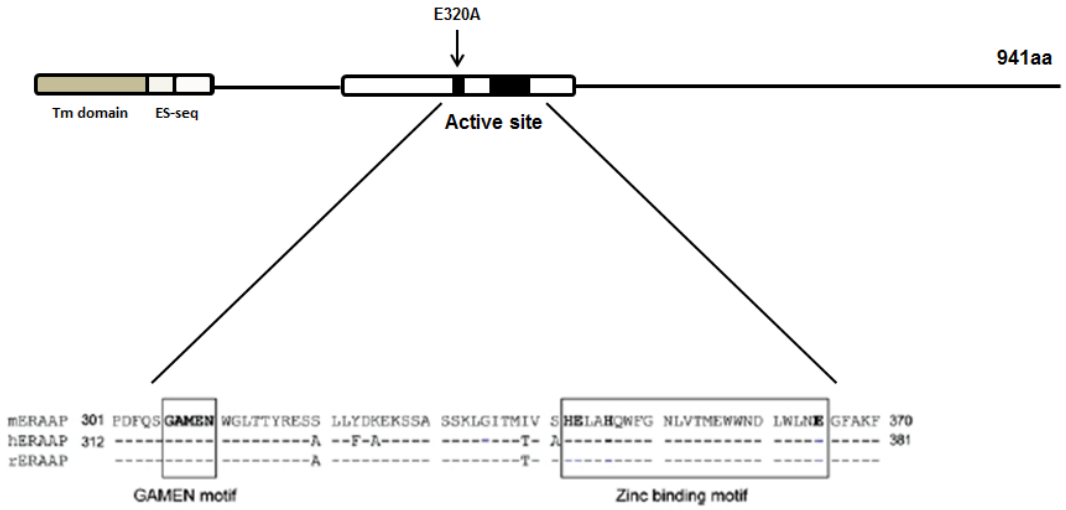

Figure 1.8. Schematic of ERAAP gene

Human ERAAP is a 941 amino acid protein containing a transmembrane domain. Here the active site region, made of two motifs common to M1 metalloproteases, is conserved between mouse, human and rat ERAAP. The E320A mutation within the active site is essential for the enzymatic function.

Recently, the crystal structure of ERAAP has been determined in both open and closed (inhibitor bound) conformations at a resolution of 2.7Å (figure 1.9). The structure suggests ERAAP is formed of four domains, with a large cavity between domain II and IV which could potentially be for substrate binding. The catalytic site containing the GAMEN and HEXXH$(X)_{18}$E motif are within domain II and domain IV is the most

variable when compared to other M1 family members. Bestatin, a broad spectrum aminopeptidase inhibitor that is bound through a bidentate interaction between its 2-hydroxy and amido oxygen atoms and the ERAAP catalytic zinc atom, is bound to ERAAP in the closed conformation. The N terminus of bestatin is bound by both Glutamine at position 183 and also glutamine at position 320, part of the GAMEN motif, confirming the role of this amino acid in forming the N terminal anchor for peptide substrates (Kochan et al., 2011; Nguyen et al., 2011).

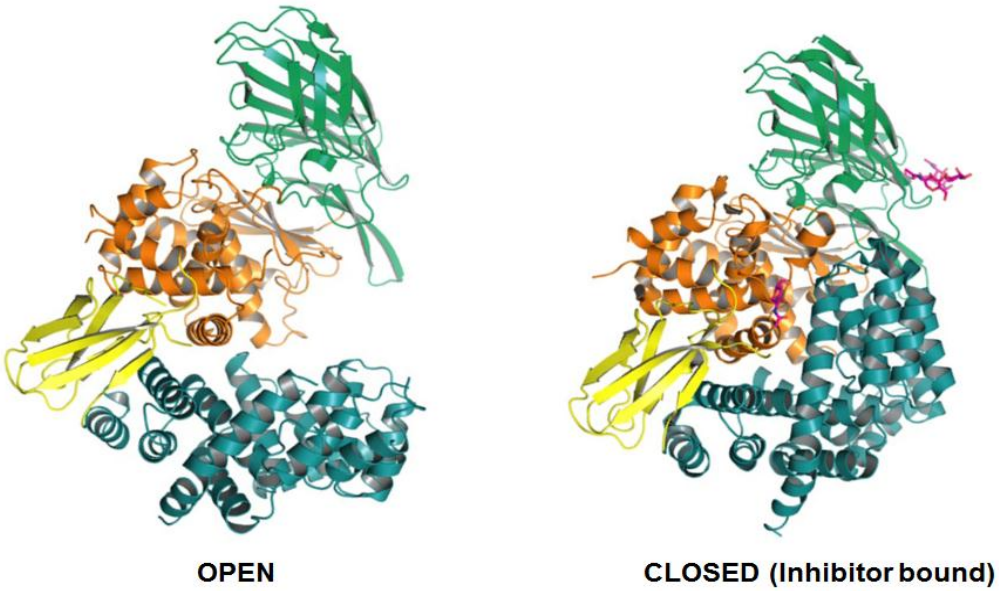

Figure 1.9. Crystal structure of ERAAP in open and closed conformations

ERAAP is a four domain protein with active site regions within domains II. Open conformation (left) demonstrates a large cavity between domains II and IV. Upon binding of an inhibitor (bestatin), ERAAP adopts a closed conformation (right) for enzymatic activity to occur (Kochan et al., 2011; Ngyuen et al., 2011).

Based on this crystal structure of the open and closed conformations, a possible mechanism of activity was suggested in that substrate can bind to ERAAP in the open conformation and once bound with peptide occupying the regulatory site, the domain closes around the substrate and catalysis can occur. Once hydrolysis has taken place the substrate is released. This proposal is more consistent with the 'molecular ruler'

mechanism, however still remains to be unclear as ERAAP can synergise with MHC I to generate optimal peptide (Kochan et al., 2011; Nguyen et al., 2011).

1.8.5. Role of ERAAP in the facilitation of cytokine receptor shedding

As well as being a key member of the antigen processing machinery, ERAAP has been identified to have roles in other biological processes and is also known as puromycin-insensitive leucyl-specific aminopeptidase (PILSAP), Aminopeptidase Regulator of TNFRSF1A Shedding 1 (ARTS1) and Adipocyte derived Leucine Aminopeptidase (A-LAP). Cytokines are small proteins secreted by many cells that act as signalling molecules, promoting intercellular signalling, effecting interactions and behaviour of cells. The cytokines include lymphokines, interleukins and cell signalling molecules such as interferons (IFN) and tumour necrosis factors (TNF). Cytokines can aid immune responses and can be pro-inflammatory or anti-inflammatory. In order to achieve their specific function, cytokines must first bind to their specific receptors on the surface of the cell, which can then initiate downstream signalling events (Legler et al., 2003; Micheau and Tschopp, 2003). Therefore, cleavage of these membrane bound receptors into soluble forms has the ability to mediate the transmission of cytokine induced signalling and responses. In this context, ERAAP (ARTS-1) has been shown to be a type II integral membrane protein, containing a transmembrane domain region that is thought to be key for its function in the facilitation of cytokine receptor shedding. The first cytokine receptor to be associated with the ability of ERAAP to act as a shedding enzyme was TNFRSF1A (Cui et al., 2002). TNFα is a multifunctional pro-inflammatory cytokine that has a role in regulating inflammatory and stress responses as well as host defense mechanisms. Regulatory mechanisms exist to control immune responses due to TNFα binding with TNFRSF1A. Firstly, Silencer of death domains bind to the TNFRSF1A intracytoplasmic domain to prevent signalling by the receptor. Secondly, the shedding of membrane TNFRSF1A to soluble TNFRSF1A (sTNFRSF1A) allows regulation of signalling. The sTNFRSF1A can compete with membrane TNFRSF1A by binding to TNFα and also soluble receptors decreasing the amount of membrane bound receptors. Soluble receptors can also act as a TNFα reservoir by reversibly binding trimeric TNF ligands and prolonging its half-life. TNFRSF1A is released into the extracellular space through two mechanisms either through exosome like vesicles releasing full length TNFRSF1A or via ectodomain cleavage of the receptor to release a soluble 27-34kDa form by a receptor sheddase such as TNFα converting enzyme, TACE / ADAM 17 (Hawari et al., 2004). ADAM 17 is a member of the metalloprotease disintegrin (ADAM) family of zinc metalloproteases

and is known to mediate TNFRSF1A shedding on the basis that ADAM 17-deficient cells have lower cell surface to soluble receptor ratio than cells expressing ADAM 17 (Reddy et al., 2000). In 2002, Cui et al used a yeast-2-hybrid system with the extracellular domain of TNFRSF1A as a bait fusion protein to identify proteins interacting with the extracellular domain of the receptor, identifying ARTS1 (ERAAP) (Cui et al., 2002). Using immunoprecipitation experiments, ERAAP was found to bind to, but not cleave, the full length membrane associated TNFRSF1A at the ectodomain region. A direct correlation has linked increased ERAAP expression with an increase in receptor shedding and decrease in membrane bound TNFRSF1A shedding was not considered to be a consequence of altered receptor mRNA levels or altered ADAM 17 expression. It was also noted that the zinc metalloprotease catalytic domain was not required for this action and cleavage by ERAAP is only specific for TNFRSF1A and not TNFRSF1B (Cui et al., 2002). The direct binding of ERAAP to membrane bound receptor suggests that ERAAP does not itself catalyse TNFRSF1A receptor ectodomain cleavage, but may act indirectly to catalyse the enzymatic activity of another sheddase, for example ADAM 17. In light of this finding, Islam et al identified a member of the nucleobindin family of DNA and calcium binding proteins, NUCB2, that binds to ERAAP in a calcium dependent manner before cleavage of TNFRSF1A can occur (Islam et al., 2006). NUCB2 contains a basic amino acid rich DNA-binding domain and is thought to mediate the release of constitutive TNFRSF1A through direct binding with ERAAP and TNFRSF1A through activity of its EF-hand domain, which is a helix-loop-helix conformation in the protein that is able to bind calcium ions (Kroll et al. 1999). NUCB2 also localises in a distinct population of vesicular structures, where it is thought to associate with ERAAP (Islam et al., 2006). More recently, RNA-binding motif gene, X chromosome (RBMX), a 43kDa binding motif protein was identified as an ERAAP binding protein which promoted the cleavage of TNFRSF1A through both pathways to the extracellular compartment (Adamik et al., 2008). RBMX has been shown to be a component of the spliceosome and it is likely that RBMX mediated TNFRSF1A release is a result of ERAAP association (Adamik et al., 2008).

Along with TNFRSF1A, ERAAP also facilitates the cleavage of IL-6R. IL-6 is a pleotropic cytokine that can act in both an anti-inflammatory and a pro-inflammatory manner. IL-6 is capable of regulating the inflammatory responses through T cell activation and production in response to infection and immunological challenge. Through its pro-inflammatory responses, dis-regulation of IL-6 and downstream response could result in diseases such as autoimmune disorders, RA, SLE and

diabetes. In contrast to its pro-inflammatory role, IL-6 plays a role in anti-inflammatory responses such as the induction of IL-1 receptor antagonist and also the expression and stimulation of TNFRSF1A shedding. The IL-6 receptor complex is formed from an 80kDa IL-6 receptor (IL-6R / CD126) and 130kDa gp130 signal transducing subunit (CD130). The IL-6R is only expressed on a small number of cells; however gp130 is ubiquitously expressed due to its role as a signal transducer for a number of other cytokines. IL-6 is able to bind to IL-6R which cannot itself initiate signal transduction pathways and therefore recruits 2 gp130 molecules which become autophosphorylated in the intracellular domain by Janus Kinases (JAK). This phosphorylation creates interaction sites for proteins containing phosphotyrosine-binding SH2 domains, such as signal transducers and activators of transcription (STAT, (Heinrich et al., 1998) . STAT1 and STAT3 are recruited to the receptor and are in turn phosphorylated by JAKs, initiating the Jak/Stat signalling pathway (Devin et al., 2000; Guschin et al., 1995). The Ras/MAP kinase and PI3kinase pathways are also known to be activated in response to IL-6 binding IL-6R. Soluble IL-6R occurs through either cleavage of the membrane bound form of the receptor releases a soluble form of the receptor, or differential mRNA splicing resulting in a soluble form of IL-6R that lacks the transmembrane domain (Mullberg et al., 1994). IL-6 is able to bind to both sIL-6R and membrane bound IL-6R. The binding of IL-6 to sIL-6R can then activate gp130 on cells that do not express membrane forms of IL-6R. This mode of ‘trans-signalling’ allows cells that lack the expression of membrane IL-6R to become responsive to IL-6 by sIL-6R produced from other cells, increasing the effect of IL-6 (Peters et al., 1996).

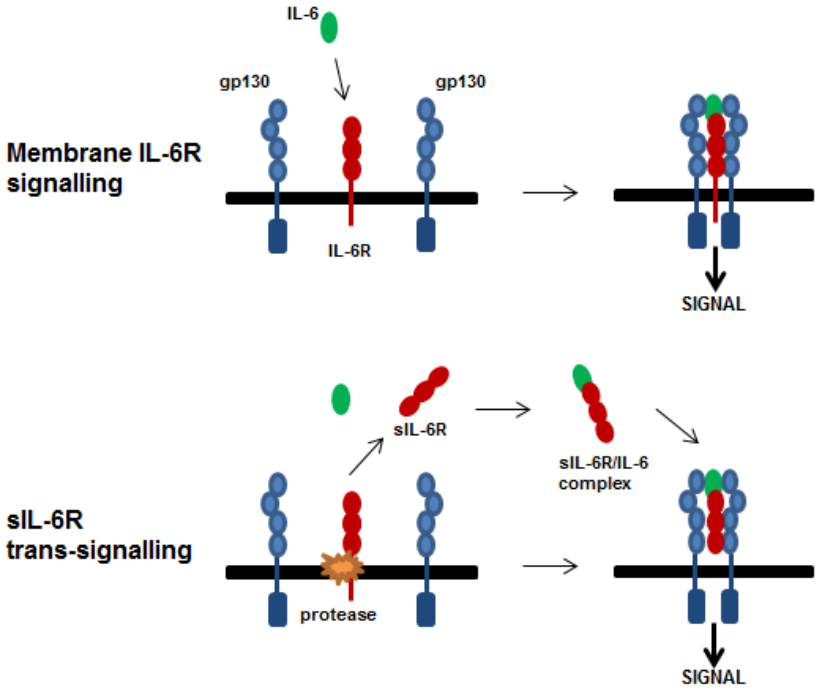

Figure 1.10 Signalling mechanism of IL-6 through IL-6R and gp130

IL-6 binds to membrane bound IL-6R which recruits 2 gp130 molecules to initiate downstream signalling. Cells not expressing IL-6R can still be activated through the formation of a sIL-6R/IL-6 complex that associates with gp130, termed 'trans-signalling'.

After previously describing a role for ERAAP in TNFRSF1A shedding, Cui et al went on to study the role of ERAAP in the shedding of membrane IL-6R. They demonstrated using immunoprecipitation experiments that, in contrast to what was shown with TNFRSF1A shedding, ERAAP could associate with the 55kDa soluble form of IL-6R. It was then shown that levels of membrane bound IL-6R was inversely correlated with ERAAP protein expression, and soluble IL6R was increased with increased ERAAP expression, shown by ELISA. The mechanism of constitutive IL-6R shedding requires the ERAAP catalytic domain to be intact, however although ERAAP catalytic activity is required for IL-6R cleavage, it has not been shown that ERAAP acts directly on IL-6R to cleave membrane bound receptor into its soluble form (Cui et al., 2003a). It is possible that ERAAP mediates its shedding activity through an indirect mechanism to promote the activity of another enzyme to cleave the receptor (Cui et al., 2003a). This mechanism is more favourable as direct shedding activity has not been documented, but an indirect mechanism has been demonstrated for TNFRSF1A cleavage (Adamik et al., 2008; Islam et al., 2006).

The third cytokine receptor that ERAAP has been suggested to facilitate cleavage of is the type II IL-1 receptor (IL-1RII). IL-1, like TNFα is a pro-inflammatory cytokine that is important in mediating inflammatory responses, host defense and has roles in the pathogenesis of inflammatory diseases, i.e. Rheumatoid Arthritis. Two different IL-1 receptors exist, type I IL-1R (IL-61I) and the type II receptor. IL-1RI is an 80kDa protein belonging to the IL-1R/toll-like receptor superfamily due to the possession of a characteristic cytoplasmic toll IL-1R domain and also immunoglobulin like domains. IL-1 is able to bind to IL-1R and recruit to form a complex with the accessory protein, IL-1RAcP. This subsequently signals via the MyD88 adaptor protein and IL-1R associated kinase to initiate a response. Conversely, IL-1RII is a 60kDa non-signally decoy receptor, due to the lack of the toll-IL-1R domain in the cytoplasmic region. Membrane bound IL-1RII can form non-signalling complexes with both IL-1 and IL-1RAcP, sequestering these components of the IL-1 signalling pathway to attenuate signalling. Soluble IL-1RII (sIL-1RII) also exists by proteloytic cleavage and release of a 47kDa extracellular domain. Furthermore, soluble IL-1RIAcP also exists, however this is only generated from alternative mRNA splicing. IL-1 is able to bind to sIL-1RII and attenuate excessive IL-1. In addition to this, sIL-1RAcP can also bind, further acting to inhibit IL-1 activity. ERAAP is thought to regulate constitutive IL-1RII shedding mediating the biological activity of IL-1 through release of sIL-1RII (Adamik et al., 2008; Cui et al., 2003b). Like IL-6R shedding, Cui et al showed that sIL-1RII co-immunoprecipitated with ERAAP and correlated changes in protein expression with changes in soluble receptor; however no effect was shown in regards to IL-1RI, suggesting ERAAP facilitation of receptor cleavage is limited to just type II IL-1R. Like IL-6R shedding, the ability of ERAAP to cleave IL-1RII is dependent on an intact Zn metalloprotease motif (Adamik et al. 2008;Cui et al. 2003b). This activity of ERAAP however, does not appear to effect ADAM 17 maturation or expression and is specific for IL-1RII member of the IL-1R family only (Adamik et al., 2008; Cui et al., 2003b).

1.8.6. Role of ERAAP in Angiogenesis

Angiogenesis is the formation of neovessels through endothelial cell proliferation and migration. Although essential for normal growth and development, the process of angiogenesis plays a crucial role in pathological diseases such as rheumatoid arthritis, tumour progression and diabetic retinopathy. The understanding of cellular and molecular mechanisms of angiogenesis is still poorly understood, however there are a number of growth factors that are important in the regulation of this process through cell proliferation, migration and networking (Conway et al., 2001). One molecule,

Vascular Endothelial Growth Factor (VEGF), is an endothelial cell (EC) tropic factor that signals to activate an array of intracellular signalling molecules such as the mitogen-activated protein kinases (MAPKs), phospholipase C with the downstream protein kinase C (PKC) and also phosphatidylinositol-3-kinase (PI3K) with its downstream Akt/protein kinase B and p70 s6 kinase (S6K) in angiogenesis. The regulation of the proliferation of endothelial cells involved in angiogenesis is aided by ERAAP and is known in these studies as puromycin-insensitive leucyl-specific aminopeptidase (PILSAP). VEGF is required for endothelial cell proliferation through a signalling cascade activating a diverse range of signalling molecules. One such molecule, phosphatidylinositol-dependent kinase 1 (PDK1), is activated upon VEGF secretion. PDK1 is known as a master kinase that is constitutively active but regulated by PI3K signalling. Within PDK1 there are 3 ligand binding sites; a substrate binding site, an ATP binding site and a docking site, with molecules such as S6K and PKC binding at the substrate binding site. Downstream activation of Akt/PKB and AGC kinases like PKC, S6K and SGK are dependent upon PDK1 signalling (Yamazaki et al., 2004).

In 2002, mouse ERAAP was shown to be expressed in ECs and reduction in expression indicated a role of ERAAP in both migration and proliferation of ECs, induced by VEGF stimulation, and in angiogenesis (Miyashita et al., 2002). It was shown that ERAAP played a crucial role in VEGF stimulated G1/S phase transition in ECs, aiding cell proliferation in the process of angiogenesis (Yamazaki et al., 2004). A reduction in ERAAP expression saw a decrease in both VEGF stimulated S6K activation and also transition from G1/S phase of the cell cycle. In cells transiently transfected with PDK1, Yamazaki et al showed that ERAAP could bind PDK1 under basal conditions and that under VEGF stimulation, S6K also associated, forming a ternary complex. The activation of S6K by PDK1 is mediated by ERAP1, which binds to PDK1 at the N-terminal non-catalytic site and is able to trim nine amino acids that block the PIF-binding pocket, the motif that recognises and binds S6K (Biondi et al., 2001). The exposure of this binding site allows the S6K to bind to PDK1 and allows phosphorylation of the threonine residue at position 229 of S6K. ERAAP is thought to be specific for S6K activation, as reduced ERAAP expression had little effect on both P13K upstream phosphorylation and activation and also on the activation of PDK1 downstream target Akt/PKB. This would suggest PDK1 activates both S6K and Akt/PKB but through different mechanisms (Akada et al., 2002; Yamazaki et al., 2004).

Mediation of cell migration and adhesion through integrin receptors is also an essential part of blood vessel formation. Integrins are transmembrane proteins required for cell migration and cell adhesion and murine ERAAP was shown to have an involvement in the regulation of integrins to promote migration of ECs (Abe and Sato, 2006; Akada et al., 2002). RhoA is a member of Rho family small GTPases and aids the re-organisation of the actin skeleton in cell migration and adhesion and ERAAP is thought to activate RhoA to aid cell migration (Suzuki et al., 2007). Alterations in ERAAP activity may therefore alter the ability of ERAAP to promote cell migration and adhesion and the formation of new blood vessels.

1.9. Endoplasmic Reticulum Aminopeptidase 2

In an effort to further elucidate the importance of the oxytocinase subfamily of M1 metalloproteases, Tanioka et al identified a novel member of this family, termed Leukocyte derived arginine aminopeptidase (L-RAP), later known as ERAP2 (Tanioka et al., 2003). In this study they used leukocytes to clone a 3.3Kb gene encoding a 960 amino acid protein with a significantly hydrophobic N terminal region, similar to ERAAP. Within this protein were two highly conserved motifs, the zinc binding site $HEXXH(X)_{18}E$ and GAMEN motif, allowing classification onto the M1 family of metalloproteases. Located on chromosome 5q15, this type II membrane spanning protein shares homology with both ERAAP (49%) and placental leucine aminopeptidase (P-RAP, 40%), with highest levels of homology around the two conserved HEXX18XE and GAMEN motifs. Northern blot analysis identified highest ERAP2 expression in spleen and leukocyte cells. To further characterize the subcellular localisation of ERAP2, immunocytochemistry was used with ERAP2 tagged with influenza HA epitope and expressed in HeLa S3 cells. This indicated that, like ERAAP, ERAP2 was localised to the ER, with the C-terminal end localising to the luminal side. ERAP2 was shown to have specific substrate activity, preferentially cleaving basic N terminal residues such as arginine and lysine however showed no preference for peptides with hydrophobic c-termini (Saveanu et al., 2005; Tanioka et al., 2003). ERAP2 can act to trim N terminally extended antigenic precursors to the correct length for optimal MHC I loading in the ER lumen, however, like ERAAP, ERAP2 trimming activity ceases at X-pro bonds. (Tanioka et al., 2003). As well as a full length form, a truncated version, termed L-RAP(s), is also localised to the lumen of the ER, however this was deemed non-functional as it exhibited no aminopeptidase activity when tested using various aminoacyl-MCAs (Saveanu et al., 2005; Tanioka et al., 2003)

ERAAP has a wide tissue distribution, correlating with MHC I expression patterns. Conversely, ERAP2 expression does not appear to be correlated with MHC I expression in tissues, suggesting that MHC I antigenic peptides are generated primarily by ERAAP with ERAP2 potentially compensating in tissues with lower ERAAP expression (Saveanu et al., 2005; Tanioka et al., 2003). Like other antigen processing machinery, ERAP2 is up regulated by IFN-γ. Both ERAAP and ERAP2 localise with CNX, but can also localise together, indicating identical subcellular distribution. To further test whether ERAAP and ERAP2 could form a heterodimeric complex, Saveanu et al used co-immunoprecipitation to detect a small amount of ERAAP/ERAP2 peptidase complex, confirming that these can form heterodimeric complexes (Saveanu et al., 2005). It is highly conceivable that these two aminopeptidases could work in concert with each other in the ER to trim N terminal extensions containing both hydrophobic residues (favoured by ERAAP) and basic residues (favoured by ERAP2), therefore combining specificities to generate peptides that the individual aminopeptidases alone cannot. This was further shown by Saveanu et al, when incubating either ERAAP or ERAP2 with the N terminally extended peptide KIRIQRGPGRAFVTI (K15I) requiring trimming *in vitro*. Both ERAAP and ERAP2 individually failed to successfully trim K15I to the final G9I. However when both ERAAP and ERAP2 were present, K15I was efficiently trimmed to generate G9I MHC I viral antigen (Saveanu et al., 2005). Although this study shows ERAAP and ERAP2 can form heterodimers, it did not prove that ERAAP and ERAP2 need to form heterodimeric complexes in order to act together to trim N terminally extended peptides. Only a small proportion of ERAAP was shown to form these complexes and *in vivo* trimming data of ERAP2 and heterodimers of ERAAP and ERAP2 is not yet available (Saveanu et al., 2005). ERAP2 is not present in mice and therefore ERAAP in mice may be sufficient to trim most amino acids and have broader peptide specificity than ERAAP in humans. ERAAP may be the dominant ER aminopeptidase, but ERAP2 may be important for trimming in those cells with a lower ERAAP expression and for longer length extensions containing both basic and hydrophobic residues. Physiologically, ERAP2 is involved in the maintenance of homeostatic states by its involvement in the cleavage of hormones kallidin and angiotensin III, however does not have any activity towards angiotensin II or vasopressin (Saveanu et al., 2005). As yet, there are no reports of ERAP2 having a role in cytokine receptor shedding, however there is an association between a haplotype in ERAAP and ERAP2 loci with increased susceptibility for AS (Tsui et al., 2010). Furthermore, a Norwegian and Australian cohort study has also

suggested a genetic link between ERAP2 and increased susceptibility to pre-eclampsia coinciding with a role for ERAP2 in hypertension (Johnson et al., 2009) (Johnson et al. 2009). More recently, a genome wide meta analysis has identified the ERAP2 loci to be linked with a risk of developing Crohn's disease (Franke et al., 2010).

1.10. Role of ERAAP in disease

ERAAP has recently been identified to be a polymorphic aminopeptidase with polymorphisms associated with the pathogenesis of many diseases including systemic arterial hypertension, diabetes mellitus and cervical carcinoma, preeclampsia and psoriasis (Yamamoto et al., 2002);(Fung et al., 2009); (Mehta et al., 2009); (Johnson et al., 2009); (Strange et al., 2010). The Lys528Arg polymorphism within ERAAP has strong associations with these diseases as well as an association with reduced bone mass density of premenopausal Japanese women (Yamada et al., 2007). Also, ERAAP, as well as MHC I surface expression varies in certain non-lymphoid cancer cell lines and functionally impaired ERAAP in tumour cell lines has also been identified, with ERAAP expression correlating with MHC I expression (Fruci et al., 2006; Fruci et al., 2008).

The role of ERAAP (A-LAP) in hypertension was first established as it shares a high level of homology with placental leucine aminopeptidase (P-LAP), another M1 metalloprotease family member, which acts to cleave peptide hormones such as vasopressin and oxytocin, thus proposing that ERAAP could also play a role in regulating peptide hormones (Hattori et al., 1999; Hattori et al., 2001). On screening for 33 polymorphisms within human ERAAP, Yamamoto et al identified the Lys528Arg polymorphism having association with essential hypertension (Yamamoto et al., 2002) . Hypertension is associated with a number of morphological changes including left ventricular hypertrophy. ERAAP aids the control of left ventricular mass through the renin-angiotensin system, essential for regulation of blood pressure (Hallberg et al., 2003) . This physiological system starts with the breakdown of angiotensinogen to angiotensin I by renin. This angiotensin I is further converted to angiotensin II by angiotensin converting enzyme (ACE). Angiotensin II can stimulate aldosterone release, which is a vasoconstrictor acting to increase blood pressure as well as increasing water retention, in turn increasing blood pressure. This bioactive hormone can also stimulate cardiac hypertrophy. ERAAP activity hydrolyses bioactive peptides such as angiotensin II and kallidin in order to regulate and control blood pressure(Hallberg et al., 2003). The association of Lys528Arg with essential

hypertension was proposed to be a consequence of this SNP having a decrease in functional activity towards the cleavage of bioactive peptide hormones compared to wild type ERAAP (Yamamoto et al., 2002). Assessment of the functional activity of this Lys528Arg mutant using L-AMC substrate showed a decreased enzymatic activity towards the L-AMC substrate. In combination with this, Lys528Arg failed to cleave both angiotensin II and kallidin hormones to angiotensin III and bradykinin respectively, providing evidence that this polymorphism is associated with essential hypertension due to its reduced enzymatic activity (Goto et al., 2006).

As well as screening for SNPs associated with essential hypertension, Mehta et al also screened 13 SNPs within TAP1, TAP2, LMP2, LMP7 and ERAAP genes, identifying polymorphisms within ERAAP as being associated with an increased risk of developing cervical carcinoma (Mehta et al., 2008; Mehta et al., 2007). Cervical carcinoma is the second most common cancer affecting women worldwide. As yet, the most important risk factor in the progression of cervical carcinoma is the infection with the human papillomavirus (HPV). There are more than 15 strains of the HPV that have risks of cancer, with type 16 and 18 being most strongly associated with cervical carcinoma, responsible for almost 70% of this cancer (Schiffman and Castle, 2003). HPV affects the epidermis and mucous membrane and it is thought to cause alterations within the cells of the cervix which ultimately leads to cervical neoplasms. Several studies have shown that various viral proteins, including the HPV E7 protein, can either physically, or at the transcriptional level interfere with APM-related cellular processes, including those mediated by TAP and the chaperone molecules (Georgopoulos et al., 2000). Mutations and down-regulation of ERAAP has been discovered in a number of cervical carcinoma cases. SNPs at position 127 and 730 in combination with polymorphisms in other components of the APM, gives rise to a three fold increase in risk of cancer and a decreases in overall survival (Mehta et al., 2009). ERAAP loss of expression has also been shown to be an independent predictor of survival in cervical carcinoma patients (Mehta et al., 2008).

It is highly conceivable that polymorphisms within ERAAP can alter the generation of correct antigenic peptides for presentation on MHC I and also affect the role of ERAAP on facilitating cytokine receptor cleavage. Defects in the activity of ERAAP caused by polymorphisms could have detrimental effects leading to the pathophysiology of disease states mentioned above. This provides interesting basis for studying the role of SNPs within ERAAP and their ability to change the functional activity of ERAAP in

peptide processing and cytokine receptor cleavage.

1.11. Association between MHC I and autoimmune disease

Autoimmune diseases are characterised by the destruction of autologous cells and tissues as a result of an inappropriate immune response. These diseases, such as rheumatoid arthritis (RA), systemic lupus erythematous (SLE), type 1 diabetes, psoriasis, multiple sclerosis (MS) and ankylosing spondylitis (AS) are influenced by both environmental and genetic factors, with increasing evidence suggesting the MHC locus is a major genetic factor contributing to disease. MHC molecules are highly polymorphic and serve to present antigenic peptide (self or foreign) to circulating T cells, eliciting an immune response where appropriate. Although the mechanisms underlying the association of MHC with autoimmune disease are largely unknown, it has been suggested that aberrant MHC presentation of peptides to autoreactive T cells results in a breakdown of immunological tolerance to self-peptides (Fernando et al., 2008). At the MHC I locus, HLA-B27 is associated with disease susceptibility in >96% AS cases (Hammer et el., 1990). HLA-Cw6, in particular the HLA-Cw0602 allele, is associated with the onset and progression of psoriasis and psoriatic arthritis (Rahman and Elder, 2012). In the majority of autoimmune disorders more than one MHC allele is associated with disease onset. In type 1 diabetes, approximately 50% of cases are attributable to the presence of HLA-DR4 and HLA-DQ, specifically HLA-DRB1, HLA-DQA1 and DQB1 alleles; with the highest risk of disease development is associated with the DR3-DQ2 and DR4-DQ8 haplotypes (Noble et al., 1996). In addition, the development and progression of MS, is strongly associated with the presence of these haplotypes (Dyment et al., 2005). The MHC locus is accountable for 30-50% susceptibility to the onset of RA, with HLA-DR4 being the main genetic risk factor and a particular emphasis on the HLA-DRB1 allele (Bax et al., 2011).

It is clear that MHC molecules have a strong genetic association with autoimmune disease often accounting for a high percentage of the genetic susceptibility to the disorder. However, in most cases the presence of a particular MHC allele alone is not sufficient to result in disease, with additional genetic and environmental factors enhancing the risk of disease development.

1.12. Ankylosing Spondylitis

Ankylosing Spondylitis (AS) is an autoimmune inflammatory disorder belonging to the family of spondyloarthropathies (SpA). This arthritic family comprises of a group of immune mediate inflammatory disorders and include rheumatoid arthritis (RA),

osteoarthritis, psoriatic arthritic and arthritis associated with crohns disease. On average, SpAs occur in 0.5 – 1% of the population, with AS attributable for 30-50% of these cases. AS is a seronegative disorder that can be characterised by an inflammation of the spine and sacroiliac joints, causing eventual erosion of the bone and vertebrae fusion (ankylosis). As well as affecting the spine, the inflammation can also be systemic at sites such as eyes, aorta, lungs, kidneys and tendon insertions. The prevalence of AS is three times more common in males than females, with an onset age between late teens and early twenties. The aetiology of the disease has long been linked to genetic factors with a high risk of heritability (de et al., 1961). With the idea that the risk of AS could be determined genetically, two groups in 1973 described a remarkable association with the risk of developing AS and the human leukocyte antigen B27 (HLA-B27) and it is now know that over 96% of AS cases present with the HLA-B27 molecule (Caffrey and James, 1973). However, not all HLA-B27 subtypes have been found to associate with AS. HLA-B2702 and B2705 are associated with AS but HLA-B2706 and B2709 are not associated with AS and may be a protective allele. Later, a genome wide linkage scan using 254 highly polymorphic satellite markers identified not only a strong genetic linkage between AS and MHC I locus but, along with another study in 2001, identified the presence of non-MHC genetic susceptibility factors in the risk of AS development (Brown et al., 1998; Laval et al., 2001). This confirmed that presence of HLA-B27 is not the only causative factor of AS, and that other genetic factors alongside this were involved in the disease development.

With the notion that HLA-B27 is not the only genetic factor in AS, genome-wide association studies have been undertaken in an effort to provide a wider picture of the genetic susceptibility towards AS. In 2007, a linkage analysis study was undertaken by the Wellcome Trust Case Control Consortium (Burton et al., 2007). This study aimed to identify new nonsynonomous polymorphisms (nsSNPs) within four disease, AS, multiple sclerosis (MS), breast cancer and autoimmune thyroid disease (AITD). The study genotyped 14,436 nsSNPS in 1000 cases and 1500 controls, revealing the strongest associations with SNPs were between genes encoded in the MHC region and the three autoimmune diseases, AS, MS and AITD (Burton et al., 2007). Strong associations were identified between AS and nsSNPs in both ERAAP (P =1 x 10^{-26}) and IL-23 receptor, resulting in 26% and 9% of AS cases respectively (Burton et al., 2007). Of these five SNPs identified within ERAAP, three have also been associated with increased risk of developing cervical carcinoma and one with an increased association with hypertension (Goto et al., 2006; Mehta et al., 2007). It was suggested

that these SNPs may impair ERAAP activity therefore resulting in AS pathogenicity. The identification of these SNPs within ERAAP has been independently replicated and several novel polymorphisms identified (Harvey et al., 2009). The discovery of the link between HLA-B27 and AS led to many hypothesis as to why this molecule is present in so many AS cases. It was first proposed that, due to their function in antigen presentation at the cell surface, HLA-B27 could be the target of auto reactive T cells, in turn resulting in inflammation. However more recently, the unusual folding properties of this allele has been the focus of many studies. HLA-B27 folding is considerably slower than other HLA molecules, with a tendency of the HLA-B27 heavy chain to misfold in the ER prior to assembly (Mear et al., 1999; Bird et al., 2003; Antoniou et al., 2004). Alongside this, after folded HLA-B27 has reached the cell surface, there is a tendency to form disulphide linked homodimers, a potential target for KIRs. The exact role in which HLA-B27 can cause AS is unknown. ERAAP has two known functions, its involvement in the ability to trim N-terminally extended peptides to optimal length for MHC I presentation and also its role in facilitation the cleavage of cytokine receptors for IL-1, IL-6 and TNF pro-inflammatory cytokines, up regulation of which are involved in many arthritic conditions. The functional significance of the SNPs identified within ERAAP is still unclear; however alterations in the activity of ERAAP could alter the ability of peptides presented at the cell surface, forming a hypothesis linking both HLA-B27 and ERAAP in the ability to generate optimal peptides for stable HLA-B27 loading. Recently, it was shown that ERAAP SNPs only affect the risk of AS in HLA-B27 positive individuals providing strong evidence that HLA-B27 and ERAAP work through a mechanism that involves the aberrant processing of peptide antigens for display (Evans et al., 2011). Conversely, an altered ability of ERAAP to cleave cytokine receptors could result in up-regulation of pro-inflammatory signalling, in turn leading to inflammation and disease progression.

1.13. HLA-B27 association with AS

In 1990 the production of a transgenic rat containing HLA-B27/human β2m provided initial evidence that the presence of HLA-B27 could result in disease when these rats presented with clinical and histological similarities to those found in HLA-B27 associated diseases in humans (Hammer et al., 1990). Shortly after this, Benjamin and Parham proposed the ‘arthritogenic peptide hypothesis’ and argues that a specific peptide only found in joint tissue may be able to bind to multiple HLA-B27 alleles and that the disease is a CTL induced response to these specific peptides only found in joint tissue. Under normal circumstances, the amount of this peptide presented is too

low to initiate a T cell response. The peptide binding specificity of HLA-B27 could select self-peptides that resemble peptides derived from pathogens and in turn initiate an auto reactive immune response (Benjamin and Parham, 1990). However, due to the unusual folding properties of HLA-B27, the relationship between HLA-B27 and AS development proved to be more complicated. HLA-B27 molecules confer unusual cell biology compared to other class I molecules. Compared to other MHC I molecules, HLA-B27 has a slower rate of folding and B2M association which dramatically alters the peptide loading efficiency of HLA-B27, requiring a considerably higher peptide supply and concentration to achieve stable peptide loading (Mear et al., 1999). Alongside this, unlike other MHC I, HLA-B27 is less dependent on tapasin for expression at the cell surface. This results in a tendency to bind and present lower affinity peptides, with more unstable cell surface molecules presented (Peh et al., 1998). Due to the slow folding nature of HLA-B27, there is an increased tendency for these immature heavy chains to misfold and aggregate within the ER. For this reason and to maintain ER homeostasis, a small number of newly formed HLA-B27 molecules are undergoing ER associated degradation (ERAD), a quality control pathway that limits the amount of unfolded or misfolded protein within the ER. In the absence of β2m or peptide, MHC I HC has a tendency to misfold, utilising ERAD to eliminate these HC. However, even in the presence of peptide and β2m, HLA- B27 can still undergo ERAD (Mear et al., 1999). The unusual characteristics of the B pocket, responsible for substrate binding, and the presence of an unpaired cysteine residue at position 67 of the α1 domain (Cys67) is thought to be essential for both peptide binding and the formation of HLA-B27 HC homodimers in the ER. Cys67, along with other B pocket residues, contributes to prolonged ER retention and dimer formation (Dangoria et al., 2002). Allen et al showed in the absence of β2m and usual peptide supply, the HLA-B27 heavy chain formed homodimers when refolded in vitro. In addition to this, both unfolded and folded dimers are capable of forming disulphide linked dimers shortly after synthesis even in cells with intact antigen processing machinery (Allen et al., 1999). The ability to distinguish between unfolded and folded dimers was through the use of conformation-specific antibodies W6/32, recognising folded heavy chain that is associated with β_2m and peptide, and HC10, specific for HLA-B and C heavy chains before they have completed folding in the absence of β2m association (Dangoria et al., 2002). HLA-B27 cell surface complexes also have the tendency to form homodimers following the dissociation of unstable heterodimeric complexes. However, these HC dimers are not likely to arise from intracellular homodimers in the ER but are shown to form from either endosomal recycling of B27 heavy chains, or through the loss of β2m and peptide at

the cell surface. Cys67 has been shown to be critical for the formation of cell surface homodimer (Bird et al., 2003).

HLA-B27 misfolding is a result of its slow folding properties and disulphide bond formation, resulting from unpaired cysteine residues exposed to the ER's oxidative environment. The presence of Cys67 significantly slows the assembly and the exit of class I from the ER. Incubation of cells at 26°C enhanced HLA-B27 dimer formation and induced the formation of HLA-A2 heavy chain dimers, suggesting that MHC I with slow folding kinetics are at higher risk of forming HC dimers in the ER (Antoniou et al., 2004). Therefore altering the peptide supply for loading onto the HLA-B27 molecules may significantly alter the assembly of HLA-B27 and in turn increase both misfolding and aggregation in the ER, but also increase the number of cell surface homodimers present. The increase in aggregation of misfolded proteins within the ER can lead to activation of the unfolded protein response. Also, cell surface HLA-B27 homodimers can act as ligands for killer-immunoglobulin like receptors present on natural killer (NK) cells.

1.14. Unfolded protein response

The ER is the site of entrance for proteins that are destined for secretory pathways and it is also the site of synthesis of many proteins. Therefore the ER relies on an efficient system including protein chaperones to prevent the aggregation and accumulation of unfolded or misfolded proteins. To ensure the protein folding capacity of the ER is not overreached a number of signalling pathways, collectively termed the unfolded protein response, have evolved to maintain a low level of ER stress. The UPR is activated in response to accumulation of unfolded proteins in the ER and acts to reduce the number of proteins translocated to the ER lumen, increase degradation of ER localised proteins and increase the folding capacity of the ER. In normal cellular conditions, ER-associated degradation, where unfolded/misfolded proteins are recognised by presence of unpaired cysteine residues and immature glycans which will be targeted for degradation by EDEM. Sec61 is the transporter believed to transport these peptides back into the cytosol where they enter the ubiquitin-proteasome system to be degraded. However, upon increase in accumulation of these unfolded/misfolded proteins, UPR is activated in response to increase ER stress.

BiP (binding immunoglobulin protein), a member of the heat shock protein 70 family, plays an important role in regulating ER homeostasis. Together with this molecule,

there are three ER transmembrane proteins that regulate the physiological responses to ER stress, inositol-requiring 1 homologue (IRE1), PERK and activating transcription factor-6 (ATF6). Under normal ER conditions, BiP binds to these three proteins preventing their activation. However, in the event of protein misfolding, BiP is released from the UPR effectors and binds to and sequesters the misfolded protein, activating the UPR effectors as a direct result of the increase in ER stress. The first function of the UPR is to try to adapt to the increase in stress by the up regulation of chaperones that aid the folding of proteins, i.e. CNX and CRT and the attenuation of new protein translation to reduce the accumulation or aggregation of misfolded proteins. The PERK and ATF6 are largely responsible for ER adaptation to stress, whilst IRE-1 promotes both survival and pro-apoptotic signalling. If this bid for ER adaptation fails, initiation of autophagy and apoptosis occurs. UPR can lead to the expression of inflammatory mediated genes through activation of IkB and NFkB pathway and also the induction of C-reactive protein and serum amyloid P-component, both of which are associated with the activation of the acute inflammatory response. Pro-inflammatory cytokines IL-6 and IL-1b can also be up regulated. (Tak and Firestein, 2001) (Zhang et al., 2006a). There is increasing evidence that the UPR is activated in cells expressing HLA-B27. This allele has been shown to display characteristics of misfolding and prolonged BiP binding, a characteristic of UPR activation. There is a strong correlation, shown with the use of transgenic rats, between HLA-B27 up regulation and UPR activation in response to increased misfolding and aggregation. (Turner et al., 2005) (Turner et al., 2007).

1.15. Killer-immunoglobulin like receptors

NK cells are a subset of lymphocytes that are an essential part of the innate immune system, having a vital role in killing virally and tumour infected cells. NK cells act by releasing cytokines and perforin/granzymes when triggered by activating receptors CD16 and NCRs. The regulation of NK function is regulated somewhat by the expression of specific receptors on the cell surface. Killer immunoglobulin-like receptors (KIRs) are expressed on NK cells and a specific T cell subset. The KIR gene family resides on chromosome 19q13.4 in the leukocyte receptor cluster and is composed of 15 genes and 2 pseudogenes. KIRs have either 2 (2D) or 3 (3D) domains and either a short (S) or long (L) cytoplasmic domain. KIRs with short cytoplasmic domains are activating, whereas long tails are inhibitory. In addition to interactions with the T cell receptor, MHC I can bind other immune receptors such as KIRs and also leukocyte immunoglobulin-like receptors (LILR). Interaction of KIRs with MHCI allows

the detection of virally infected cells or tumour cells, and can detect levels of MHC I on these cells and in turn promote the necessary response for NK cells. Therefore there is a potential role for the activation of NK cells through KIRs in recognition of HLA-B27 homodimers at the cell surface, which is known to occur in AS patients. KIR3DL1 has been shown to bind HLA class I alleles containing Bw4 motif, which includes HLA-B27 (Peruzzi et al., 1996). It was later shown that HLA-B27 homodimers are recognised by both KIR3DL1 and KIR3DL2 and is not dependent on the peptide sequence bound to MHC I and inhibited NK and T cell IFN-γ production. In contrast to this, HLA-B27 heterotrimers only binds with KIR3DL1 and this interaction is dependent on the peptide sequence (Kollnberger et al., 2002). In combination with this, Chan et al found a significant up regulation of KIR3DL2 on NK and CD4+ cells in patients with SpAs and specifically NK cells from patients with AS had an increase in cytolytic functions. This was up regulation was also shown to be confined to HLA-B27 positive individuals, with an increase in KIR3DL2 in B27 SpA positive patients compared to negative patients (Chan et al., 2005). The strength of the HLA-KIR interaction has become functionally significant and evidence suggests this can play a role in disease. The KIR3DS1 has been shown to be associated with AS and was found in combination with HLA-B alleles compared to controls in a study undertaken in two separate Caucasian cohorts. Alongside this, the inhibitory KIR, KIR3DL1 had a decrease in frequency in patients verses controls (Lopez-Larrea et al., 2006). More recently, however, McCappin et al also undertook a similar study in a Caucasian cohort and found no association of either the stimulatory KIR, KIR3DS1 or the inhibitory KIR, KIR3DL1 (McCappin et al., 2010). KIR interactions with MHC I can promote T cell survival, therefore interactions with B27 homodimers and KIR3DL2 could promote the survival of self-reactive T cells. Also, loss of recognition of inhibitory KIRs or an up regulation in recognition of stimulatory KIRs could be a result of unstable heterotrimeric HLA-B27 complexes at the cell surface or an increase in the expression of HLA-B27 homodimers.

1.16. Aims and objectives

ERAAP is required for efficient processing of N-terminally extended peptides within the antigen processing pathway. Alongside this, ERAAP was identified to facilitate in the cleavage of cytokine receptors for IL-6, TNF-α and IL-1 however the mechanism by which ERAAP exerts its activity has not yet been elucidated. Recently, single nucleotide polymorphisms within ERAAP have been associated with the susceptibility of several autoimmune diseases. The effect of these SNPs on the functional role of ERAAP (antigen processing and cytokine receptor shedding) and how this relates to

these diseases is currently unknown. Therefore elucidating the effect of SNPs (associated with ankylosing spondylitis) on ERAAP function will provide important information in determining the mechanism of action linking these SNPs with disease. Determining the SNPs within alleles and haplotypes from a cohort of AS patients versus controls may provide essential information into the frequency of specific SNPs, and together with functional studies may provide further evidence in understanding the mechanism by which these SNPs may act resulting in disease.

Specifically, the objectives within this study are: to investigate;

1. To investigate the role of SNPs within ERAAP on peptide processing function.
2. To identify the SNPs present within a population of AS patient alleles compared to controls and the effect these have on ERAAP function.
3. To investigate the function of ERAAP in facilitating cytokine receptor shedding.

Chapter 2: Materials and Methods

2.1 Cell culture and maintenance

K89 and B3Z T cell hybridoma (Nilabh Shastri, University of Berkeley, California), CEM (LGC Promochem) and ERAAP$^{-/-}$ were maintained in RPMI 1640 culture medium (without glutamine, Lonza, UK) and U937 cells maintained in DMEM (without glutamine, Lonza, UK). Both RPMI 1640 and DMEM were supplemented with 10% heat inactivated foetal calf serum (FCS, PAA, UK), 2mM L-glutamine (Lonza, UK), 50U/ml Streptomycin (Lonza, UK), 50U/ml Penicillin (Lonza, UK), 1% Hepes (1M, PAA, UK) and 500nM β2-mercaptoethanol (Sigma; complete RPMI/complete DMEM) at 37°C/5% CO_2. Adherent cell lines, K89 and ERAAP$^{-/-}$ were harvested using 1mM EDTA and removed from the cell culture vessel by pastette.

2.1.1 Subcloning of SHL8/H-2K^b specific B3Z T cell hybridoma

B3Z T cell hybridoma was subcloned to obtain clones with higher sensitivity to SIINFEHL (SHL8) bound to H-2K^b presented at the cell surface. Cells were counted and seeded at 1 cell per well in 200µl of a 96 well flat bottomed cell culture plate. After 8-10 days, wells positive for B3Z cell growth were harvested and tested for sensitivity to SHL8 peptide. 10nM SHL8 peptide was added to each well along with K89 APCs and each B3Z hybridoma requiring testing and incubated overnight before the replacing the supernatant with CPRG (section 2.4).

2.2. Reduction of ERAAP expression using RNA interference

2.2.1. Annealing oligonucleotides

Small interfering RNA oligonucleotide (siRNA, Sigma, UK) were used to achieve successful knock-down of mouse ERAAP in K89 cells expressing endogenous ERAAP (table 2.1) Both sense and anti-sense strands contained 3' dTdT overhangs. siRNA duplexes were annealed using 5x siRNA annealing buffer containing 1M potassium acetate, 300mM Hepes, 1M magnesium acetate and RNase free H_2O. The oligonucleotides were annealed by adding 30µl of both sense and anti-sense and 15µl 5x annealing buffer and incubated at 90ºC for 1 minute (Table 2.1) followed by incubation at 37°C for 1 hour, generating a 20mM stock of siRNA oligonucleotides.

Name	Strand	Target sequence
ERAAP	Sense	AGCUAGUAAUGGAGACUCAdTdT
ERAAP	Anti-sense	UGAGUCUCCAUUACUAGCUdTdT
Lamin B1	Sense	CGCGCUUGGUAGAGGUGGATTdTdT
Lamin B1	Anti-sense	UCCACCUCUACCAAGCGCGTTdTdT
hERAAP	Sense	AACGUAGUGAUGGGACACCAUdTdT
hERAAP	Anti-sense	AUGGUGUCCCAUCACUACGdTdT

Table 2.1. siRNA oligonucleotide primers for ERAAP knock-down

2.2.2. siRNA mediated ERAAP knock-down

K89 cells were seeded at a concentration of 5 x 10^4 cells per ml in 2ml complete RPMI in a 6-well cell culture plate and left to incubate overnight to achieve 30-50% confluency at the time of transfection. U937 cells were seeded at a concentration of 5 x 10^4 cells/ml in 2ml complete DMEM and used to transfect immediately. INTERFERin transfection reagent (Polyplus transfection Inc.) was used to achieve knock-down of ERAAP gene expression with siRNA duplexes in both K89 and U937 cells.
The concentration of siRNA first used ranged from 0.6pmoles (8.4ng) to 4.4pmoles (62ng) per well and was optimised to 2.2pmoles (31ng) per well. 2.2pmoles siRNA duplexes (20mM stock) were added to 200µl serum free cell culture medium followed by 8µl INTERFERin reagent and homogenised by vortexing for 10 seconds. This was then incubated at room temperature for 15 minutes to allow formation of complexes between INTERFERin and siRNA. During this time, the media on the cells was replaced with fresh complete RPMI or DMEM. The transfection mixture was added to the correct well at a final concentration of 1nM siRNA. After 48 hours cells were harvested and gene silencing measured by RT-PCR for mRNA ERAAP expression.

2.3 Transfection of human ERAAP and minigene constructs

Human ERAAP variants and minigene constructs were introduced into cells using either Fugene 6 or Fugene HD transfection reagents (Roche, Germany). K89 and ERAAP$^{-/-}$ cells were seeded at a concentration of 10^5 cells/ml in 2ml complete RPMI per well of a six well cell culture plate and incubated overnight to achieve 50-80% confluency. U937 cells were plated at 10^5 cells/per ml in 2ml complete DMEM on the day of transfection. In some experiments using K89 and U937, INTERFERin reagent

was used as above to achieve knock-down of ERAAP gene expression before transfection of ERAAP variants. In these instances, cells were plated and treated with INTERFERin as above and subsequently transfected 5 hours post siRNA knock-down. Serum free RPMI (97µl) was added to 1.5ml eppendorf tubes followed by 3µl Fugene 6 transfection reagent (a ratio of 3µl Fugene 6 to every 1µg plasmid DNA sample used) and incubated at room temperature for 5 minutes. After this, 1µg of the plasmid DNA sample was added and incubated at room temperature for 15 minutes. To generate peptide extracts, ERAAP$^{-/-}$ and K89 cells were seeded as above in 10cm cell culture dishes. In this case, a ratio of 18µl Fugene 6 to 6µg plasmid DNA was used in 600µl serum free RPMI as above. The transfection mix was added drop wise to cells which were harvested after 48 hours for use in the appropriate assay. When transfecting U937 cells, Fugene HD transfection reagent was used at a 3:2 ratio (3µl Fugene HD: 2µg plasmid DNA). The DNA was added to 100µl serum free DMEM, followed by 3µl Fugene HD, briefly mixed and left to incubate for 15 minutes at room temperature. The transfection mixture was added to the U937 cells and harvested after 48 hours for the appropriate assay.

2.4 T cell activation assay

Following 48 hour incubation, K89 or ERAAP$^{-/-}$ transfected cells were harvested, titrated in 96 well plates and incubated overnight with the LacZ inducible B3Z T cell hybridoma at 10^5 cells per well. After incubation, cells were centrifuged at 1500rpm for 2 minutes, the supernatant discarded and replaced with 100µl per well of chlorophenol red-beta-D-galactopyranoside (CPRG; 91mg CPRG, Roche), 1.25ml Nonidet-p40 (Sigma), 9ml 1M $MgCl_2$ (Sigma) per 1 litre phosphate buffered saline and incubated at room temperature. CPRG is a substrate for β-galactosidase and the response is generated through the activation of T cells by the recognition of SHL8 peptide in complex with the H-2K^b which transcribes the LacZ reporter gene generating β-galactosidase. In the presence of β-galactosidase, CPRG is cleaved releasing a substrate causing colour change from yellow to increasing intensities of red upon cleavage. This colour change correlates to the number of SHL8-H-2K^b complexes at the cell surface and is determined by a Biorad 680 microplate reader. Readings were taken at 8 and 24 hours at a reference wavelength of A595nm with an additional wavelength of A695nm used to subtract background levels from the result. The data was then analysed using GraphPad Prism 4.0 software. Statistical analysis was undertaken where possible using a two-tailed paired T test with 99% confidence

interval, or with one-way ANOVA with Dunnett post-test with a confidence interval of 99%.

2.5 Generation of stable ERAAP knock down K89 cells

siRNA oligonucleotides specific for mouse ERAAP were cloned into the p*Silencer* 4.1/CMV/hygro siRNA expression vector system (Ambion) using the digest sites BamHI and HindIII. This vector uses a CMV promoter and an SV40 polyadenylation signal which is able to drive a high level of ERAAP siRNA expression under the selection of the hygromycin antibiotic (0.5mg/ml). This system could then generate K89 cells with stable ERAAP gene silencing and be used to test for hERAAP function. K89 cells were transfected with this p*Silencer* vector as above and 24 hours post transfection the antibiotic hygromycin (0.5mg/ml, Sigma) was introduced to the cells to select for positively transfected K89 cells containing the p*Silencer* vector system. Cells that survived in the presence of hygromycin were expanded and maintained in 10cm culture dishes with complete RPMI under the selection of hygromycin and used in subsequent experiments testing for hERAAP function. Successful reduction in ERAAP gene expression through the stable expression of siRNA specific for ERAAP was assessed by RT-PCR of cDNA created from RNA isolated from these cells (below).

2.6 RT-PCR

In order to determine the expression of either endogenous ERAAP in cells treated with siRNA specific for ERAAP to reduce endogenous gene expression, or to determine successful transfection and transcription of hERAAP mRNA when testing hERAAP and polymorphic hERAAP, RT-PCR was carried out using primers in table 2.4. The PCR was carried out using cDNA from transfected cells as the template and KOD hot start DNA polymerase (Novagen, USA) with 40 cycles of 95°C for 20 seconds, 65°C for 10 seconds and 70°C for 8 seconds. The PCR was analysed on 1% agarose gel electrophoresis for expression.

2.7 Leucinethiol inhibition of ERAAP activity

U937 cells were treated with 0.5mM dithiothreitol (DTT) alone or DTT plus 30µM leucinethiol (Sigma), a potent aminopeptidase inhibitor, for 6 hours before harvesting and analysing by flow cytometry (Serwold et al., 2001).

2.8 Immunoblotting

2.8.1 Preparation of cell lysates

U937 cells used in imunoprecipitation experiments were treated with 160units/ml human IFN-γ (PeproTech, UK) 48 hours prior to harvesting. To create cell lysates, cells were counted and centrifuged 1200rpm for 5 minutes and re-suspended in 500µl PBS to wash the cells before being transferred to an eppendorf and centrifuged at 2,000rpm for a further 3 minutes, generating a pellet of cells. The PBS was discarded and the pellet of cells re-suspended in NP40 lysis buffer; 150mM NaCl (Sigma), 5mM EDTA (Fisher), 20mM Tris-HCl pH7.4 (Sigma) and 1% Nonidet-P40 (NP40, US Biological, USA). Added to the lysis buffer were protease inhibitors iodoacetamide (IAA, 5%, Sigma) and phenylmethyl sulfonyl fluoride (PMSF, 5%, Sigma). Cells were incubated in lysis buffer on ice for 30 minutes before being centrifuged at 13,000rpm for 15 minutes at 4^{o}C to pellet the unwanted cell debris. The resulting supernatant was collected and stored at -20^{o}C until required.

2.8.2 SDS-PAGE gel

1mm gel cassettes (Invitrogen) were used in order to cast a 10% SDS-PAGE gel. Firstly, a 10% resolving gel was generated, using 500µl water saturated butanol to create an even surface at the top of the gel. After this had set, the butanol was removed and replaced with a 5% stacking gel along with a comb containing the appropriate number of wells for samples required to be analysed (table 2.2). 3x Non reducing sample buffer containing 50% glycerol, 1M Tris pH 6, 10% SDS and H_2O, was added to each sample and loaded onto the gel, along with 1ul Magic Marker (Sigma) and 3µl Pro sieve marker (Invitrogen). The samples were run in 1x SDS running buffer at 200V for approximately 1 hour. After this, the resolving gel was used to transfer the protein onto nitrocellulose membrane, hybond C (Amersham, UK). Layers of sponges, whatman paper, hybond C, resolving gel, whatman paper and sponges was assembled and 5x transfer buffer (72.05g glycine, 15.15g Tris base made to 1litre with H_2O) diluted to 1x transfer buffer using 50ml 5x transfer buffer, 150ml H_2O and 50ml ethanol, added to the tank. This was run at 23V for 1 hour at room temperature.

Reagents	10% resolving gel (ml)	5% stacking gel (ml)
dH_2O	4.6	2.1
30% Acrylamide/Bis (37:5:1, BioRad, UK)	3.3	0.5
1.5M Tris HCl (pH 8.8)	2.5	-
0.5M Tris HCl (pH 6.8)	-	0.38
10% SDS	0.1	0.03
10% APS	0.1	0.03
TEMED (Sigma, UK)	0.004	0.002

Table 2.2. Contents of the resolving and stacking gels

2.8.3 Blocking and immunodetection

The nitrocellulose membrane was incubated at 4°C overnight in blocking buffer; 5% milk (Marvel) in PBS with 0.1% Tween 20 (Sigma), in order to block non-specific binding sites. The primary antibodies were diluted in blocking buffer, and secondary antibodies diluted in PBS with 0.1% tween 20 and incubated for 1 hour at room temperature with gentle rocking. The membranes were washed (1x 10 minute followed by 2x 5 minute) with 10ml fresh wash buffer (PBS with 0.1% tween 20) in between antibody incubations with the addition of 2x 5 minute washes after the secondary antibody.

In order to detect the presence of proteins, the membrane was developed using equal volumes of super signal enhancer and super signal stable peroxide (Super signal West Pico and Femto Chemiluminescence Substrate Kit; Pierce) were mixed and incubated for 5 minutes on the membrane then exposed and imaged with Fluor-S Multi-imager (Biorad, UK).

Antibody	Specificity	Species	Assay/Dilution	Source
IL-6R [B-R6]	IL-6R	Mouse mAb	I.P: 10ug FACS: 1:100 WB: 1:1,000	Abcam 1mg/ml
ARTS-1	ERAAP	Rabbit pAb	WB: 1:1,000	Abcam 1mg/ml
H-120	ERAAP	Rabbit pAb	I.P: 10ug	Santa Cruz 200mg/ml
TNF Receptor I	TNFRSF1A	Rabbit pAb	FACS: 1:100	Abcam 1mg/ml
GAPDH	GAPDH	Mouse mAb	WB: 1:10,000	Abcam 1mg/ml
Fluorescin Isothiocyanate (FITC)	Anti-rabbit IgG	Goat	FACS: 1:100	Sigma
Phycoerythrin (PE)	Anti-mouse IgG	Goat	FACS: 1:100	Abcam
Allophycocyanin (APC)	Anti-mouse IgG	Goat	FACS: 1:100	Sigma
Anti-mouse IgG-HRP	Fc specific	Goat	WB: 1:20,000	Sigma
Anti-rabbit IgG-HRP	Fc specific	Goat	WB: 1:20,000	Sigma

Table 2.3. Antibodies used in immunodetection, IP and flow cytometry

2.9 Co-immunoprecipitation for IL-6R and ERAAP

During the creation of cell lysates, 10µg of either IL-6R antibody (Abcam), ARTS1 (Abcam) antibody or mouse IgG as control was added to the lysates and incubated for 30 minutes at 4°C in order to bind to the target protein. During this time, Dynabeads protein G (Invitrogen, UK) were prepared by completely resuspending the beads and transferring 50µl to an eppendorf. The tube containing dynabeads was placed on the supplied magnet to separate the beads from the solution, removing the supernatant to leave the beads. The resulting supernatant from the lysates created, also containing

the appropriate antibody (as in section 2.8.1) was added to the dynabeads. This solution was incubated with rotation at 37°C for 15 minutes in order to bind the antibody to the dynabeads via the Fc region of the antibody. The tube was subsequently placed on the magnet and the supernatant removed, leaving the dynabeads bound with the antibody-target protein (either IL-6R or ERAAP) complex. The removed supernatant was retained for further analysis.

The dynabeads-antibody-protein complex was washed three times resuspending in 200µl PBS for each wash step. Between each wash, the sample was separated on the magnet and supernatant was discarded. Finally the complex was resuspended in 100µl PBS and transferred into a clean tube to avoid co-elution of proteins bound to the tube wall. To elute the target proteins, the 100µl PBS was removed using the magnet and replaced with 20µl 1x NRSB, resuspending the complex by pipetting before heating for 10 minutes at 70°C to dissociate the dynabead complex. After heating, the sample was placed on the magnet and the supernatant remaining was used to analyse by immunoblotting (above).

2.10 Cloning

2.10.1 RNA isolation

The isolation of RNA from cells was carried out using the Zyppy mini RNA isolation II kit (Zymo research, USA) and used according to manufacturers' instructions. RNA was eluted from the zymo IC column in 35µl RNase free H_2O. Any remaining DNA contamination was cleared from the sample using Turbo DNase (Applied Biosystems). This enzyme non-specifically cleaves double-stranded DNA, leaving 5' phosphorylated oligodeoxynucleotides in a reaction consisting of 35µl RNA, 1x turbo DNase buffer and 1µl Turbo DNase enzyme. After incubation for 30 minutes at 37°C, 10x stop buffer was added, vortexed and incubated at 37°C for 1 minute before centrifuging at 13,000 rpm for 1 minute. The DNase treated RNA was transferred to a clean RNase free tube and stored at -80°C until required.

2.10.2 cDNA synthesis

Transcriptor High Fidelity cDNA synthesis kit (Roche) was used to synthesise cDNA using 100ng – 500ng isolated RNA as the template and carried out according to manufacturers' instructions. Briefly, RNA along with anchored-oligo(dT)18 primer were added to sterile, nuclease free PCR tubes on ice and template primer denatured at

65°C for 10 minutes. After this, the 20µl final reaction mixture consisting of 1x reaction buffer, 20 units protector RNase inhibitor, 1mM deoxynucleotide mix , 5mM DTT and 10units reverse transcriptase, was heated to 55ºC for 30 minutes and 85ºC for 5 minutes and reaction stopped by placing at 4°C. The cDNA was directly used for amplification by PCR before being stored at -20ºC until further use.

2.10.3 Amplification of target genes by PCR

Human ERAAP and IL-6R were amplified from 1µg CEM and U937 cDNA using KOD Hot Start polymerase (Novagen, USA) and specific primers incorporating EcoRI restriction sites at the 5' and XhoI site in the hERAAP and XbaI site in the IL-6R at the 3' ends (table 2.4) The PCR reaction was carried out according to manufacturers' instructions with 35 cycles of 95ºC for 20 seconds, 55ºC for 10 seconds and 70ºC for 65 seconds. A small sample was run on a 1% agarose electrophoresis gel to confirm the PCR had been successful and the correct size of DNA was present. When successful, the PCR product was purified using Qiagen PCR Purification kit (Qiagen, UK) according to manufacturers' instructions and eluted in 30µl H_2O. The amount of DNA present was determined using Nanodrop (Thermo Scientific).

Target	Sequence	Restriction site
5' hERAAP	**GACGAATTCATGGTGTTTCTGCCCCTCAAATG**	**EcoRI**
3' hERAAP	**GACCTCGAGCATACGTTCAAGCTTTTCAC**	**XhoI**
5' IL-6 receptor	**GACGAATTCATGCTGGCCGTCGGTGCG**	**EcoRI**
3' IL-6 receptor	**GACTCTAGATCTGGGGAAGAAGTAGTC**	**XbaI**
5' hERAAP RT-PCR	**ACGTAGTGATGGGACACCATTTC**	**-**
3' hERAAP RT-PCR	**TCCCGAACCACTGGTGAGCCA**	**-**

Table 2.4. hERAAP and IL-6R primers used to amplify the genes

2.10.4 Restriction enzyme digest

The plasmid vector pcDNA3 and DNA required to be ligated into the vectors were digested in standard 30µl reactions consisting of dH_2O, 1x BSA, 1x buffer, 2µg DNA, 1µl EcoR1 (12 units/µl; Promega) and 1µl Xho1 (10units/µl; Promega) for hERAAP or 1µl Xba1 (12units/µl; Promega) for IL-6R. A single restriction digest used for analysis

consists of 10ul dH2O, 1x BSA, 1x buffer, 2µg DNA and 1µl restriction enzyme. Reactions were incubated at 37ºC for one hour before being run on 1% agarose electrophoresis gel. The DNA was excised from the gel and purified according to the QIAEX II extraction instructions (Qiagen).

2.10.5 DNA Ligation

Optimal ligation of the digested PCR product into the digested pcDNA3 vector was undertaken by using a ratio of 3:1 of DNA to vector. T4 DNA ligase (NEB, UK) and 10x ligase buffer were used for ligation in a final reaction of either 15µl or 20µl. The reaction was incubated overnight at 16°C

2.10.6 Bacterial Transformation

Top 10 competent cells (Invitrogen, UK) were used to transform cloned hERAAP and IL-6R in the pcDNA3 vector. JM109 cells were used to transform all constructs generated by site directed mutagenesis (section 2.11). 5µl of ligated plasmid DNA or 1ul SDM product was added to bacterial cells and incubated on ice for 30 minutes. Subsequently, the bacteria were heat shocked at 42ºC for 35 seconds and placed on ice. After the addition of 250µl SOC media (table 2.5), the bacteria were incubated for 1 hour at 37ºC with shaking at 220rpm before plating on agar plates containing ampicillin (100µg/ml) and incubated overnight.

Medium	Component
LB	**0.5% Yeast Extract**
	2% Tryptone
	10mM NaCl
SOC	**2.5mM KCl**
	10mM MgCl2
	10mM MgSO4
	20mM Glucose

Table 2.5. Components of LB and SOC medium

2.10.7 Screening of bacterial colonies

To screen the bacterial colonies a selection were picked using 200ul pipette tips and incubated in LB medium containing ampicillin (100µg/ml) at 37°C with 220rpm shacking for 6 hours. The plasmid DNA was purified using the Zyppy plasmid miniprep kit (Zymo Research, USA) and was followed according to manufacturers' instructions. To assess whether the correct insert was present within the plasmid, restriction enzyme digests were carried out as in section 2.10.4 and subsequently run on a 1% agarose gel to determine the presence of the correct insert.

2.10.8 Maxiprep

The bacterial culture containing the correct DNA sequences was amplified overnight in 100ml LB medium containing ampicillin (100µg/ml) at 37ºC with 220rpm shaking. The Zyppy plasmid maxiprep kit (Zymo Research, USA) was followed according to manufacturers' instructions to purify a greater quantity of plasmid DNA for use in functional assays.

2.10.9 Sequencing of DNA

Plasmid DNA containing the correct insert was sent for sequencing at Oxford Geneservice Sequencing Ltd (Sourcebioscience Sequencing Ltd, Oxford, UK) to determine the correct gene sequence or in the case of generation of polymorphic ERAAP, to determine whether a single base change had been incorporated. Primers used for sequencing are shown in table 2.6.

Name	Target sequence	Tm (°C)
T7 Forward	**TAATACGACTCACTATAGG**	**44.6**
SP6 Reverse	**ATTTAGGTGACACTATAG**	**41.2**
hERAAPseq2	**CGATGCTGCGGTGACTCTTCTA**	**56.7**
hDNseq	**GGCATCAGGAAGGGGTGGATG**	**58.3**

Table 2.6. Primers designed for sequencing of ERAAP

2.11 Site Directed Mutagenesis

Human ERAAP in pcDNA3 was used as the template for the site directed mutagenesis. Recently, six single nucleotide polymorphisms were identified by GWAS to have

association with the autoimmune disorder, AS. In order to assess the functional role these SNPs have within hERAAP, we needed to generate constructs of hERAAP containing the individual SNP. Therefore, primers were designed for site directed mutagenesis (SDM), which utilises a PCR reaction to incorporate a single base change into the template DNA. The forward and reverse primers were designed to have a GC content of 55-60% and Tm of 60°C-70°C, consisting of 10-15 nucleotide bases either side of the mutated nucleotide (table 2.7). Two sequencing primers were also designed, positioned 40-50 nucleotides upstream of the M349V mutation (hERAAPseq2) and D575N mutation (hDNseq), in order to identify correctly generated polymorphisms (table 2.6).

The 50µl PCR reaction was set up using KOD Hot Start polymerase as above with forward and reverse primers designed to incorporate a single base change (table 2.7). The reaction conditions were 95ºC for 2 minutes followed by 18 cycles of 95ºC x 20 seconds, 65ºC x 10 seconds and 70ºC x 3 minutes. After SDM, the product was digested with 1µl Dpn1 (10units/µl; Promega) for 1 hour at 37ºC to digest any methylated adenine residues in the original DNA, leaving only mutated ERAAP.

Target	Sequence	Tm (°C)
5' E320A	**CTGGTGCTATGGCAAACTGGGGACTG**	**75.2**
3' E320A	**CAGTCCCCAGTTTGCCATAGCACCAG**	**75.2**
5' R127P	**CTGGAACACCCCCCTCAGGAGCAAATG**	**78.4**
3' R127P	**CAATTTGCTCCTGAGGGGGGTGTTCCAG**	**78.4**
5' M349V	**CTTGGCATCACAGTGACTGTGG**	**67.9**
3' M349V	**CCACAGTCACTGTGATGCCAAG**	**67.9**
5' K528R	**GGACACTGCAGAGGGGCTTTCCTCTG**	**75.9**
3' K528R	**CAGAGGAAAGCCCCTCTGCAGTGTCC**	**75.9**
5' D575N	**CAGCAAATCCAACATGGTCCATC**	**69.3**
3' D575N	**GATGGACCATGTTGGATTTGCTG**	**69.3**
5' R725Q	**GCTCAGTCTCAGAGCAAATGCTGCGGAG**	**77.3**
3' R725Q	**CTCCGCAGCATTTGCTCTGAGACTGAGC**	**77.3**
5' Q730E	**GCTGCGGAGTGAACTACTACTCC**	**65.1**
3' Q730E	**GGAGTAGTAGTTCACTCCGCAGC**	**65.1**

Table 2.7. Primers for the generation of individual polymorphic hERAAP constructs

2.11.1 Ethanol Precipitation

SDM PCR (50µl) reaction was made to 100µl using dH_2O. 0.1x 3M sodium acetate (pH5.5) was added followed by 2.5x 100% ethanol (-20ºC) and incubated at 37ºC for 1 hour. The reaction was centrifuged at 13,000rpm/4ºC for 20 minutes before removing the supernatant and adding 200µl 70% ethanol (-20ºC). This was centrifuged as above for 5 minutes and once again supernatant removed and the DNA pellet left to air dry before re-suspending in 10µl H_2O.

2.12 Peptide Extracts

Cells were seeded into 10cm culture dishes and transfected as above. After 48 hours, cells were harvested and washed with PBS before being resuspended in 400µl 10% formic acid containing 10µM influenza nucleoprotein ASNENMETM to act as an irrelevant 'martyr' peptide for binding to the HPLC column to allow a higher concentration of SIINFEHL or N-terminally extended precursors to be eluted rather than bind to the column. This was heated to 95°C for 10 minutes followed by centrifuging at 13,000rpm/5 minutes. The supernatant was transferred to a 10kD cut-off filter (Millipore, UK) and centrifuged for a further 40 minutes. These extracts were either immediately analysed using RP-HPLC or dried overnight in a speed vac concentrator (Thermo, UK).

2.13 Peptide extracts T cell activation assay

Extracts dried overnight were resuspended in 50µl phenol red in PBS. 0.1M NaOH was used to increase the pH of the sample until the colour returned to red. Samples were incubated for 30 minutes on ice to allow peptides to enter solution. After this, 50µg trypsin or the equivalent amount of PBS as control was added to samples and incubated for 37°C for 5 hours. This was titrated in a 1 in 2 dilution across 8 wells of a 96 well flat bottom plate before the addition of K89 cells as antigen presenting cells at 5 x 10^4 cells per well and B3Z T cell hybridoma at 10^5 cells per well. These cells were incubated with peptide overnight before the addition of CPRG as above.

2.14 Reverse-Phase High Performance Liquid Chromatography

To determine specific fractions of peptides, 50fmoles synthetic peptides (GL Biochem, China) in 400µl dH_2O were fractionated (AIVMK-, MK-, K- and SHL8) on a C18 (0.5µM, 2.1mm ID, 250mm, Vydac) column with 23%-45% acetonitrile/dH_2O gradient at a flow

rate of 0.25ml/min. Peptides extracts from transfected cells were also fractionated and collected in 150µl fractions in a flat bottomed 96 well plate. Fractionated sample plates were dried overnight in a speed vac concentrator (Thermo Scientific, UK). In some experiments 50µg trypsin (Sigma, UK) was added to each well containing sample and incubated at 37°C for 5 hours in order to cleave the lysine residue within the AIVMK-SHL8 peptide, releasing the final SHL8 peptide. K89 cells were used as antigen presenting cells and added at 5 x 10^4 cells per well, along with B3Z T cell hybridoma at 10^5 cells per well for the detection of the peptide. The cells were incubated overnight before the addition of CPRG as above.

2.15 Flow Cytometry

When looking at the effect of ERAAP on cytokine receptor shedding, hERAAP was transfected into U937 cells or hERAAP together with IL-6R into ERAAP$^{-/-}$ fibroblasts and harvested for analysis after 48 hours. Also, U937 treated with siRNA specific for ERAAP or leucinethiol were harvested after 48 hours for analysis of cytokine receptor expression. To determine levels of IL-6R and TNFRS1A surface expression, cells were incubated with the appropriate antibodies as in table 2.3. Cells were washed and analysed with FACs Canto (BD Bioscience) and FlowJo software.

2.16 Enzyme linked-immuno-sorbent assay (ELISA).

ERAAP$^{-/-}$ cells were transfected as in section 2.3 with IL-6R along with vector only, hERAAP or Mutant. 200µl cell culture supernatant was harvest at 0, 6, 11, 24, 30, 36 and 48 hour time points post transfection and stored at -20°C until use. In order to determine concentrations of sIL-6R in cell culture supernatants, ELISA was carried out according to human IL-6R ELISA Kit (Abcam) manufacturers' instructions using harvested supernatants. Firstly, 100µl standard diluent was added to all wells of microstrips pre-coated with IL6R monoclonal antibody. 200µl IL-6R standard was then added to the first well and then titrated at a 1 in 2 dilution over subsequent wells, generating IL-6R standard dilutions ranging between 1000pg/ml to 31.25pg/ml. Following this, 100µl cell culture supernatant samples were added to designated samples wells alongside control samples before the addition of biotinylated anti-IL-6R to all wells. This was incubated for 60 minutes at room temperature before aspirating the liquid from each well and washing 3 times using supplied was buffer. Streptavidin-HRP solution was added to all wells and incubated for a further 30 minutes at room temperature before washing as before. 100µl TMB substrate was pipetted into each well and incubated in the dark for 12-15 minutes at room temperature. The addition

of100µl H_2SO_4 stopped the reaction and the absorbance of each well determined immediately using Biorad 680 Microplate reader at a wavelength of 450nm and 610nm as the reference wavelength.

2.17 AS cases and control patient study

All patient samples were recruited from AS, SLE and RA patients attending the Wellcome Trust Clinical Centre, Southampton General Hospital. The study was performed according to the protocol approved by the National Research Ethics services and Southampton Research Ethics committee (study reference number RHM MED 0869 and rec reference 09/H0504/88). Study title 'ERAP1 (ERAAP) polymorphisms linked to disease susceptibility'.

2.17.1 Isolation of genomic DNA

Total genomic DNA was purified from whole-blood samples obtained from patients using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturers' spin-column protocol. Briefly, 50µl whole-blood was added to 20µl proteinase K and the volume adjusted to 220µl using PBS in order to lyse the samples. Buffering conditions were adjusted to provide optimal binding to the DNeasy spin column. Contaminants and enzyme inhibitors were removed through two was steps and the DNA was eluted in 200µl buffer AE and stored at -80°C until further use.

2.17.2. Isolation of RNA

200µl whole blood was used to isolate RNA from patient samples using ZR whole-blood total RNA kit (Zymo Research R1020,) and was followed according to manufacturers' instructions, eluting RNA in 50µl DNase/RNase free H_2O into an RNase-free 1.5ml tube. Turbo DNase reaction (section 2.10.1) was carried out immediately after RNA isolation.

2.17.3. Cloning hERAAP from patient samples

cDNA synthesis was carried out as in section 2.10.2 immediately after turbo DNase treatment of RNA, using 10.4µl patient RNA sample. cDNA was subsequently used to amplify hERAAP using PCR protocol as above and primers in table 2.4 to amplify full length 2.7Kb ERAAP. Amplification of 2.7Kb ERAAP was not always successful, therefore in these instances, RT-PCR forward primer was used with the hERAAP reverse primer, amplifying a 2.6Kb ERAAP fragment, analysed by 1% agarose gel

electrophoresis. The hERAAP was cloned into pcDNA3 as above (section 2.10) and initially four bacterial colonies picked, processed and sent for sequencing to determine the two allele ERAAP sequences within each patient sample to determine the patient haplotype. Once different ERAAP alleles were determined, they were maxipreped as above (2.10.8) to provide a stock of ERAAP from each patient to use in functional studies to assess the activity of each allele on the ability to process N-terminally extended peptides.

Chapter 3: The role of ERAAP in processing antigenic peptide precursors

Proteolysis of antigenic precursors in the cytoplasm frequently generates N-terminally extended peptides which require further processing before optimal loading onto the MHC molecule for stable cell surface presentation can occur. These peptides are translocated into the ER for loading through TAP which has been shown to transport peptides between 8-21 amino acids in length into the ER in vitro and preferentially transports 8-15 amino acid peptides in vivo (Paulsson et al., 2001; Schumacher et al., 1994). Therefore, a proportion of the peptides transported into the ER will be N-terminally extended, requiring trimming to the appropriate length for MHC I loading (Goldberg et al., 2002); (Cascio et al., 2001). ERAAP was identified as a key member of the antigen processing machinery through its ability to trim N-terminally extended peptide precursors to the optimal length for stable loading on to MHC (Driscoll et al., 1993; Saric et al., 2002; Serwold et al., 2002). At present, the mechanisms and interactions of ERAAP with other molecules within the ER, including the peptide loading complex, are largely unknown. The role of ERAAP in antigen processing was further reinforced when an increase in peptide trimming activity of ERAAP occurred with the up-regulation of IFN-γ, consistent with the up-regulation of members of the PLC (Saric et al., 2002; Serwold et al., 2002; York et al., 2002). The absence of ERAAP activity alters the antigenic repertoire for presentation at the cell surface and in turn affects the activation of the $CD8^+$ T cell immune response (Blanchard et al., 2008; Hammer et al., 2006; Hammer et al., 2007). Polymorphisms within in the ERAAP sequence have shown a strong linkage to AS as well as other diseases such as hypertension, psoriasis, diabetes and cervical carcinoma (Burton et al., 2007; Fung et al., 2009; Mehta et al., 2009; Strange et al., 2010; Yamamoto et al., 2002). The greatest genetic link to AS, which is an autoimmune inflammatory disorder, is the presence of the HLA-B27 molecule. The exact role of HLA-B27 within the disease is unknown, however it has been postulated that the propensity of the molecule to form dimers in the ER and at the cell surface contributes to disease progression (Antoniou et al., 2004; Burton et al., 2007; Dangoria et al., 2002). Despite this, no satisfactory explanation exists for the fact the HLA-B27 is normally non-pathogenic (99% of HLA-B27 positive individuals never develop AS) and that some other factors, genetic or environmental, must be required to precipitate HLA-B27 linked pathogenicity in AS. The role of ERAAP and the six identified SNPs in the disease is unknown, although the ability to generate peptides for loading onto HLA-B27 molecules may affect its ability to dimerise. For this reason it was important to elucidate the effects of these polymorphisms within ERAAP on the ability to generate final peptide epitopes for presentation. These polymorphisms may alter ERAAP activity in the generation of

optimal peptide epitopes for stable MHC I loading, resulting in a change in peptide repertoire presented at the cell surface. This provides a functional link between peptide trimming in the MHC I antigen processing pathway and ERAAP polymorphisms as a first step towards associating ERAAP and HLA-B27 in AS pathogenicity.

3.1. ERAAP is required for processing of N-terminally extended peptides

In this study, a well characterised murine model system (Shastri and Gonzalez, 1993) was utilised to determine the role of ERAAP on processing of N-terminally extended peptide precursors within the endoplasmic reticulum. The B3Z T cell hybridoma express a specific TCR at the cell surface that recognise the modified peptide derived from OVA(257-264), SIINFEHL, bound and presented on H-2K^b (SHL8/H-2K^b complex). Recognition of this complex at the cell surface by B3Z transcribes the β-galactosidase (lacZ) construct which is driven by elements from the IL-2 promoter in these cells. A chemiluminescent substrate, CPRG, is cleaved in the presence of β-galactosidase, resulting in a colour change and is used to detect the lacZ activity of B3Z cells. This colour change correlates with the amount of SHL8 peptide presented by H-2K^b at the cell surface and can determine the level of processing of N-terminally extended peptides to the final SHL8. Minigene constructs with differing N-termini were introduced into mouse L-cells (K89 cells) that stably express the H-2K^b molecule at the cell surface. These minigene constructs consist of SIINFEHL (SHL8), a modified version of the immunodominant epitope of OVA. This 8 amino acid peptide is of optimal length and therefore does not require trimming prior to stable MHC I loading. Also an N-terminally extended 13 amino acid peptide was used, AIVMK-SIINFEHL (X5-SHL8), that requires the removal of 5 amino acids to yield the final SHL8 prior to loading onto MHC I. In addition, these minigene constructs are preceded by an ER signal sequence peptide (ES) which allows translocation directly into the ER lumen following protein translation where the signal peptide is cleaved by signal peptidases to release either the SHL8 or X5-SHL8 peptides. This allows any aminopeptidase activity to be limited to the ER only.

Knock-down of ERAAP gene expression in K89 cells was necessary to allow the assessment of ERAAP on trimming the additional N-terminal amino acids. To successfully reduce ERAAP expression, different concentrations of siRNA oligonucleotides (siRNA) complementary for mouse ERAAP or Lamin B1 (control) were introduced into K89 cells. RT-PCR from these cells allowed optimal conditions to

reduce ERAAP gene expression to be identified (figure 3.1a). The optimal concentration of siRNA to reduce the gene expression was 2.2pmoles, reducing the endogenous ERAAP expression by approximately 90%, whereas transfection of Lamin B1 siRNA into K89 cells had little effect on ERAAP expression in comparison to untreated cells (figure 3.1a).

To evaluate the requirement of ERAAP to process the N-terminally extended peptide precursor to its final form, SHL8 or X5-SHL8 were introduced into K89 cells, figure 3.1b. These cells were shown to stimulate the SHL8/H-2K^b specific T cell hybridoma, B3Z, equally well. As N-terminally extended precursors are unable to bind to H-2K^b and therefore do not stimulate the B3Z, the reconstitution of a functional ERAAP was identified as being able to trim the additional N-terminal 5 amino acids, generating the final SHL8 peptide for presentation on H-2K^b (figure 3.1b). Next siRNA was used to reduce ERAAP expression, as in figure 3.1a. Transfection of SHL8 into K89 cells with reduced ERAAP expression showed little difference compared to normal K89 (ERAAP positive); however transfection of X5-SHL8 revealed a dramatically reduced ability to stimulate the B3Z (figure 3.1c). This confirms the requirement for ERAAP to trim N-terminally extended precursors to the correct epitope length for its presentation. The response shown with the X5-SHL8 was not completely reduced, which may indicate that either the knock-down of ERAAP expression was not 100% complete, providing some trimming activity, or peptide trimming that is not limited to the ER may occur at low levels, being transported into the cytosol, processed and re-entering the ER through TAP transporter to be loaded onto H-2K^b, providing pMHC I to stimulate the B3Z hybridoma.

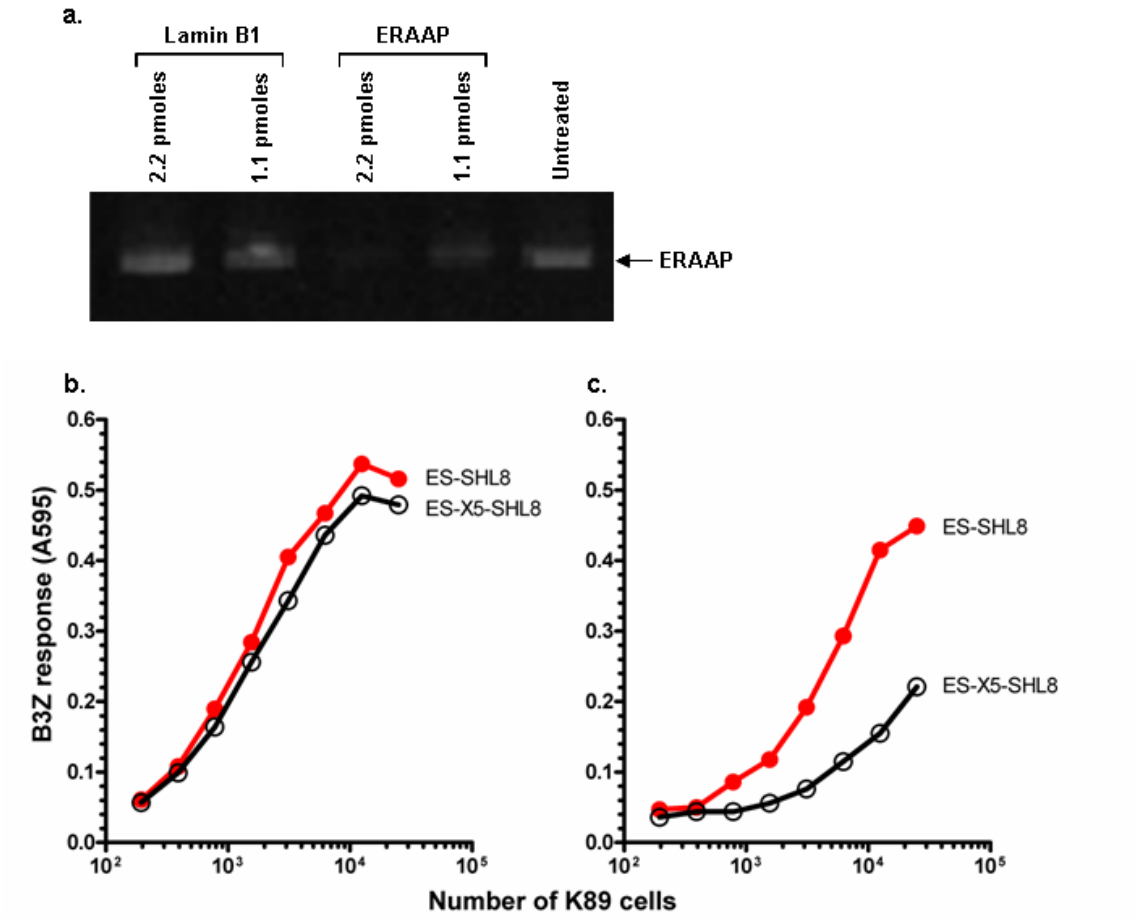

Figure 3.1. ERAAP is required for trimming of N-terminally extended precursors.

K89 cells were treated with different concentrations of siRNA oligonucleotides specific for ERAAP or Lamin B1 (control). RT-PCR for ERAAP shows a successful reduction in ERAAP expression (2.2pmoles, a). K89 cells (ERAAP positive) were transfected with either 1µg ES-SHL8 or ES-X5-SHL8 (b). K89 cells were transfected with ERAAP specific siRNA oligonucleotides together with 1µg ES-SHL8 or ES-X5-SHL8 (c). In the presence of ERAAP, the final SHL8 peptide can be generated from the precursor X5-SHL8. However, in the absence of ERAAP, the B3Z response is reduced when X5-SHL8 is introduced.

3.2 Reconstitution with human ERAAP restores peptide trimming activity

Wild type human ERAAP (hERAAP), encoding a 941 amino acid protein, was successfully isolated from human CEM cells, a leukaemic lymphoblastoid cell line. The gene was cloned into the pcDNA3 plasmid expression vector and sequenced prior to use to ensure the correct sequence was present with no variations compared to

published wild type hERAAP. To determine the role of hERAAP in processing the additional N-terminal amino acids from the X5-SHL8 to release the final SHL8 peptide, hERAAP was introduced into K89 cells with reduced ERAAP expression (figure 3.2). This was possible since the ERAAP siRNA were mouse specific, therefore the addition of hERAAP would not be targeted by this siRNA (figure 3.2a). Firstly, the optimal concentration of hERAAP to restore peptide processing activity in these cells was determined. K89 cells treated with siRNA for ERAAP were further transfected with X5-SHL8 along with different concentrations of hERAAP. Cells treated with Lamin B1 and therefore maintained endogenous ERAAP expression were transfected with X5-SHL8 as a positive control (figure 3.2b). Transfection with 1µg of hERAAP restores trimming activity to the same level as positive control containing endogenous ERAAP expression, indicated by the B3Z response comparable with Lamin B1. Transfection with other concentrations hERAAP generated a reduced B3Z response in comparison to that shown with 1µg hERAAP. Therefore in subsequent experiments 1µg hERAAP is used to restore peptide trimming activity to physiological levels

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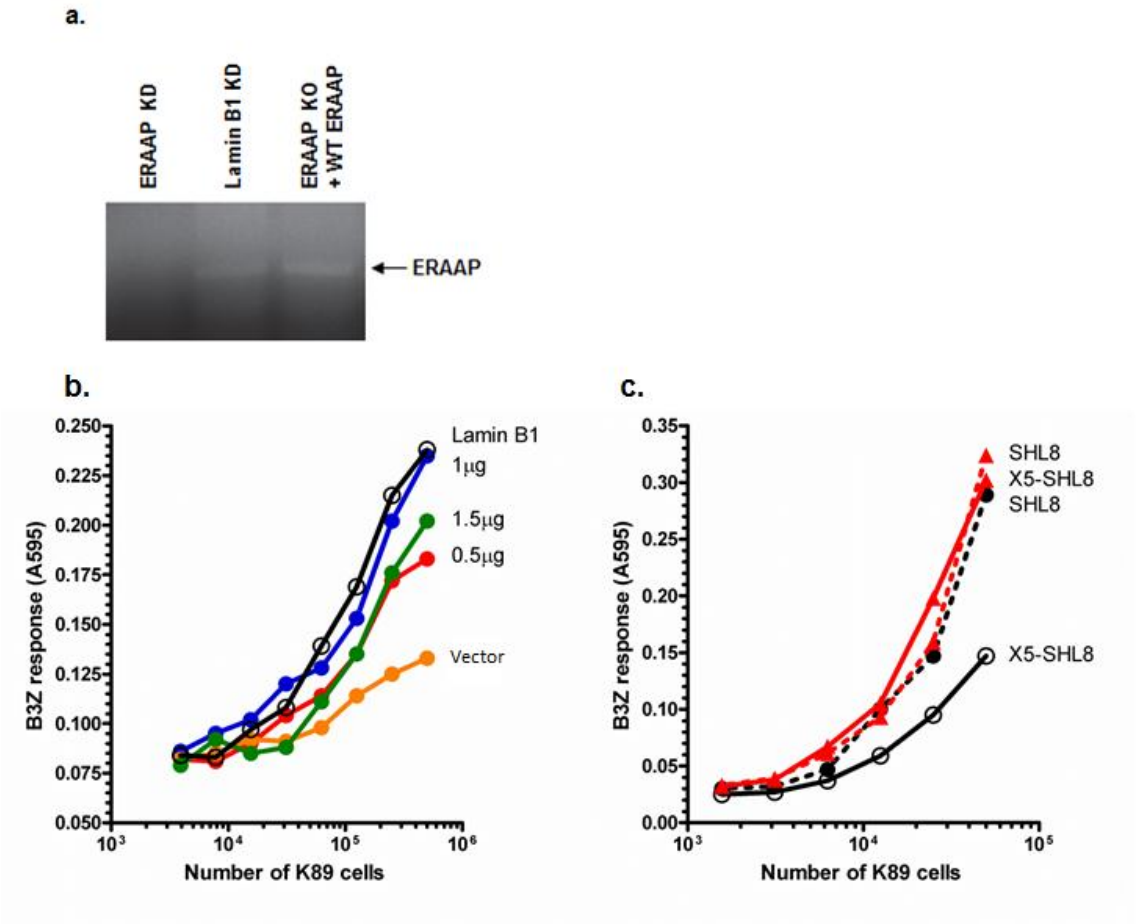

Figure 3.2 Reconstitution with human ERAAP restores peptide processing

K89 cells were treated with ERAAP specific siRNA oligonucleotides to reduce ERAAP expression (a). These cells were transfected with 1µg X5-SHL8 along with 1µg pcDNA3 or either 0.5µg, 1µg or 1.5µg hERAAP (b). B3Z response indicates optimal ERAAP expression and activity is generated from 1µg hERAAP. K89 cells treated with ERAAP siRNA were then transfected with either 1µg SHL8 (dashed line) or X5-SHL8 (solid line) with either pcDNA3 (black) or hERAAP (red, c). Addition of hERAAP into cells with reduced ERAAP expression restores peptide trimming activity.

The addition of hERAAP into cells with reduced ERAAP expression restores peptide processing activity to generate the final optimal 8 residue peptide, SHL8 from X5-SHL8 (figure 3.2c). The B3Z response observed in hERAAP X5-SHL8 ERAAP knock-down transfected cells is the same as that from the SHL8 transfected cells in the presence and absence of ERAAP, when tested over a range of E:T ratio (figure 3.2).

Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) was used to further confirm the ability of hERAAP to process X5-SHL8. The SHL8 system has previously been optimised (Serwold et al. 2002) to identify trimmed products of the X5-

SHL8 precursor, eluted from X5-SHL8 expressing target cells. Extracts were fractionated before the addition of APCs and B3Z to detect the level of SHL8 generated. From this I was able to determine hERAAP trimming activity by the elution of SHL8 peptide at a specific fraction (fraction 20). These fractions were identified by using the SHL8 synthetic peptide (figure 3.3a). Peptide extracts created from K89 cells previously treated with ERAAP or Lamin B1 siRNA and transfected with X5-SHL8 or SHL8 (figure 3.3b) along with hERAAP or vector only (figure 3.3c) were fractionated on HPLC. Analysis of processed peptides from cells containing either endogenous ERAAP or reduced ERAAP expression shows a distinct peak at fraction 20/21 co-eluting with SHL8. Transfection of final SHL8 peptide into these cells demonstrated a comparable B3Z response. Conversely, the addition of the N-terminally extended precursor, failed to generate a response in the absence of ERAAP, consistent with a reduction in the production of the final SHL8 epitope. A small level of B3Z stimulation was observed in ERAAP knock-down K89 X5-SHL8 cells, shown by a small SHL8 peak (figure 3.3b). This is consistent with the previous observations in which the addition of an N-terminally extended precursor into cells with reduced ERAAP still generates a small level of B3Z stimulation (figure 3.1c). In cells with reduced ERAAP expression, reconstitution with hERAAP restores peptide processing activity, generating a response similar to SHL8. Thus, the reconstitution of hERAAP can generate the same level of B3Z stimulation from the release of SHL8 as the addition of SHL8 which does not require processing.

These data show that ERAAP expression is essential for the generation of pMHC I molecules from the N-terminally extended precursor and are consistent with the results observed from the T cell activation studies (figure 3.2b).

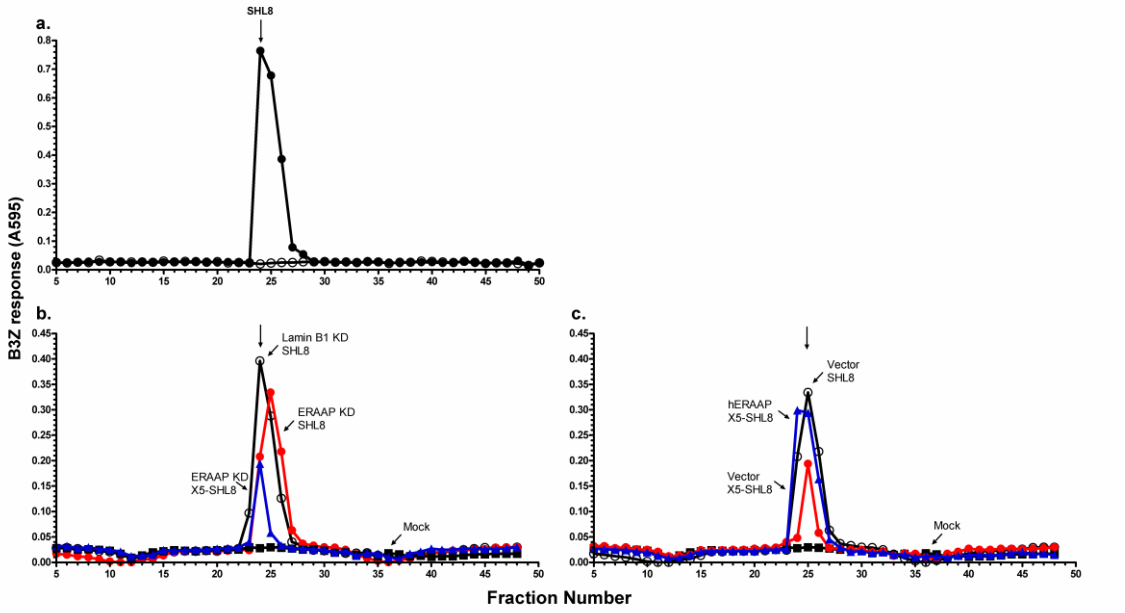

Figure 3.3 Human ERAAP restores trimming activity

RP-HPLC identified SHL8 elution in fraction 24 (a). Lamin B1 or ERAAP siRNA treated K89 cells were transfected with either X5-SHL8 or SHL8 (b) and ERAAP siRNA treated K89 cells transfected with either X5-SHL8 or SHL8 along with either pcDNA3 or hERAAP (c). Peptide extracts from these cells were run on RP-HPLC. ERAAP is required for generation of the final peptide epitope, with the addition of hERAAP able to generate the final peptide from the precursor.

3.3. Isolation of hERAAP variants

During the isolation of hERAAP, two variants were cloned from CEM and U937 leukaemic lymphoma cells. Sequencing of these variants was carried out to determine the differences between wild type hERAAP (hERAAP) and the unknown cloned hERAAP (figure 3.4). The first variant of hERAAP contained six SNPs that were previously identified in the linkage analysis study with AS, (Burton et al., 2007). This full length hERAAP variant contains intact active site domains and has no other alterations compared to hERAAP. This strongly suggested that this allele is present in the human population. The second variant of hERAAP was found to be a truncated splice variant. This variant consists of the same N-terminus and active site domains; however the protein is truncated, following the failure to accept the splice site of exon 11, leading to the extended translation of exon 10 and results in a premature stop codon generating a 514 amino acid protein. There were no SNPs present within this second variant of hERAAP. For the purpose of this study these variants are termed mutant and

hERAAPtr respectively. These variants had not been previously documented within the literature and therefore their activity was unknown. It was previously shown that the GAMEN active site domain was important for the enzymatic activity of ERAAP. Using the hERAAP-pcDNA3 construct, site directed mutagenesis was undertaken to generate a mutation at amino acid position 320 from a glutamic acid to an alanine (E320A). This mutation occurs within the GAMEN active site motif common to M1 family of metalloproteases, resulting in a GAMAN motif previously shown cease ERAAP activity (Kanaseki et al., 2006).

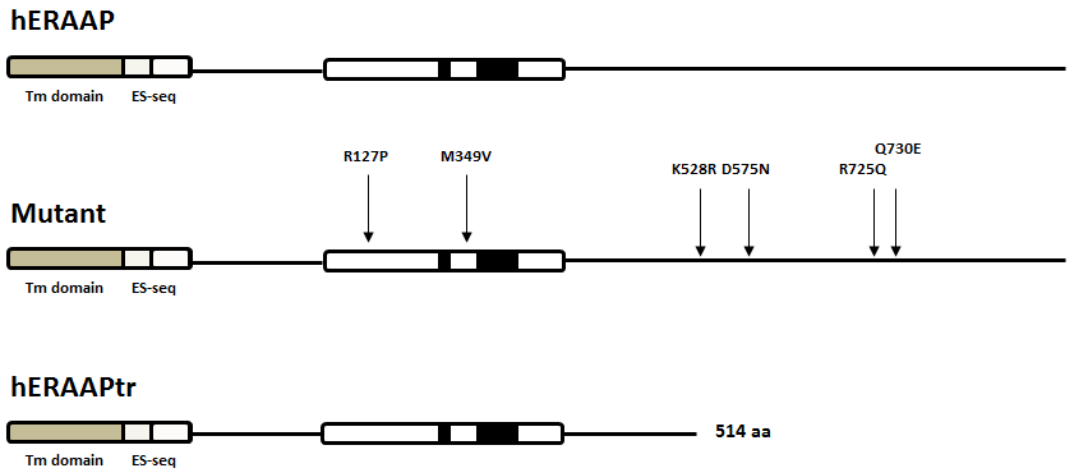

Figure 3.4. Schematic of cloned hERAAP variants

During the cloning process, two variants were identified, corresponding to hERAAP containing six SNPs (mutant) and a truncated splice variant (hERAAPtr).

3.4. Human ERAAP variants reduce peptide processing activity

As the requirement of ERAAP to process N-terminally extended peptides had been demonstrated, the activity of the novel hERAAP variants (mutant and hERAAPtr) identified during the cloning process would be assessed for their ability to process N-terminally extended peptides. The E320A mutation is known to have no enzymatic activity and for this reason, the E320A hERAAP was used as a negative control in subsequent experiments, allowing comparison of enzymatic activity with the hERAAP and hERAAP variants (Kanaseki et al., 2006).

The use of transient knock-down of ERAAP in K89 cells with siRNA followed by reconstitution with the minigene constructs SHL8 and X5-SHL8 provided variable and inconsistent results between experiments. Therefore, a stable cell line with reduced ERAAP expression was generated, which provided a more consistent and long-term solution to the variation in siRNA ERAAP knock-down efficiency. Using the p*Silencer* 4.1 CMV plasmid expression vector system (Ambion), containing an SV40 polyadenylation signal that can express high levels of ERAAP siRNA, stable K89 cells with reduced ERAAP gene expression were generated under hygromycin antibiotic selection. These cells were shown to have reduced ERAAP protein expression compared to normal K89 (figure 3.5a). To first determine the peptide processing activity of hERAAP, K89 cells with p*Silencer* stable knock-down of ERAAP gene expression (ERAAP knock-down K89 cells) were transfected with either empty vector or hERAAP along with either SHL8 or X5-SHL8 (figure 3.5b). The addition of hERAAP into these ERAAP knock-down K89 cells was shown to restore the ERAAP expression to normal levels. SHL8 transfected cells were able to stimulate the B3Z, whereas X5-SHL8 transfected cells failed to stimulate the B3Z to the same extent, generating a response consistent with previous experiments (figure 3.1). Reconstitution of ERAAP knock-down K89 with hERAAP recovered the ability to trim the precursor to the final SHL8 epitope, shown by a high ability to stimulate a B3Z response comparable to SHL8 with no ERAAP present.

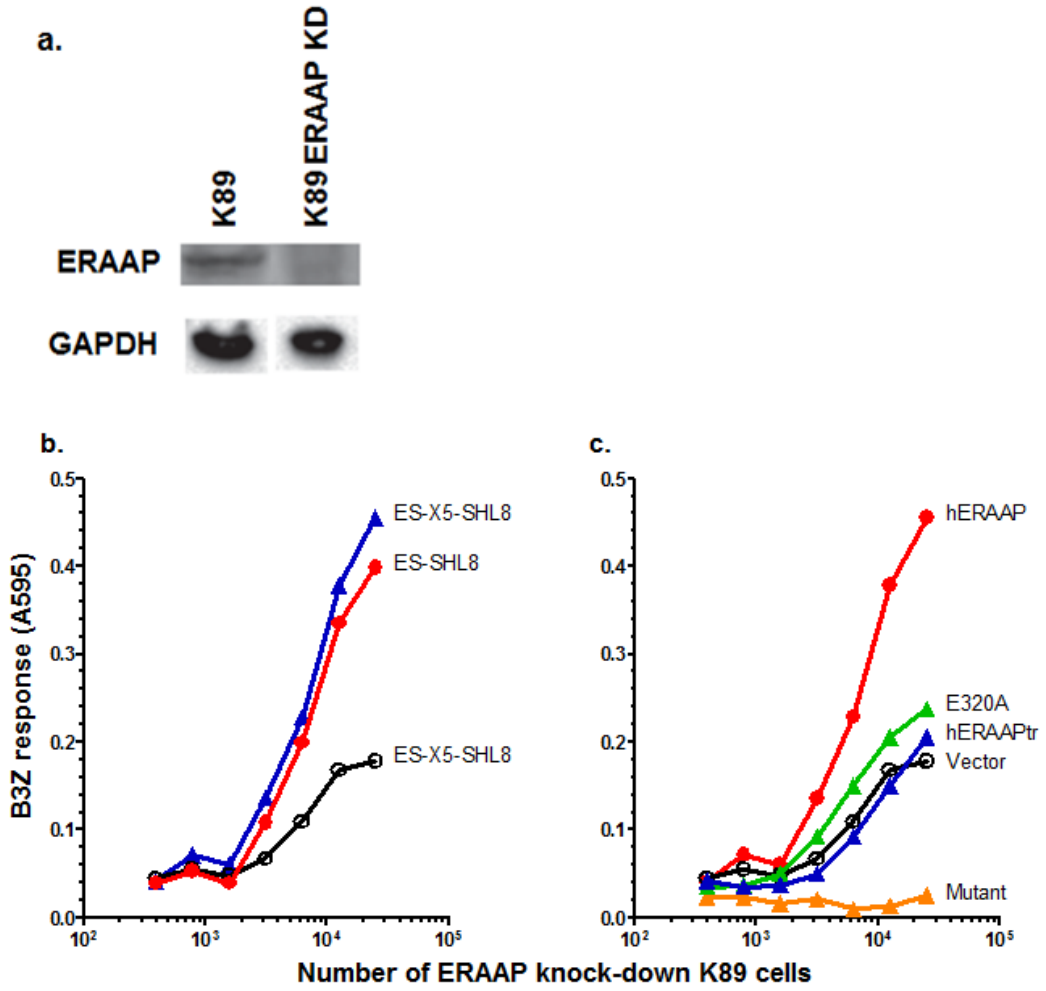

Figure 3.5. Addition of human ERAAP variants in cells with reduced expression of ERAAP alters ability to recover peptide processing activity.

K89 cells were stably transfected with p*Silencer* vector containing ERAAP specific siRNA oligonucleotides and show a successful reduction in ERAAP protein expression (a). These cells were transfected with either SHL8 or X5-SHL8 along with pcDNA3 or hERAAP (b). ERAAP knock-down K89 cells were then transfected with X5-SHL8 along with pcDNA3, hERAAP, E320A, mutant or hERAAPtr (c). Reconstitution of hERAAP restores peptide processing; however the addition of mutant abrogates any B3Z response, with E320A and hERAAPtr having reduced activity comparable with vector only.

To further elucidate the trimming phenotypes of the cloned hERAAP variants, these cells were transfected with X5-SHL8 together with the hERAAP variants (figure 3.5c). By contrast to hERAAP (figure 3.5b and c), reconstitution with the E320A hERAAP failed to recover precursor trimming and generated a response similar to vector alone

(figure 3.5c). This result is consistent with that observed by Kanaseki et al in which this mutation at position 320 ceases the peptide processing capability of ERAAP (Kanaseki et al., 2006). The two hERAAP variants, hERAAPtr and mutant also failed to generate a response, indicating an absence of function. Interestingly, the addition of mutant into these cells with the N-terminally extended precursor completely reduces the B3Z response to a greater extent than vector alone (figure 3.5c).

3.4.1. AS associated SNPs alter peptide processing activity

The autoimmune inflammatory disorder AS is genetically linked to the presence of an HLA-B27 allele, which has a propensity to form miss-assembled HC homodimers, especially when the supply of peptides to the ER is limited ($TAP^{-/-}$) or when the peptide editing machinery in the ER is non-functional ($Tpn^{-/-}$). It is therefore possible that polymorphisms in ERAAP may alter its ability to generate an optimal supply of peptides to stably support assembly of HLA-B27/β_2m heterodimers. The SNPs identified in the 2007 GWAS study are present collectively within the mutant allele isolated during the cloning hERAAP process (Burton et al., 2007). This haplotype has already been shown to be non-functional at restoring B3Z response in X5-SHL8 expressing cells (figure 3.5). Therefore, in an attempt to map this loss of function to specific individual amino acid variants, the extent to which they alter the ability of hERAAP to generate the final epitope from the N-terminally extended precursor was assessed. It was also of important to identify whether these SNPs in combination generated the phenotype shown by mutant hERAAP, or whether it was an individual SNP alone that was responsible for the abrogation of activity. Not only was it important to identify the function of these SNPs in relation to the mutant hERAAP phenotype, but identifying their ability to generate the correct epitope for stable MHC I may identify the role they play within AS susceptibility.

In order to determine the function of the SNPs, hERAAP constructs containing the six AS-associated individual SNPs were generated. Using the hERAAP-pcDNA3 construct, site directed mutagenesis that utilises PCR to incorporate the single nucleotide change by primers designed specifically for each mutation was carried out and each construct sequenced to ensure a single base change which would result in the amino acid change (table 3.1).

Amino Acid Position	Nucleotide change	Amino Acid change	Name
127	**G > C**	**Arg > Pro**	**R127P**
349	**A > G**	**Met > Val**	**M349V**
528	**A > G**	**Lys > Arg**	**K528R**
575	**G > A**	**Asp > Asn**	**D575N**
725	**C > A**	**Arg > Gln**	**R725Q**
730	**C > G**	**Gln > Glu**	**Q730E**
320	**A > C**	**Glu > Ala**	**E320A (control)**

Table 3.1. Single nucleotide polymorphisms in ERAAP

As before, ERAAP knock-down K89 cells were transfected with X5-SHL8 and reconstituted with hERAAP or E320A as controls (positive and negative ERAAP activity respectively) and also with the hERAAP containing SNPs, M349V, D575N, R725Q or Q730E to assess their function (figure 3.6). The M349V and D575N SNPs show little difference in B3Z stimulation compared to wild type hERAAP, suggesting they are able to trim the N-terminally extended precursor to the same extent as hERAAP. Both R725Q and Q730E show some reduction in B3Z response, which would be consistent with a reduction in enzymatic activity, suggesting these two SNPs have a greater impact on the role of ERAAP to generate MHC I peptide epitopes compared to the M349V and D575N. However, it is noticeable that none of these 4 SNPs generate the similar phenotype to mutant, suggesting it may be either K528R or R127P that reduces the activity, or that a combination of more than one SNP is required. These preliminary data into the activity of the SNPs on ERAAP function suggest that a) R725Q and Q730E have the most effect on trimming activity and b) individual variants do not act independently to alter ERAAP function.

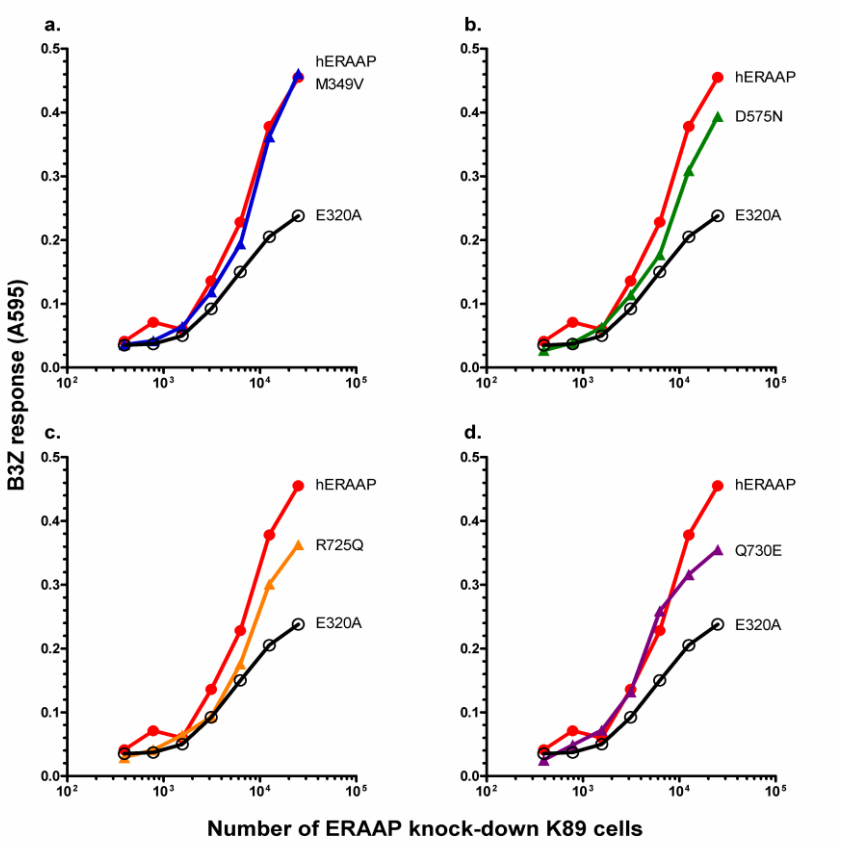

Figure 3.6. Addition of ERAAP containing SNPs alters peptide processing activity

K89 cells stably transfected with p*Silencer* vector were transfected with X5-SHL8 along with hERAAP, E320A or ERAAP containing SNPs M349V (a), D575N (b), R725Q (c) or Q730E (d). The SNPs indicate a hierarchy of peptide trimming with R725Q and Q730E SNPs reducing trimming activity.

3.4.2. Refining the X5-SHL8 trimming assay using ERAAP$^{-/-}$ cells

The results generated so far have demonstrated that reconstitution of ERAAP knock-down K89 with hERAAP is able to completely restore peptide trimming in these cells. We obtained ERAAP$^{-/-}$ fibroblasts generated from ERAAP$^{-/-}$ mice. ERAAP knock-out mice were generated by the targeted deletion of exons 4-8 in the gene encoding ERAAP, resulting in a loss of functional ERAAP protein expression (Yan et al., 2006). To compare which cell line was best to use in later experiments, ERAAP knock-down K89 and ERAAP$^{-/-}$ cells were transfected with the minigene constructs (figure 3.7). Although both cell lines reveal an inability to trim X5-SHL8 to SHL8, the difference was more pronounced in ERAAP$^{-/-}$ cells (figure 3.7c). Therefore, ERAAP$^{-/-}$ cells would be

used in all further experiments to highlight the differences in processing activity of the hERAAP variants.

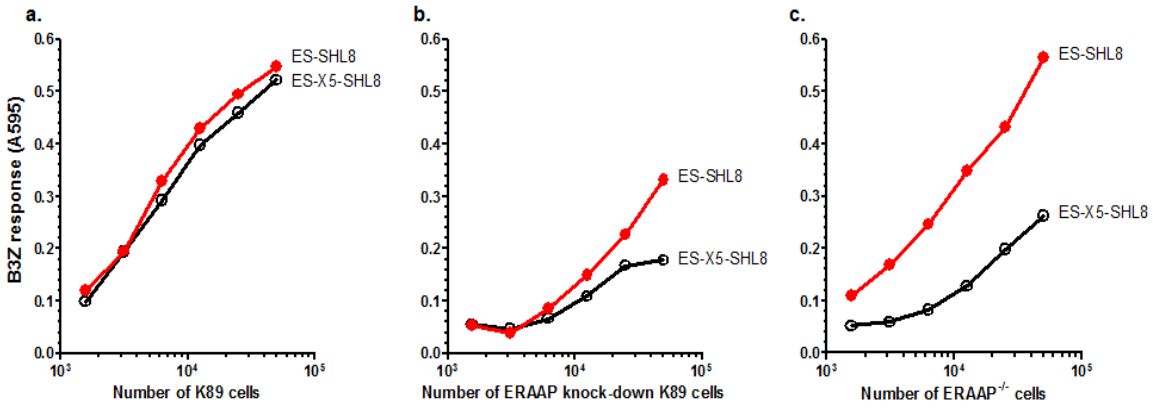

Figure 3.7. ERAAP$^{-/-}$ cells show greater variation in response to peptide requiring processing compared to final length peptide.

ERAAP expressing K89 (a), ERAAP knock-down K89 (b) or ERAAP$^{-/-}$ cells (c) were transfected with SHL8 or X5-SHL8. ERAAP is required to trim N-terminally extended peptide from the precursor X5-SHL8 to SHL8, however the difference in response between X5-SHL8 and SHL8 is greater in ERAAP$^{-/-}$ cells (b).

3.4.3. Human ERAAP variants reduce trimming activity in ERAAP$^{-/-}$ cells

To further investigate the functions of hERAAP and variants mutant and hERAAPtr, ERAAP$^{-/-}$ cells were transfected with X5-SHL8 together with these variants. Western blot shows expression of the hERAAP variants after transfection into these cells (figure 3.8a). Reconstitution of hERAAP once again restored peptide trimming activity, shown by the increased B3Z stimulation. The vector only generated some level of B3Z stimulation, however when Mutant and hERAAPtr were introduced into these cells, the B3Z response is negative, generating a phenotype comparable to E320A (figure 3.8b). This difference between these variants and vector only is consistent with previous findings (figure 3.5), suggesting ER independent trimming of X5-SHL8 may occur, however the variants may act to inhibit this mechanism by sequestering the peptide from further trimming not limited to the ER. The difference observed between these variants and hERAAP was found to be statistically significant (figure 3.8c, $p=<0.01$) when presented as a percentage of maximal responses (hERAAP=100%, figure 3.8c). This suggests that both mutant and hERAAPtr do not have functional peptide trimming

ability. These results are consistent with our previous experiments using K89 ERAAP knock-down cells.

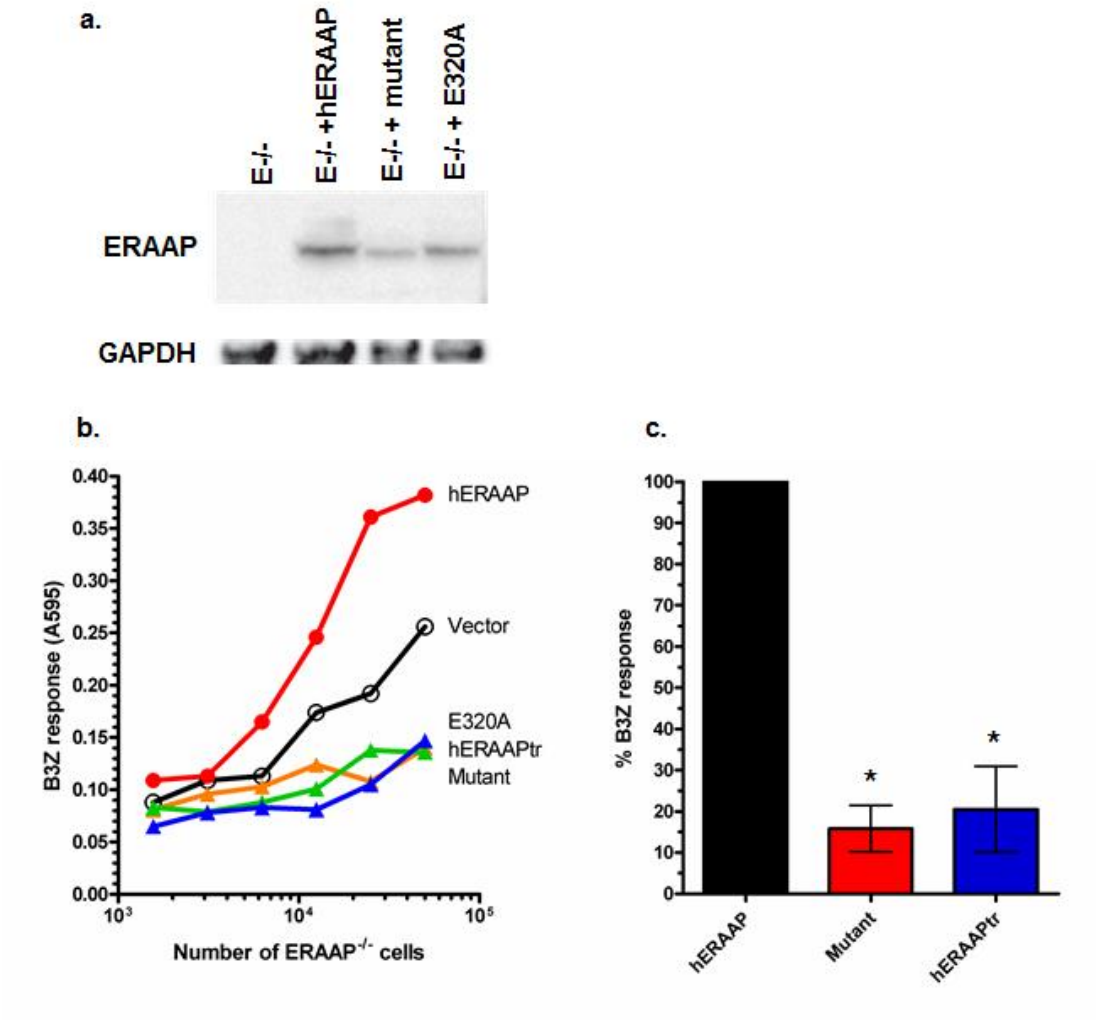

Figure 3.8. Addition of different hERAAP variants into ERAAP$^{-/-}$ cells shows a reduction in trimming of peptide precursors.

Western blot shows the expression of transfected ERAAP variants (a). ERAAP$^{-/-}$ cells were transfected with X5-SHL8 together with vector, hERAAP, mutant, E320A or hERAAPtr (b). Reconstitution with hERAAP restores peptide processing from the X5-SHL8 precursor to the final SHL8 peptide. However the addition of mutant, E320A and hERAAPtr show a significant reduction in trimming activity (c, * *p*=<0.01). Data is a representation of ten experiments.

To confirm the absence of SHL8 trimmed peptide from these variants, peptide extracts were generated from ERAAP$^{-/-}$ cells transfected with X5-SHL8 along with hERAAP and variants E320A, mutant and hERAAPtr. These extracts were either treated with PBS or

trypsin before the addition of K89 cells as APCs and B3Z hybridoma. Treating extracts with trypsin will cleave the final lysine residue of the N-terminal extension releasing the final SHL8 peptide which can then bind H-2K^{b} and stimulate B3Z response. Using trypsin allows us to determine the presence of the untrimmed peptide precursor within the cell extracts. In the absence of trypsin, only extracts from cells reconstituted with hERAAP can stimulate the B3Z, with mutant, vector and E320A having similar reduced responses, comparable to untransfected cells (figure 3.9a). However, the addition of trypsin generated B3Z responses in all transfected cell samples but requires a much higher number of cell equivalents to generate a response similar to hERAAP (figure 3.9b). This further shows the ability of hERAAP to restore peptide processing and reveals that peptide precursors are present and therefore not trimmed by the hERAAP variants mutant, E320A and hERAAPtr. These data are consistent with a hypo-active trimming phenotype. Also evident is that in the presence of hERAAP there is approximately 1 log more SHL8 after trypsin treatment, which suggests that the substrate (X5-SHL8) is not limiting.

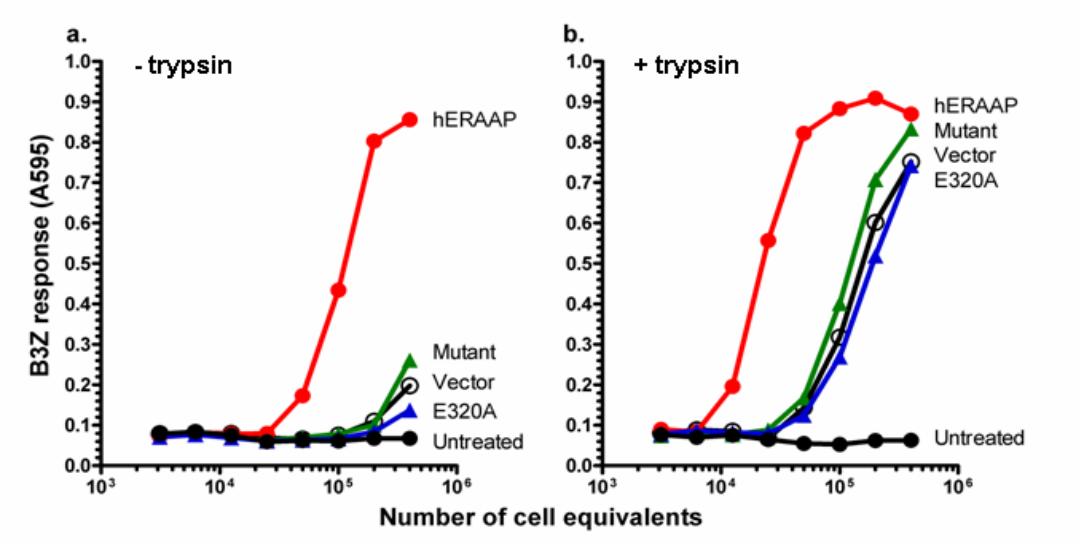

Figure 3.9. Addition of different hERAAP variants into ERAAP$^{-/-}$ cells reduces the generation of final peptide SHL8

Peptide extracts were created from cells transfected as in figure 3.8 and treated with PBS (a) or trypsin (b) before the addition of antigen presenting cells, K89 and T cell hybridoma, B3Z. Mutant and E320A fail to generate the final SHL8 epitope from the N-terminally extended precursor, whilst hERAAP restores trimming activity.

RP-HPLC was utilised to further identify the ability of hERAAP and hERAAP variants to remove the additional 5 N-terminal amino acids. In addition, a trypsin incubation step following fractionation of cell extracts was included to allow the identification of the presence of SHL8 precursor peptides (X5-SHL8). Peptide extracts from ERAAP$^{-/-}$ cells transfected with X5-SHL8 and different ERAAP variants were fractionated by RP-HPLC.

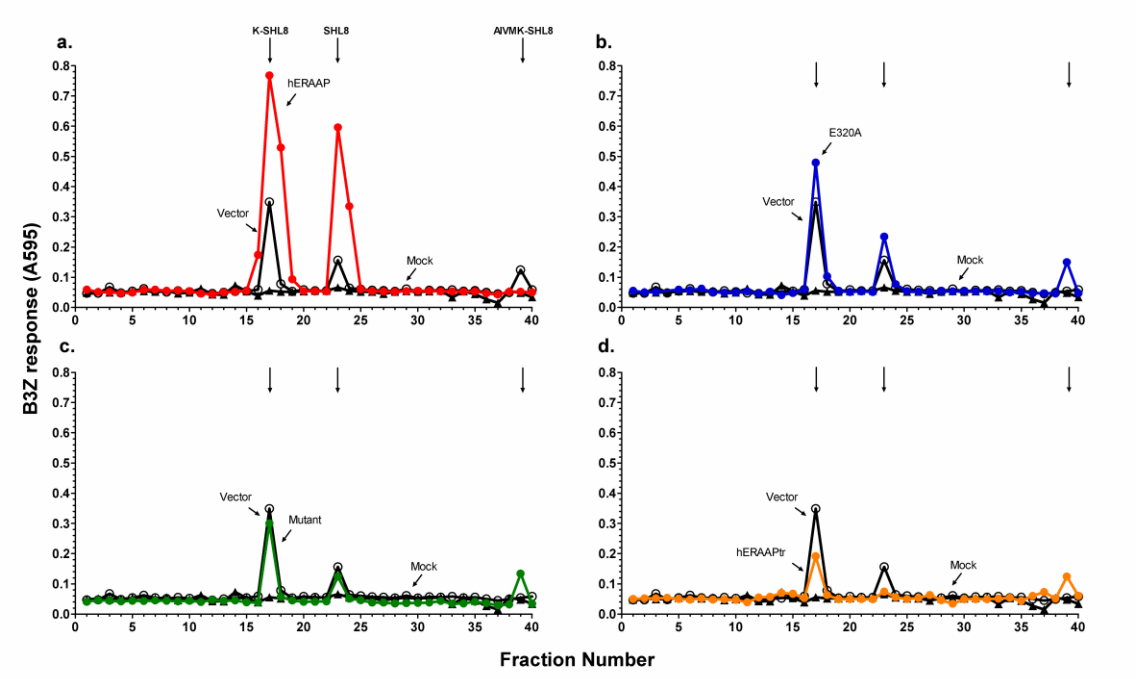

Figure 3.10. HPLC fractionation of peptide extracts from cells containing hERAAP variants

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 and either vector alone, hERAAP (a), E320A (b), mutant (c) or hERAAPtr (d) and peptide extracts from these cells run on HPLC. Synthetic peptide runs, K-SHL8 and SHL8, eluted in fractions 17 and 23. Human ERAAP can trim the precursor to the correct length, whereas E320A, mutant and hERAAPtr generates a response similar or less than vector alone.

Analysis of processed peptides in HPLC fractionated extracts showed two distinct peaks corresponding to SHL8 and K-SHL8. K-SHL8 stabilises the H-2D^{b} molecule and therefore is detected following trypsin treatment of extracts from ERAAP$^{-/-}$ cells. This is not observed in K89 since they do not express H-2D^{b} molecules. As expected the

amount of K-SHL8 and SHL8 observed in ERAAP transfected cells was much greater than that seen when other hERAAP variants were present, indicating reconstitution of peptide trimming (figure 3.10a). Cells transfected with E320A generated some B3Z response indicating low level trimming, however as E320A has no trimming activity, and the results are the same as vector alone. These results are consistent with non-ER restricted trimming (figure 3.10b). The addition of mutant and hERAAPtr revealed B3Z stimulation comparable with vector alone and E320A (figure 3.10 c and d). The absence of B3Z responses by mutant and hERAAPtr indicate a hypo-active trimming profile.

Overall these data suggest that hERAAP can restore peptide processing activity. Two novel hERAAP variants, mutant and hERAAPtr, have been identified, and demonstrate a significantly reduced ability to process N-terminally extended precursors to their final optimal peptide stable for loading.

3.5 Single Nucleotide Polymorphisms in ERAAP alter the trimming activity

Preliminary data undertaken using K89 ERAAP knock-down K89 cells suggested the SNPs associated with AS were able to alter the processing of N-terminally extended peptides. However at that time not all SNPs constructs had been created and K89 ERAAP knock-down cells showed limited difference between positive and negative controls. Therefore it was necessary to further characterise the role of these polymorphisms within hERAAP using ERAAP$^{-/-}$ cells. These ERAAP SNPs were transfected into the ERAAP$^{-/-}$ cells and their ability to trim X5-SHL8 to the final SHL8 peptide was assessed. As it has previously been shown E320A ERAAP has a minimal trimming activity and was therefore used throughout as a negative control. Figure 3.11 shows a representative T cell activation assay (from five experiments) indicating the trimming activity of the individual SNPs within hERAAP. Of the six SNPs tested, three generate a significant reduction in response compared to hERAAP activity (K528R, R725Q, Q730E, figure 3.11g).

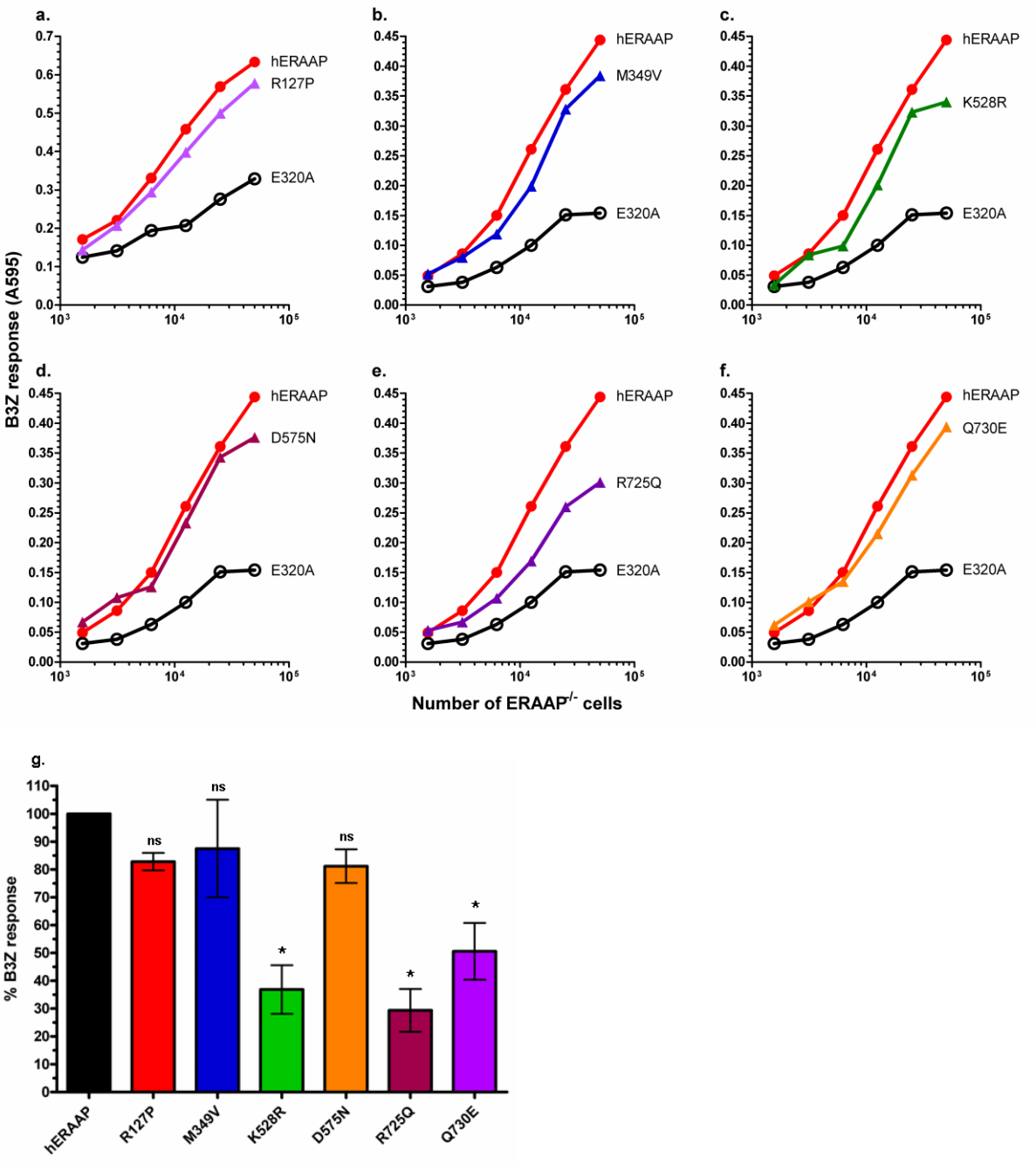

Figure 3.11. The addition of individual SNPs hERAAP variants alters peptide processing.

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 together with hERAAP, E320A or ERAAP containing SNPs R127P (a), M394V (b), K528R (c), D575N (d), R725Q (e), and Q730E (f). The SNPs indicate a hierarchy of peptide trimming with D575N able to process peptides similar to hERAAP; however K528R and R725Q ERAAP have a reduced processing ability (* *p*=<0.01) . Data is a representation of five experiments.

R127P, M349V and D575N show only a slight reduction in trimming activity which do not have a statistically significant difference (~15-20%, figure 3.11 a, b, d and g). Q730E shows a significant reduction in B3Z response (p= <0.01) with an average reduction of 50% activity (figure 3.11f and g). However, R725Q and K528R SNPs show the greatest phenotype of the individual SNPs and has a statistically significant reduction in B3Z response (p=<0.01, ~60-70%, figure 3.11b, e and g). These positions within ERAAP may therefore be responsible for the peptide processing activity. However, although the SNPs have a significant reduction in response, none of the SNPs generate a response that is completely abrogated (E320A negative control).

3.6. Double SNPs suggest a cumulative effect on trimming activity

The preliminary data (section 3.4.1) suggested the possibility that SNP may not act alone in altering ERAAP function, but that they might interact as an allele to alter function since none of the single SNPs generated a loss of function phenotype as profound as mutant (containing all six SNPs). As a result of this observation, it was important to investigate whether this phenotype could result from combinations of more than one SNP. Double SNP variants were created in a similar manner to the individual SNPs, using the SNP ERAAP variants as templates and site directed mutagenesis to incorporate a second polymorphism within the ERAAP. The different combinations of double SNPs were assessed in the same way as described above for their ability to process N-terminally extended peptide.

3.6.1. Double SNPs containing R725Q have a reduced trimming ability

Firstly, all double SNPs containing the R725Q polymorphisms were tested for their ability to trim the N-terminally extended precursor to the final peptide. As this SNP individually generated the most reduction in trimming activity of all the SNPs tested compared to wild type hERAAP, it was of interest to assess this polymorphism in combination with the other SNPs (figure 3.12).

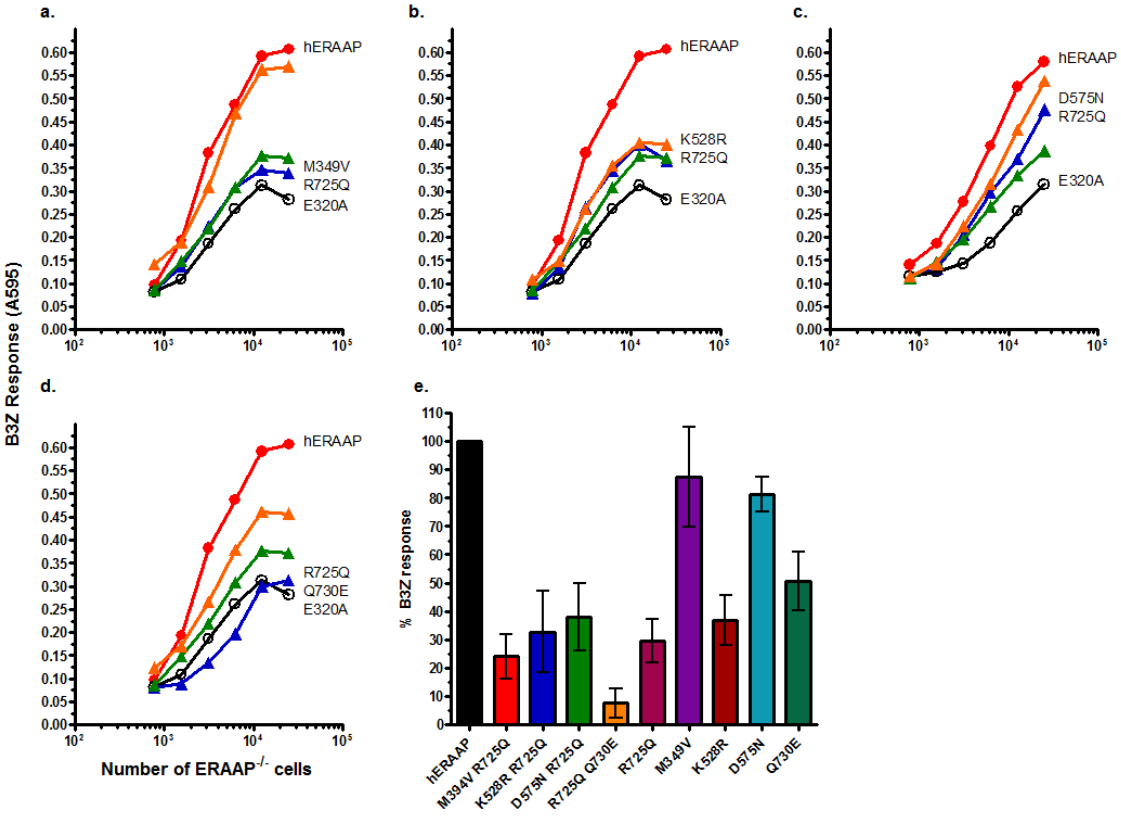

Figure 3.12. R725Q family of double SNPs reduces peptide precursor processing

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 together with hERAAP, E320A or double SNPs M349V / R725Q (a), K528R / R725Q (b), D575N / R725Q (c) or R725Q / Q730E (d). These SNPs in combination with R725Q SNP reduce the processing ability of ERAAP compared to wild type hERAAP. The R725Q / Q730E has an abrogated response which is lower than E320A control (* *p*=<0.01) Data is a representation of six experiments.

Overall, the findings observed show that R725Q double SNPs have a more pronounced reduction in trimming activity compared to both wild type and individual SNPs. R725Q in combination with M349V, K528R, D575N and Q730E show phenotypes similar to E320A and appear to have reduced trimming of the N-terminal extension resulting in a reduced B3Z response (figure 3.12). All double R725Q SNPs generate a statistical significant reduction in B3Z response compared to hERAAP (*p*=<0.01, figure 3.12e). R725Q in combination with M349V or K528R show a reduction in response compared with the activity of R725Q individually, however do not completely abrogate the response as seen with mutant (figure 3.11, 3.12a, b and e). The addition of D575N with R725Q has little change in the trimming activity of this

ERAAP compared to R725Q activity alone; however the addition of D575N does not restore the trimming activity to wild type ERAAP, still generating a statistically significant reduction in trimming (~60%). Interestingly, the R725Q / Q730E double SNP showed a complete abrogation of B3Z response; having the greatest phenotype of all the double SNPs (p=<0.01; figure 3.12d). This was comparable to the complete loss of function observed with the mutant phenotype. Further to this, the combination of SNPs (M349V, K528R and Q730E) with R725Q results in a reduction in response regardless of their individual trimming capacity. This implies that the region of 725 – 730 is a key area of ERAAP required for trimming of N-terminal extensions.

3.6.2. Double SNPs containing K528R have reduced trimming ability

As K528R also caused a significant reduction in processing of X5-SHL8 to SHL8 (figure 3.11c and g), it was important to identify whether SNPs in combination with this polymorphism generate more cumulative effect as shown with R725Q double SNPS (figure 3.12). Overall the findings observed show that like R725Q, K528R in combination with any other of the three SNPs reduced the trimming activity of hERAAP (figure 3.13). However, none of these double SNPs completely abrogated trimming activity as seen with R725Q / Q730E. All SNPs in combination with K528R exhibit a greater reduction in trimming activity compared to the K528R alone, with all the K528R double SNPs having a statistically significant reduction (p=<0.01) in the ability to generate the final SHL8 peptide compared to hERAAP. The K528R / Q730E shows the greatest trimming activity with only ~50% reduction compared to hERAAP, whereas the K528R / D575N only has 20% activity compared to hERAAP which is a dramatic decrease in trimming compared to D575N alone, which has ~80% trimming activity compared to hERAAP. Interestingly, none of the K528R double SNP ERAAPs generated a complete abrogation of trimming similar to E320A. However, it shows that a combination of more than one polymorphism has a cumulative effect on reduction in ERAAP function. These results suggest that K528R region of ERAAP is important for the functional activity of ERAAP in peptide processing.

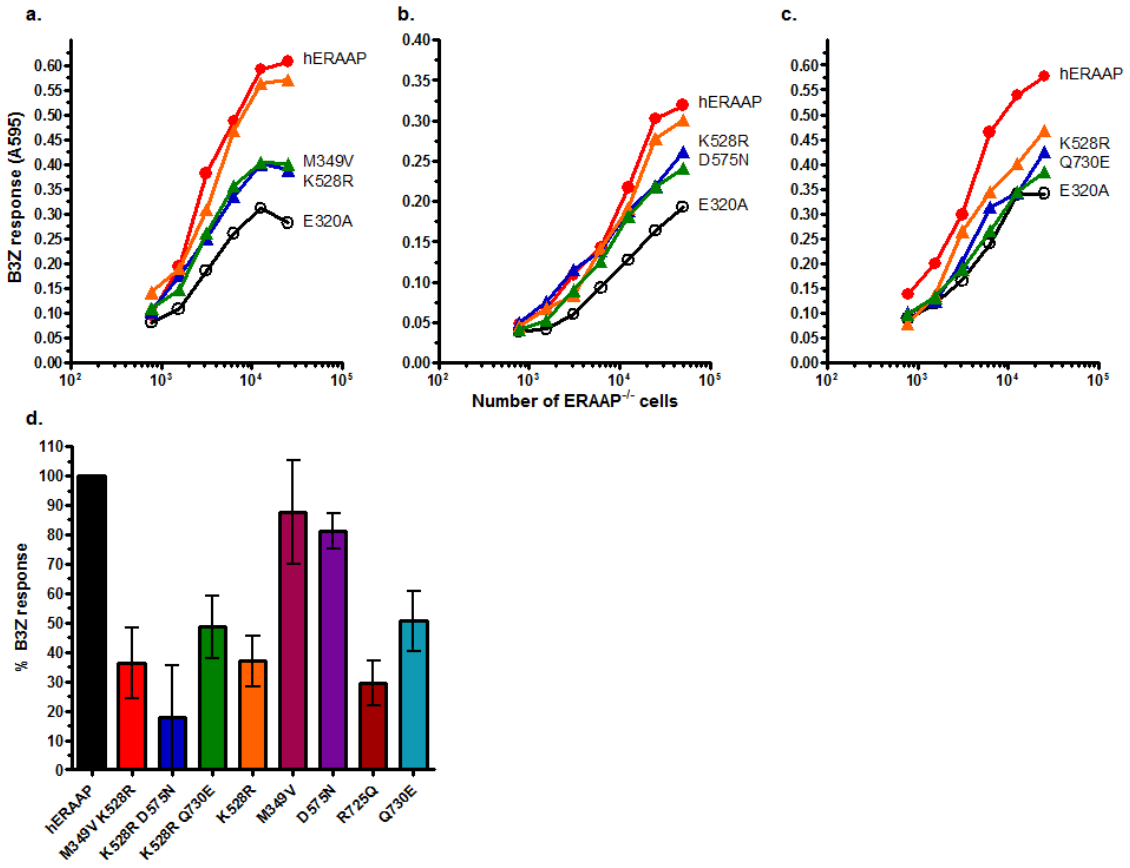

Figure 3.13 K528R family of double SNPs reduces peptide precursor processing

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 together with hERAAP, E320A or double SNPs M349V / K528R (a), K528R / D575N (b) or K528R / Q730E (c). These SNPs in combination with K528R SNP reduce the peptide processing function of ERAAP (* *p*=<0.01). Data is a representation of six experiments.

3.6.3. Selective double SNPs have functional trimming activity

The combination of any individual SNP with K528R and R725Q all show statistically significant reduction in trimming activity compared to hERAAP. However not all double SNP combinations were shown to reduce trimming activity to the same extent. The three remaining double SNP combinations M349V / D575N, M349V / Q730E and D575N / Q730E did not show a significant reduction in trimming activity in comparison with hERAAP activity (figure 3.14). Therefore the activity of these double SNPs that do not contain either K528R or R725Q further implies that maintaining functional processing activity may be dependent on the K528R and R725Q regions.

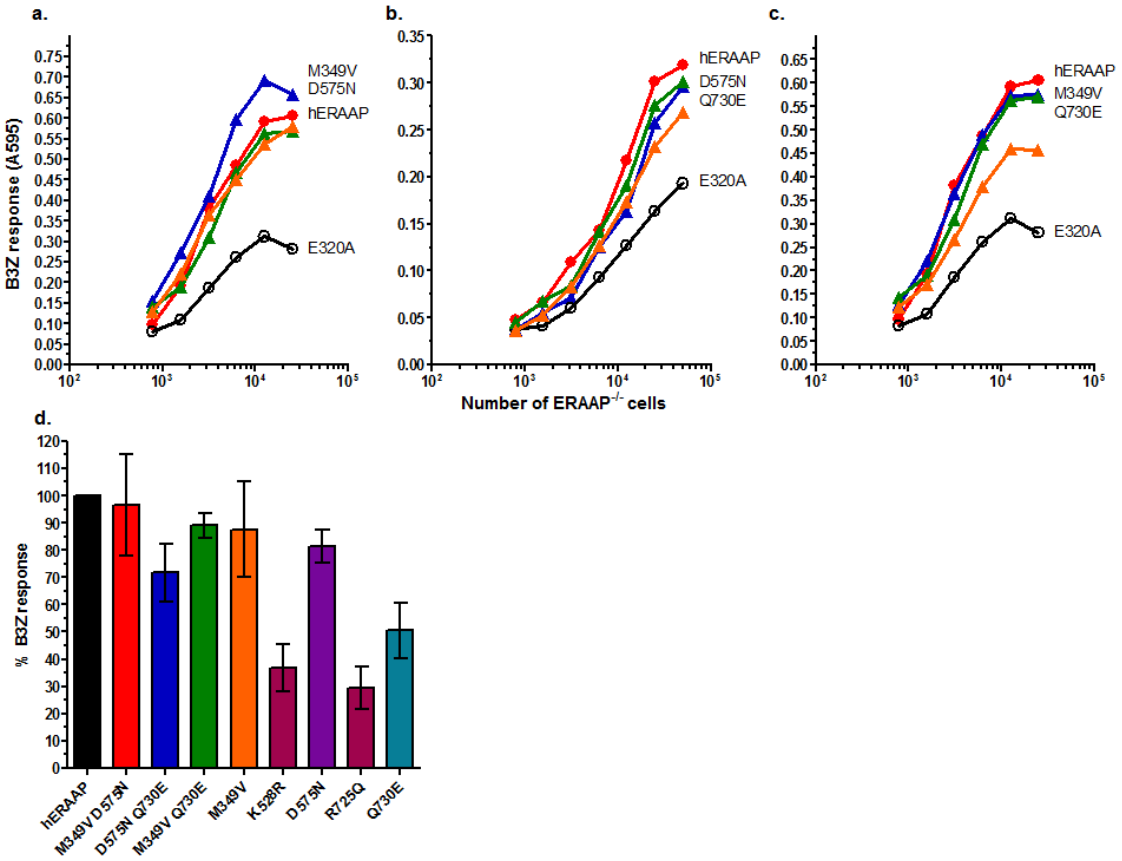

Figure 3.14. Functional peptide processing activity of ERAAP containing double SNPs

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 together with hERAAP, E320A or double SNPs M349V / D575N (a), D575N / Q730E (b) or M349V / Q730E (c). These double SNPs show the ability to trim N-terminally extended precursor to the same extent as wild type human ERAAP, indicating that these SNPs do not alter the function of ERAAP in peptide processing activity. Data is a representation of six experiments.

These data suggests that combinations of double SNPs have a cumulative effect on peptide processing of N-terminally extended peptides to generate the final epitope. Overall, a reduction has been shown with double SNP ERAAP variants and these results suggest that the consistent lack of B3Z stimulation with SNPs in combination with R725Q and K528R indicate that these two areas are important for ERAAP activity in its role in the antigen processing pathway to generate final peptide antigens.

3.7. An alternative substrate indicates ERAAP variants have reduced activity

ERAAP has been shown to have substrate specificity, preferentially cleaving hydrophobic and basic residues. As X5-SHL8 required 5 amino acids to be cleaved before release of the final epitope; we wanted to investigate whether ERAAP and its variants could trim an alternative N-terminal extension, LEQLEK-SHL8. This precursor contains an extra N-terminal amino acid and is less hydrophobic, with more charged amino acids. The hERAAP variants were tested as before, substituting X5-SHL8 for X6-SHL8 (figure 3.15). The results observed show that wild type hERAAP can restore peptide processing activity, generating the final SHL8 epitope. However, as observed in the previous experiments, the hERAAP variants, E320A, mutant and hERAAPtr are unable to cleave this peptide precursor to the final epitope, shown by B3Z stimulation consistent with vector only. The significant reduction in enzymatic activity of mutant and hERAAPtr ($p=<0.01$) is consistent with the significant reduction in the ability of these two variants to cleave the AIVMK N-terminal extension (figure 3.8b). This suggests that the addition of an amino acid and an altered N-terminal region does not change the inability of mutant and hERAAPtr to cleave this N-terminal extension. In addition, hERAAP was able to trim X5-SHL8 showing that wild type hERAAP is able to process both AIVMK and LEQLEK.

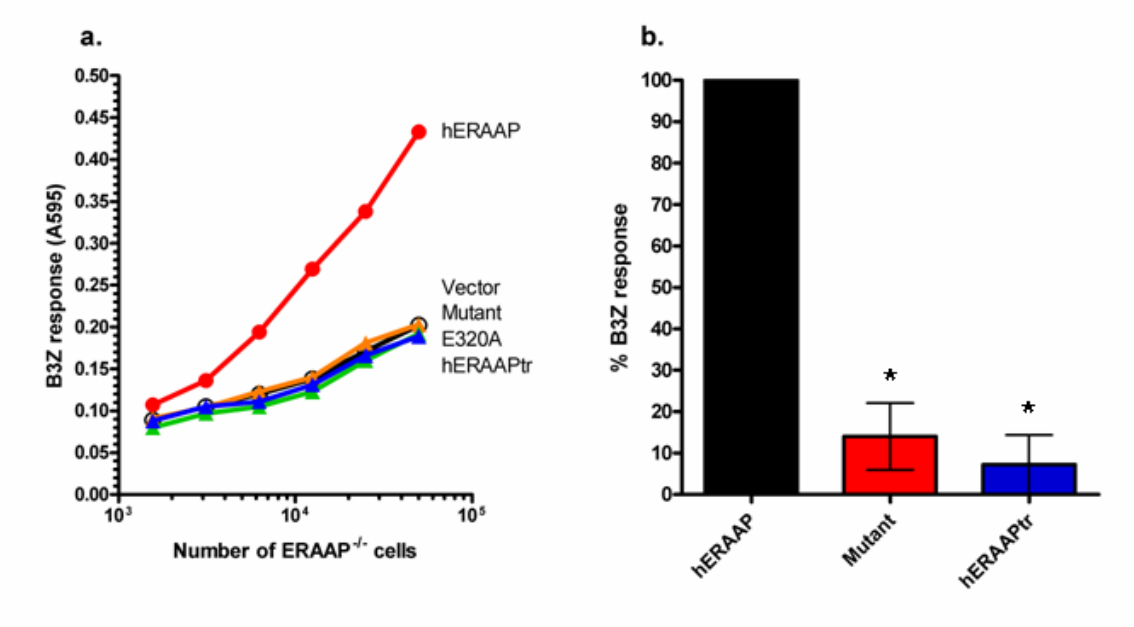

Figure 3.15. Addition of different hERAAP variants into ERAAP$^{-/-}$ cells indicates a reduction in trimming of a different peptide precursor substrate

ERAAP$^{-/-}$ cells were transfected with X6-SHL8 together with vector, hERAAP, Mutant, E320A or hERAAPtr. Reconstitution with hERAAP restores peptide processing from the X6-SHL8 precursor to the final SHL8 peptide. However, like the processing activity for X5-SHL8, the addition of mutant, E320A and hERAAPtr show a reduction in trimming activity to generate the final SHL8 from X6-SHL8 precursor (* p=<0.01). Data is a representation of four experiments.

3.7.1. Individual SNPs have more pronounced trimming phenotypes with altered substrate

In order to begin to investigate substrate specificity of hERAAP and its naturally occurring variants, trimming of LEQLEK-SHL8 (X6-SHL8) with AIVMK-SHL8 (X5-SHL8) were compared. Using the X5-SHL8, three of the individual SNPs conferred a significant reduction in trimming activity (K528R, R735Q and Q730E), whereas three did not alter trimming activity compared to hERAAP (R127P, M349V, D575N). The addition of the individual SNP hERAAP alters the ability to process LEQLEK-SHL8, however, like processing of X5-SHL8, not all the SNPs show a reduction in trimming activity, with D575N having B3Z stimulation comparable to hERAAP. Q730E shows a small reduction in trimming; however this reduction does not appear to be significant when compared to hERAAP activity. This is different to its activity on X5-SHL8 which showed a significant reduction in trimming (figure 3.16e and f).

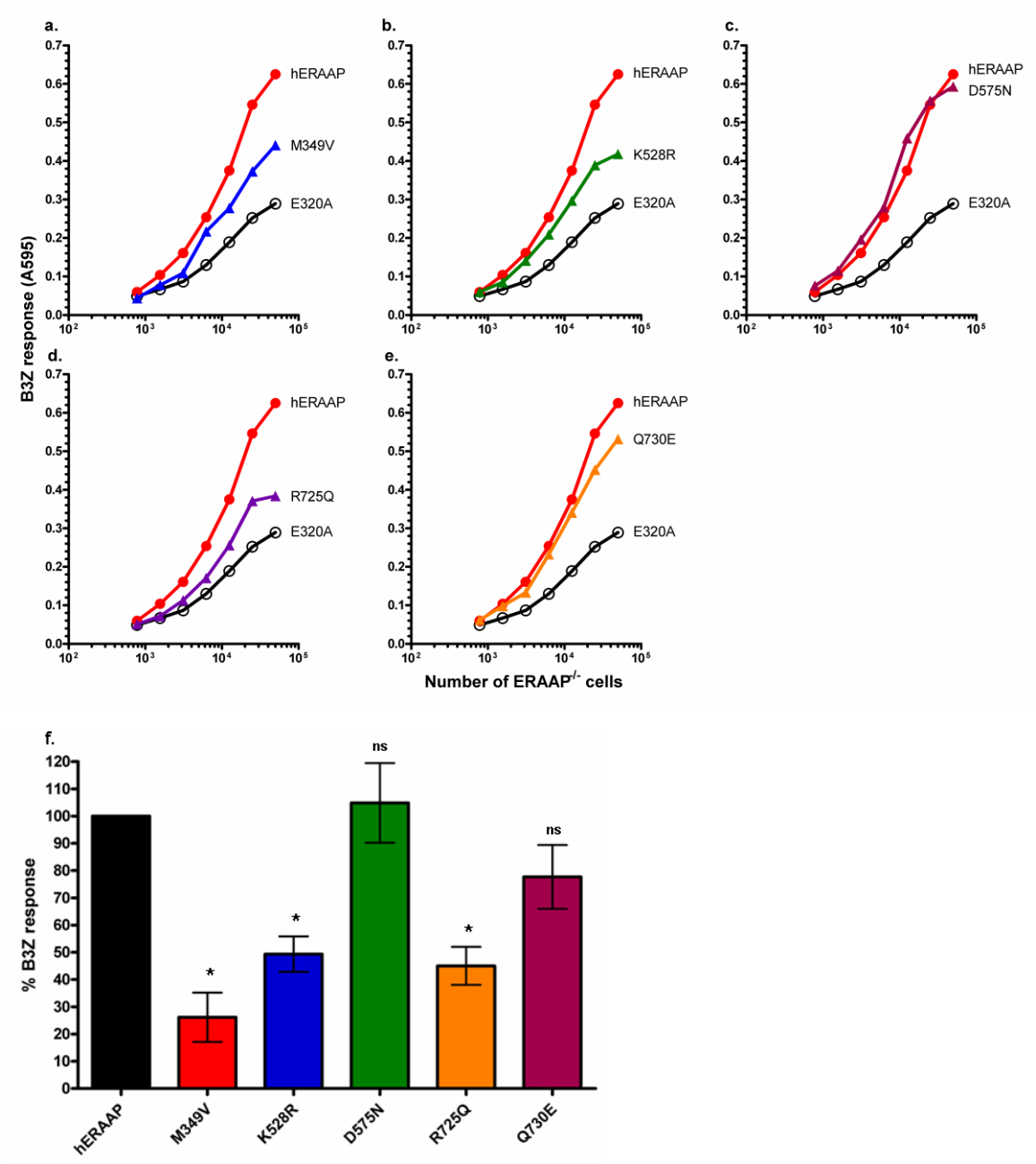

Figure 3.16. The addition of individual SNPs hERAAP variants alters peptide processing of a different peptide substrate

ERAAP$^{-/-}$ cells were transfected with X6-SHL8 along with hERAAP, E320A or ERAAP containing SNPs M349V (a), K528R (b), D575N (c), R725Q (d) or Q730E (e). The SNPs indicate a hierarchy of peptide trimming with D575N still able to process peptides similar to hERAAP, with Q730E having a small reduction; however the processing of this alternative substrate show M349V, K528R, R725Q have a reduced processing ability (* *p*=<0.01) Data is a representation of four repeats.

Three of the five SNPs tested (M349V, K528R and R725Q) show a significant reduction in trimming activity compared to hERAAP, with M349V generating the lowest response (~25% activity, *p*=<0.01, figure 3.16). The reduction in activity of K528R and R725Q is consistent with the activity seen when processing X5-SHL8 (figure 3.11). However there is a significant reduction in the ability of M349V to process a 6 amino acid N-terminal extension (~80% reduction) compared to almost normal trimming function of the 5 amino acid AIVMK extension (~15-20% reduction). These results show that K528R and R725Q behave similarly with either X5- or X6-SHL8 and also demonstrate a role for M349V in defective peptide processing of a more charged and longer N-terminal extension. Therefore it is possible that the individual SNPs have altered processing activity towards different peptide substrates depending on the size and properties of the precursor. But nevertheless, K528R and R725Q are shown to dominate function.

3.8. The addition of two ERAAP alleles alters peptide trimming activity

Here this study has shown that the addition of SNPs within ERAAP into cells lacking ERAAP activity alters the ability to trim N-terminally extended peptides, in most cases failing to generate the final optimal peptide for MHC I loading to the same extent as hERAAP. As two copies of the ERAAP gene are present within cells, it is highly conceivable that these two copies may differ in their gene sequence. Therefore, as the activity of individual alleles had been identified, it was important to identify the overall trimming activity of potential ERAAP haplotypes (two ERAAP alleles expressed) and whether the addition of defective alleles containing certain polymorphisms, in this case both mutant and R725Q / Q730E (both having an abrogated phenotype) would be restored by the addition of a fully functional wild type hERAAP. ERAAP$^{-/-}$ were transfected with X5-SHL8 along with hERAAP and either mutant or R725Q / Q730E. As previously described, the mutant and R725Q / Q730E have a negative B3Z response, similar to the negative control (figure 3.8 and 3.12e). The addition of wild type hERAAP alongside mutant restored trimming activity to the same level as wild type hERAAP (figure 3.17a). In contrast to this, when R725Q / Q730E is transfected together with hERAAP, trimming activity is still significantly reduced, to approximately 40% of wild type (*p*=<0.01, figure 3.17b). While this combination gave rise to a significant increase in trimming compared to that seen with R725Q / Q730E alone (10%, figure 3.12d and e), hERAAP did not restore normal trimming function.

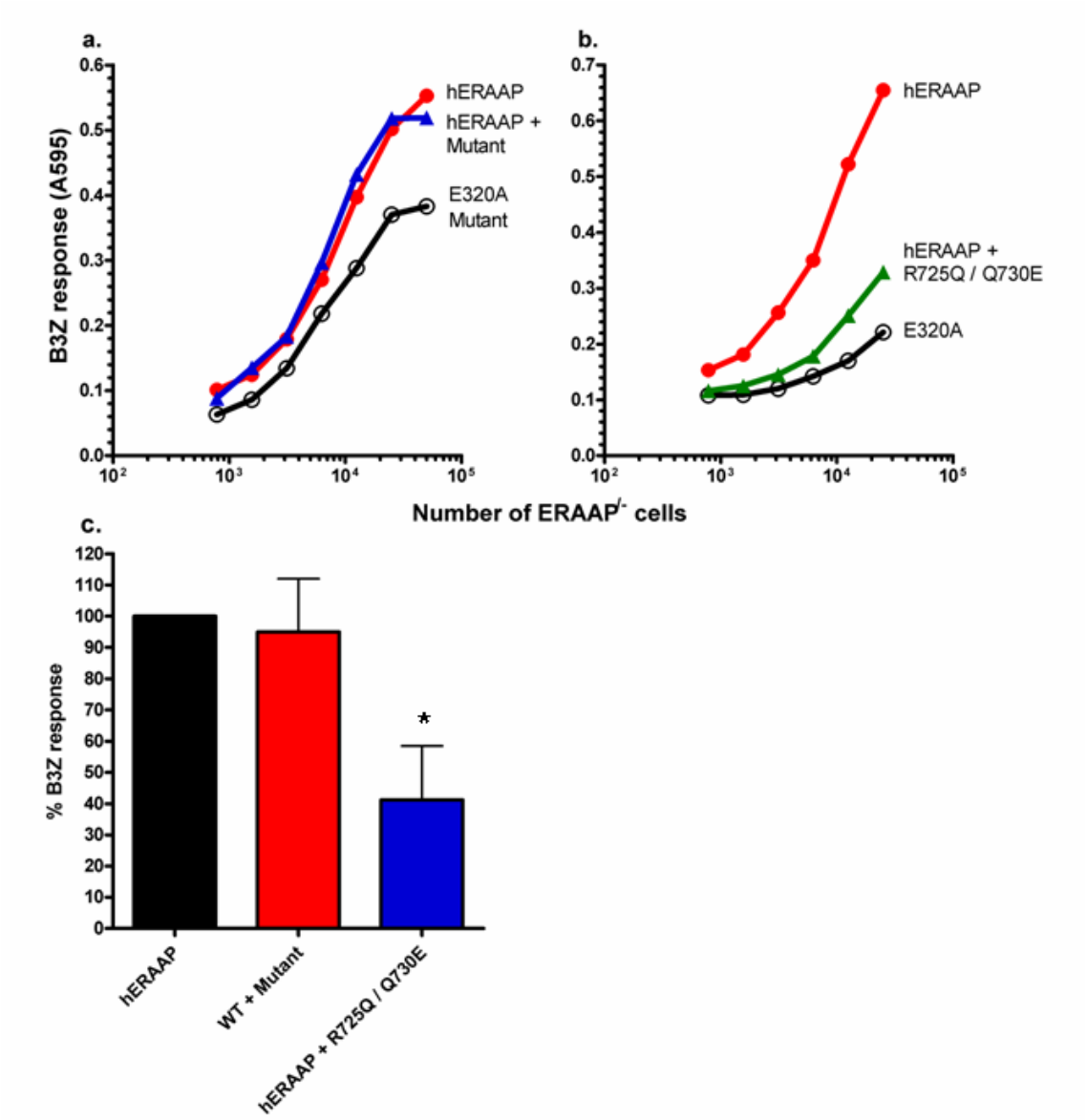

Figure 3.17. Reconstitution with two hERAAP alleles alters trimming activity

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 along with 1ug hERAAP, E320A or 0.5µg both hERAAP and mutant (a) and 0.5µg both hERAAP and R725Q / Q730E (b). The addition of hERAAP alongside mutant restores trimming activity to wild type hERAAP, however the addition of hERAAP with R725Q / Q730E reduces trimming activity (* p=<0.01). Data is representative of four experiments.

These data indicate the importance of understanding allele function in the context of a haplotype, suggesting that combinations of alleles may result in different trimming activity compared to the individual alleles alone. The lack of trimming of hERAAP and

R725Q / Q730E further highlights this polymorphic region (amino acids 725-730) as important for trimming activity. These results suggest that haplotype trimming activity of two alleles in combination will prove more important for the assessment of the generation of final length peptides than the activity of individual ERAAP alleles. The findings shown here demonstrate the importance of assessing ERAAP allele combinations to ascertain the true trimming ability seen in vivo.

3.9. Summary

The results shown in this chapter, through the use of a mouse model system, identify that ERAAP is required to process N-terminally extended peptides. In addition to this, it is demonstrated that variations in the ERAAP sequence have severe consequences on the ability to efficiently generate the final peptide epitope for stable presentation on MHC I. These findings suggest that SNPs within ERAAP can alter the generation of peptides within the ER, providing a possible link with defective peptide processing and AS development. Using T cell activation assays, peptide extracts and HPLC;

1. Efficient trimming of a 13 residue precursor for SIINFEHL (X5-SHL8) to SHL8 peptide in the presence of wild type hERAAP is demonstrated.

2. Reducing the expression of ERAAP in mouse cells inhibited the generation of SHL8 from its N-terminally extended precursor, however the SHL8 peptide requiring no trimming was unaffected, providing evidence for the requirement of ERAAP in trimming of N-terminally extended peptide in the ER.

3. A truncated splice variant (hERAAPtr) and a polymorphic ERAAP (mutant) resulted in an abrogated B3Z response indicating no presentation of SHL8 at the cell surface, therefore unable to successfully process the peptide precursor into it final epitope. Alongside this, consistent with previous studies, mutating the GAMEN active site motif to GAMAN (E320A) ceased all trimming activity showing this region is essential for the enzymatic function of ERAAP (Kanaseki et al., 2006).

4. The lack of processing ability demonstrated by hERAAPtr suggests a role for the missing C-terminal region of this protein for the ability to trim precursors. It raises potential questions as to whether this protein is able to fold correctly or if it is a protein that is functionally relevant in humans. The presence of six SNPs within mutant also has a detrimental effect on the ability of the aminopeptidase to process peptides to their final optimal length. As individual SNPs have shown a high linkage with AS, it was thought possible that the phenotype generated by mutant was a result of one of these SNPs rather than a combination.

5. K528R, R725Q and Q730E SNPs have a significant reduction in trimming activity; however some degree of trimming activity occurred. This correlates with previous

findings that ERAAP containing the position 528 SNP is unable to cleave peptide hormones involved in hypertension (Goto et al., 2006). Therefore these results would indicate that the K528, R725 and Q730 regions are important in the peptide processing activity. An important observation identified that none of the individual SNPs were able to generate a phenotype demonstrated by mutant ERAAP.

6. Double SNP mutants were confirmed to have a cumulative effect on the processing activity compared to individual SNPs. Any SNP in combination with either K528R or R725Q had a detrimental effect on the ability to process X5-SHL8 to the final peptide giving a strong indication that these positions are important for peptide trimming. Further to this, double SNPs without either one of these SNPs did not reduce the trimming activity more than the individual SNPs. One double SNP (R725Q / Q730E) was shown to reduce the processing activity to the extent of mutant ERAAP, suggesting the R725 to Q730 amino acid region as being functionally important for the activity of hERAAP in peptide processing.

7. A modified six amino acid N-terminal extension creating a more charged peptide precursor (X6-SHL8), was still processed by wild type hERAAP. Most of the hERAAP variants displayed a similar trimming activity compared to X5-SHL8. However one SNP, M349V, had a significant reduction in trimming activity similar to R725Q. Unlike X5-SHL8, Q730E had processing activity comparable to hERAAP, suggesting the N-terminal extension properties of the precursor may alter the activity of the SNPs towards their ability to process the peptide.

8. In physiological conditions, two ERAAP alleles are expressed within a cell. Two ERAAP variants, mutant and R725Q / Q730E, showed an abrogated trimming activity individually. When mutant was introduced in combination with wild type hERAAP, the ability to generate the final peptide epitope was restored, indicating hERAAP has a dominant phenotype compared to mutant and that the mutant phenotype is a loss of function phenotype.

9. Trimming activity was not restored when wild type hERAAP was in combination with R725Q / Q730E, suggesting that this double SNP ERAAP conveys a dominant-negative phenotype. This further suggests that mutant may have a hypo-

active trimming activity, whereas R725Q / Q730E is likely to have a hyper-active trimming activity.

Chapter 4: Functional activity of ERAAP alleles identified from ankylosing spondylitis patients

Spondyloarthropathies (SpA) are a group of immune mediated inflammatory disorders that occur in 0.5-1% of the population. One member of this family of diseases, AS, is responsible for 30-50% of SpA diseases and affects numerous areas of the body such as the spine, eyes, aorta, lungs, kidneys and tendon insertions. AS was shown to be highly heritable and genetically linked to the presence of HLA-B27, with >96% cases positive for the expression of this molecule (de Blecourt et al., 1961, (Brown et al., 1998; Caffrey and James, 1973). HLA-B27 demonstrates unusual folding properties leading to a high level of misfolding and aggregation within the ER as well as the tendency to form homodimers at the cell surface following the dissociation of unstable heterodimeric complexes (Bird et al., 2003; Mear et al., 1999). Generation of optimal peptide ligands are important in both of these processes as a) restricted peptide supply or editing can result in ER homodimers and b) suboptimal peptides with an increased k_{off} have the ability to form surface homodimers. It has recently been shown that the presence of non-MHC I genetic susceptibility factors may contribute to the risk of developing AS and subsequently, strong associations were identified between AS and nsSNPs within the ERAAP gene ($p=1 \times 10^{-26}$, (Brown et al., 1998; Burton et al., 2007; Laval et al., 2001). More recently, however, it was revealed that these SNPs only affect the risk of AS development in patients that are HLA-B27 positive, indicating that disease is associated with linked functions of these two molecules known to intersect in the antigen processing pathway (Evans et al., 2011). Here it is shown that ERAAP is required for processing of N-terminal amino acids to generate the final optimal peptide. Polymorphisms within ERAAP that are associated with AS susceptibility were shown to alter the ability to generate final peptide antigen and that a combination of more than one SNP resulted in a cumulative effect. Consistent with individual SNP trimming data, Evnouchidou et al also revealed ERAAP containing either K528R or Q730E altered the enzymatic processing activity. Both K528R and Q730E ERAAP were able to generate final antigenic peptide but K528R demonstrated lower activity than wild type when assessed for trimming activity in vitro (Evnouchidou et al., 2011)

Recent studies have highlighted individual SNPs within ERAAP as being associated with AS in a number of different populations, however as yet no study has investigated disease association at the level of the individual ERAAP allele, or the patient haplotype. Due to the possible role of SNPs within ERAAP being associated with AS by its ability to process peptides for stable loading onto the HLA-B27 molecule, a cohort of AS positive patient samples were obtained and the combinations of polymorphisms present on both of their ERAAP alleles determined in order to compare this to control

patient ERAAP alleles. This would allow the identification of any common ERAAP alleles and haplotypes that are present in the population of AS positive individuals and assess the ability of these alleles individually and as a complete haplotype to process N-terminally extended peptides. This may provide vital information into the ability of AS patients to generate the peptide epitopes for loading onto MHC I.

4.1. Peptide processing activity of HapMap identified AS alleles

A number of studies screening the ERAAP gene have identified SNPs within ERAAP to be associated with risk of AS in different populations (Chen et al., 2011; Davidson et al., 2009; Pimentel-Santos et al., 2009; Szczypiorska et al., 2011). In 2009, a study was undertaken using data from an AS patient cohort combined with data from HapMap which predicted ERAAP alleles comprising of various combinations of SNPs (table 4.1, Harvey et al., 2009). From this data, only six different ERAAP alleles were identified within patients and controls. The most common allele was shown to be wild type hERAAP, with a similar frequency in both case and control cohorts. Only two SNPs, K528R and Q730E were present individually, with K528R occurring in both cases and controls, and Q730E only present in a small number of controls. In addition, no allele was shown to be present that contained all the AS associated SNPs identified.

Two alleles were found to have differential frequency between cases and controls, which both contained multiple SNPs. One allele termed ‘susceptible’ was present in more cases than controls and contains K528R and Q730E SNPs. The other allele, termed ‘protective’, had a higher frequency in controls compared to cases and contains M349V, D575N and R725Q SNPs (table 4.1). However, it is important to identify that this study did not contain HLA-B27 positive but AS negative control samples and the HapMap data assumes no functional interaction between the SNPs identified. This is interesting as K528R individually demonstrates a loss of function phenotype, however is not overrepresented in cases in comparison to controls. Also, K528R / Q730E is suggested to be a susceptible allele, however Q730E individually did not show a reduction in trimming and was not the most affected SNP by the addition of K528R. Also, R725Q was shown to have a loss of function but is not identified in the HapMap data, and in contrast the cumulative effect on loss of function observed, this allele is suggested to be protective when in combination with both M349V and D575N. Finally, a variant of hERAAP containing six SNPs was identified from human cells in this study and generates a loss of function in activity but was not identified within the HapMap data. Therefore assessing the activity of the susceptible and protective alleles on their

ability to process peptide precursors was essential for understanding the proposed role these may play in disease susceptibility.

Allele	Cases	Controls	Comment
Wild Type	**0.436**	**0.411**	
K528R/Q730E	**0.263**	**0.325**	**Susceptible**
M349V/D575N/R725Q	**0.220**	**0.175**	**Protective**
K528R	**0.074**	**0.079**	
M349V/D575N/R725Q/Q730E	**0.006**	**0.006**	
Q730E	**0.000**	**0.002**	
M349V/K528R/D575N/R725Q	**0.000**	**0.000**	
M349V/K528R/D575N/R725Q/Q730E	**0.000**	**0.000**	

Table 4.1. HapMap ERAAP patient allele data (Harvey et al. 2009)

4.1.1 Peptide processing activity of the susceptible and protective alleles

Firstly, the apparent anomaly of this sequence data to the functional studies (chapter 3), in that R725Q (in combination with M349V and D575N) is protective and not susceptible, since R725Q containing mutants so far have shown to demonstrate a loss of function, was assessed (figure 3.12). Therefore level of activity of both the protective and the susceptible alleles towards processing of N-terminally extended peptides was determined. As in chapter 3, site directed mutagenesis was used to incorporate a single base change in hERAAP, resulting in the change in amino acids to generate M349V / D575N / R725Q. The K528R / Q730E ERAAP had previously been created through site directed mutagenesis (chapter 3) and analysed this for its processing function. These ERAAP alleles were introduced into ERAAP$^{-/-}$ cells alongside X5-SHL8 and assessed for their ability to generate the final SHL8 peptide from its precursor. It was previously demonstrated that K528R / Q730E had a defective trimming activity towards X5-SHL8 and again a statistically significant reduction in trimming activity with this allele is shown here (figure 4.1a and c). However, the addition of M349V / D575N / R725Q demonstrated trimming activity comparable with the hERAAP response and therefore is able to restore trimming activity in cells deficient in ERAAP (figure 4.1b and c). The defective nature of K528R / Q730E in contrast to M349V / D575N / R725Q is consistent with this allele being linked with AS susceptibility since it is present in more

AS positive patients than controls. Therefore it may be the inability of this allele to generate final peptides which leads to the onset/progression of the disease.

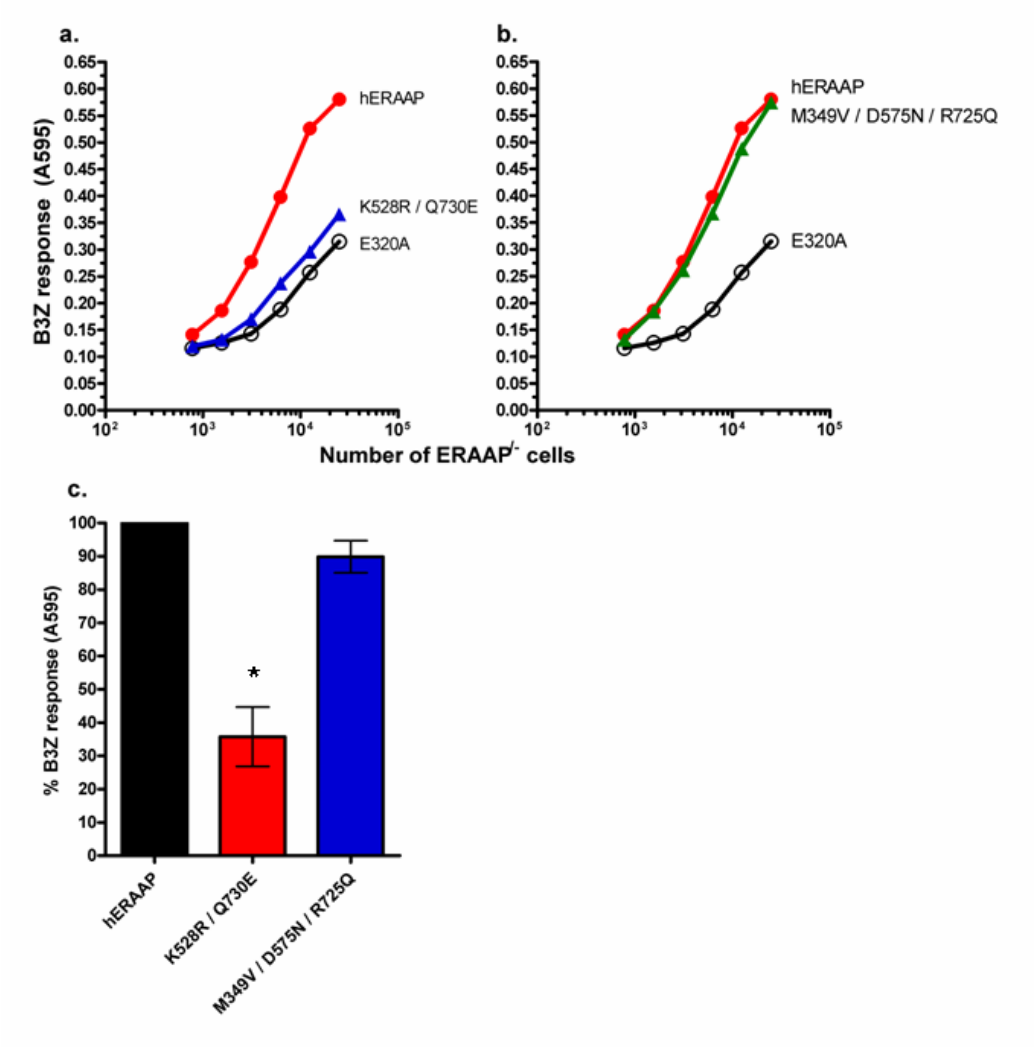

Figure 4.1. Susceptible and protective ERAAP alleles alter peptide processing

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 along with 1µg hERAAP, E320A, K528R / Q730E (a) or M349V / D575N / R725Q (b). The addition of K528R / Q730E reduces activity, whereas the M349V / D575N / R725Q has little effect on trimming compared to wild type. Percentage B3Z response shows K528R / Q730E has a significant reduction in activity (c, *p=<0.01). Data is a representation of five experiments.

These findings suggest that the susceptible allele is less functional in peptide processing than the protective allele.

4.1.2 Altering the peptide substrate properties alters processing activity

Previously demonstrated here is that altering both the length and properties of the amino acid extension alters the ability of some polymorphic ERAAP to process the N-terminal precursor peptide (chapter 3). To further determine the processing activity of the susceptible and protective alleles, LEQLEK-SHL8 (X6-SHL8), a longer and more charged precursor, was introduced into ERAAP$^{-/-}$ cells along with either the susceptible or protective alleles (figure 4.2). Surprisingly, and in contrast with activity towards X5-SHL8, the results obtained show the K528R / Q730E is able to restore trimming activity towards this altered substrate, with an activity comparable to hERAAP. However, the protective allele in this instance is unable to process the LEQLEK extension to the same extent as AIVMK, shown by a reduction in B3Z response (~60%) which is statistically significant in comparison to hERAAP (p=<0.01, figure 4.2b and c).

These results suggest that the addition of an N-terminal amino acid in combination with an increase in charge changes the activity of these ERAAP alleles towards the processing of this N-terminal extension in comparison to X5-SHL8. This would also suggest that these two alleles may be able to functionally trim certain N-terminal extensions, depending on their properties, conferring substrate specificity.

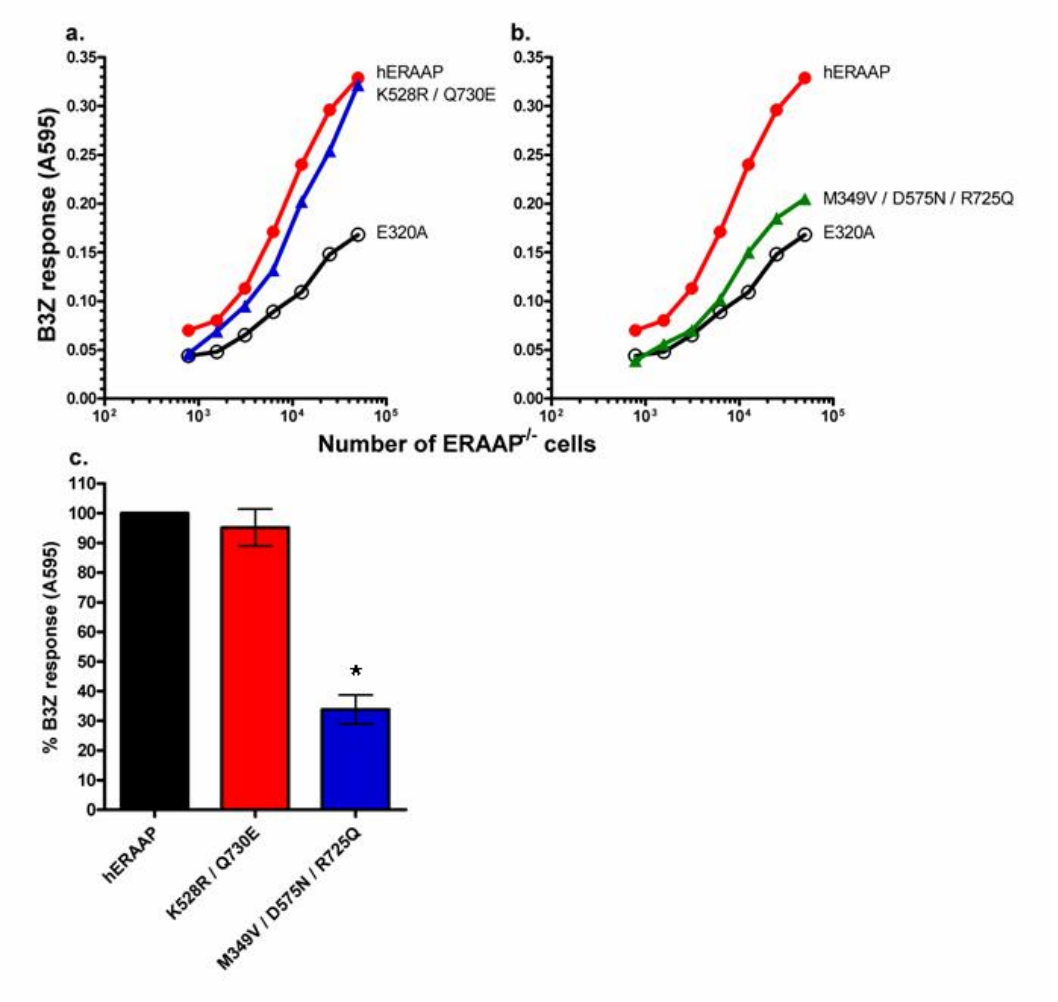

Figure 4.2. Susceptible and protective alleles have different processing activity with different substrates.

ERAAP$^{-/-}$ cells were transfected with X6-SHL8 along with 1µg hERAAP, E320A, K528R / Q730E (a) or M349V / D575N / R725Q (b). The addition of K528R / Q730E has little effect on trimming, whereas M349V / D575N / R725Q reduces activity compared to hERAAP. Percentage B3Z response shows M349V / D575N / R725Q has a significant reduction in activity (c, *p=<0.01). Data is a representation of four experiments.

4.2 AS patient cohort identified common ERAAP SNP alleles

The previous ERAAP allele data was obtained from HapMap analysis (Harvey et al., 2009). As shown previously in chapter 3, the SNPs identified in having a strong linkage with AS alter the ability to process N-terminally extended peptides to different levels compared to hERAAP. To date, no study has determined ERAAP alleles and haplotypes from AS patients and have only identified susceptible SNPs or potential combinations based on linkage scores. Therefore a cohort of AS patient and control

samples were obtained to identify the polymorphisms present within their ERAAP alleles and also identify the combinations of two alleles present (haplotype) and assess these for the ability to process N-terminally extended peptides.

After obtaining whole blood samples from patients mRNA was extracted in order to determine their ERAAP sequence from cDNA. These patient alleles were subsequently cloned into the pcDNA3 expression vector to use in functional studies to assess their ability to process N-terminal extensions (table 4.2). To date 18 AS patients and 13 control patients (RA, SLE and oesophageal cancer patients) have been obtained, and are currently in the process of obtaining inflammatory non-AS, non-inflammatory osteoarthritis and normal control cohort (table 4.2). In contrast to the alleles predicted by the HapMap analysis, 9 ERAAP alleles are identified here. These include wild type hERAAP, which unlike the findings from the HapMap study, is more frequent in control samples than AS positive cases. In addition, ERAAP alleles that are only present in AS positive individuals and have not yet been shown to be present within the control population. Only two alleles were shown to contain individual SNPS, K528R and M249V, and all other alleles contain multiple SNP combinations. Here the presence of mutant allele (containing six SNPs) is also identified, which is more frequent in cases than controls and is also the most frequent case allele. Unexpectedly, the K528R / Q730E, shown in the HapMap analysis to be present in more cases than controls, is only present in control patients within this study (in combination with a mutant allele, see below). The frequency of the proposed susceptible and protective alleles in this study is less than that shown within the HapMap analysis. In addition, a greater number of alleles were observed in this cohort of samples than previously identified (Harvey et al., 2009).

Allele	Cases (n=27)	Controls (n=18)
Wild Type	**0.111**	**0.444**
Mutant	**0.444**	**0.278**
R725Q / Q730E	**0.074**	**0.000**
M347V	**0.037**	**0.000**
K528R	**0.222**	**0.166**
K528R / R725Q	**0.074**	**0.000**
M349V / K528R	**0.037**	**0.000**
K528R / Q730E	**0.000**	**0.056**
M349V / D575N / R725Q	**0.000**	**0.056**

Table 4.2. Study of AS vs control ERAAP alleles

4.2.1 Peptide processing activity of the identified patient alleles

As ERAAP alleles within AS patients were identified, most of which contain more than one SNP, further assessment into the role they may play within AS susceptibility was required by determining their ability to process N-terminally extended peptides to generate the final antigenic peptide.

Comparison of sequences from identical patient alleles revealed identical ERAAP sequences; therefore only one representative sample of the allele was used in trimming analysis. As before, ERAAP$^{-/-}$ cells were transfected with X5-SHL8 along with each of the cloned patient ERAAP alleles. The trimming activity of M349V, K528R / Q730E and M349V / D575N / R725Q using X5-SHL8 had previously been determined (figure 3.11 and figure 4.1). The wild type hERAAP, M349V / D575N / R725Q and M349V show trimming activity comparable with hERAAP and are able to trim the N-terminally extended precursor (figure 4.1b, figure 4.3a and g). All the other alleles identified within the study generate a significant reduction in response, with less than 50% activity towards the processing of X5-SHL8 (p=<0.01, figure 4.3).

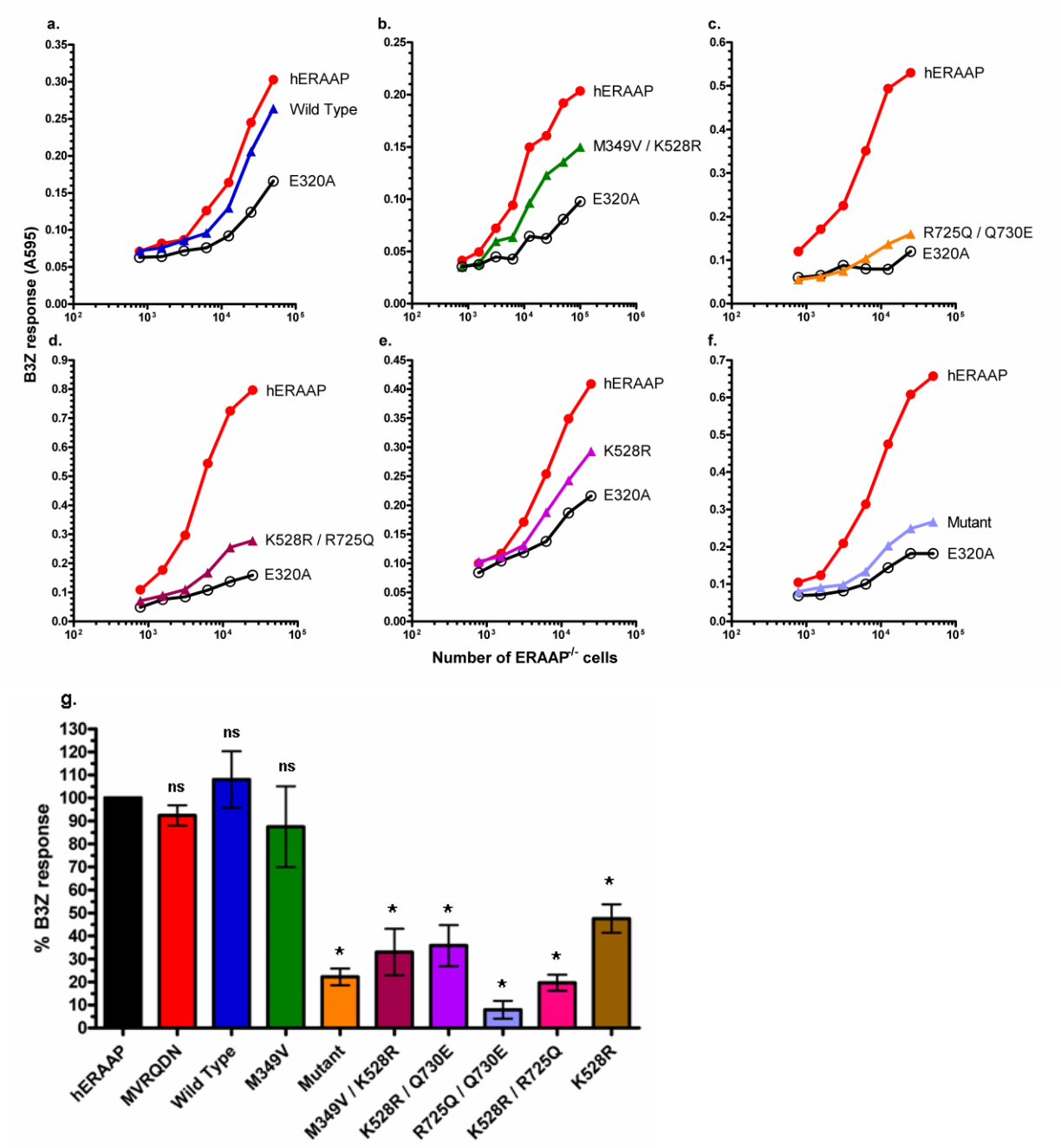

Figure 4.3 Patient ERAAP alleles alter peptide processing activity

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 along with 1µg hERAAP, E320A or each of the patient ERAAP alleles identified in the study. Both wild type (a), M349V / D575N / R725Q (figure 4.1) and M349V (chapter 3) did not decrease in trimming activity, however the other identified alleles have a significant reduction in trimming activity (c, * *p*=<0.01). Data is a representation of four experiments.

Interestingly, these findings show that all the SNP alleles that result in a reduction in activity contain either the K528R or R725Q SNP. The greatest reduction in activity is shown with the patient R725Q / Q730E allele generating a response comparable with E320A, consistent with our findings described in chapter 3. Of the alleles which exhibit normal activity, M349V / D575N, R725Q is only found in control samples and wild type is more frequent in controls compared to cases. By contrast, M349V is only seen in case samples. ERAAP alleles with a normal activity have a higher representation in control samples compared to cases, whereas defective ERAAP alleles have a greater representation in cases compared to controls.

To further identify the ability of these ERAAP alleles to process X5-SHL8, peptide extracts were generated from transfected cells and RP-HPLC utilised to fractionate these peptides. In order to determine the amount of peptide processing activity occurring, the fractionated peptides were dried overnight and treated with trypsin to cleave the lysine residue in the N-terminal extension, releasing final SHL8 peptide which can be recognised by B3Z in complex with H-2K^{b} at the cell surface. This would release the SHL8 peptide in fractions corresponding to N-terminally extended precursors. Firstly the fractions in which N-terminally extended peptides (AIVMK-, MK-, K- and SHL8) were eluted was established by using synthetic peptides. Trypsin treatment of fractions revealed AIVMK-SHL8 (X5-SHL8), K-SHL8 and SHL8 in fractions 39, 21 and 28 respectively (figure 4.4).

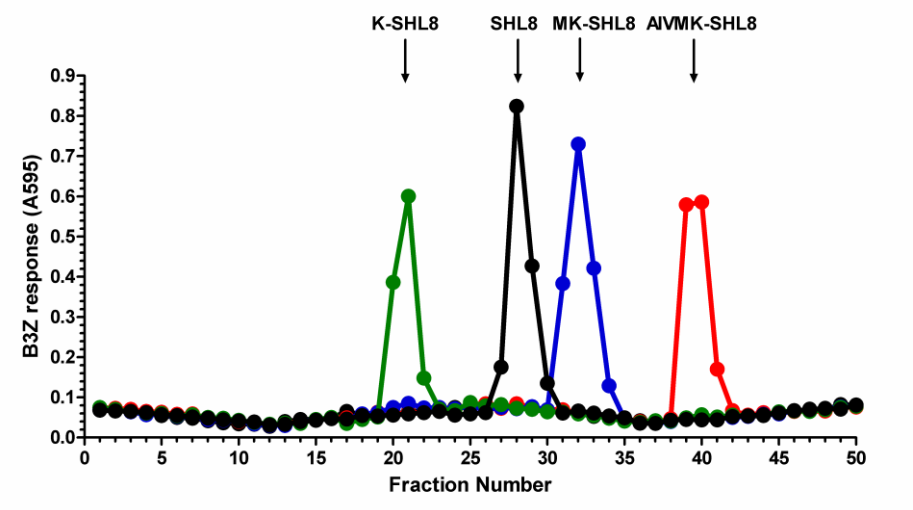

Figure 4.4. HPLC analysis of synthetic peptides

Synthetic peptides AIVMK-SHL8, K-SHL8 and SHL8 were fractionated by RP-HPLC and subsequently treated with 50µg trypsin for 5 hours. Fractions revealed these peptides eluted in fractions 30/40, 21/22 and 27/28 respectively.

Analysis of processed peptides in fractionated extracts revealed three peaks, corresponding to X5-SHL8, K-SHL8 and SHL8. The hERAAP shows functional processing, with the presence of both K-SHL8 and SHL8 peaks, but the absence of a peak corresponding to X5-SHL8. Once again the presence of a peak at K-SHL8 is due to the binding of this peptide to the H-2D^{b} molecule. In contrast, the E320A shows only a peak at X5-SHL8, consistent with lack of trimming activity. The two alleles R725Q / Q730E and K528R / R725Q only show a reduced peak corresponding to K-SHL8, with no SHL8 or X5-SHL8 present in comparison to hERAAP and E320A (figure 4.5b and c). This suggests that these alleles may have a hyper-active trimming phenotype consistent with the lack of peptide precursor and final peptide (K-SHL8 and SHL8).

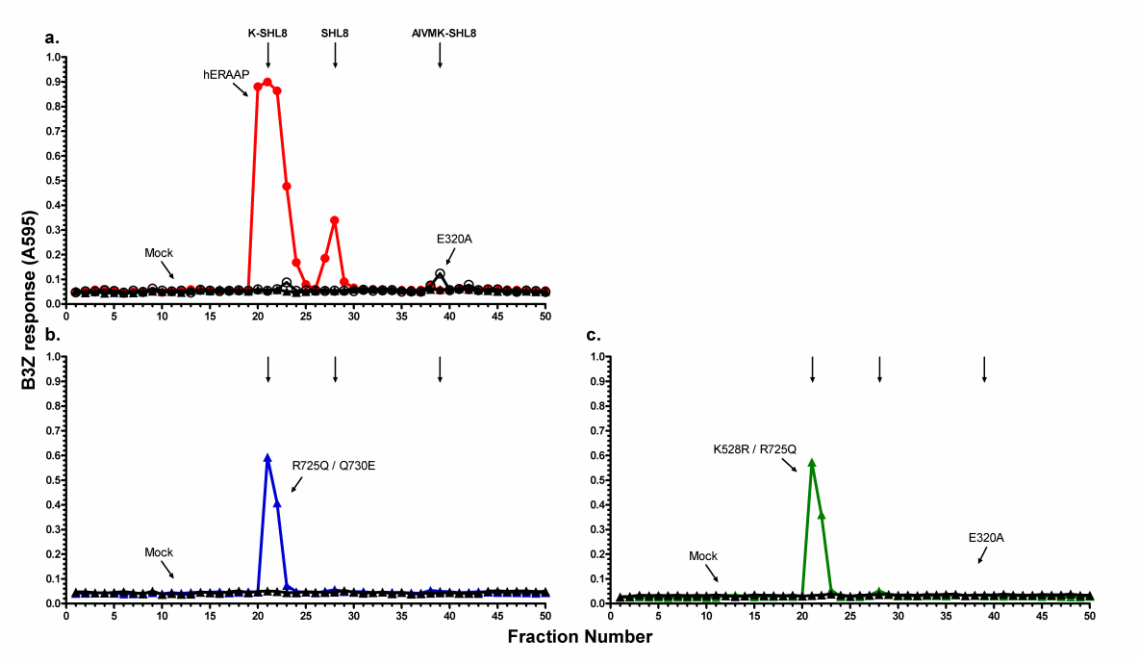

Figure 4.5. HPLC analysis of potential hyper-active AS patient alleles

ERAAP$^{-/-}$ cells were transfected as in figure 4.5 creating peptide extracts after 48 hours and run on RP-HPLC. Eluted peptides were treated with 50µg trypsin for 5 hours prior to incubation with K89 and B3Z. hERAAP is able to generate final SHL8 and K-SHL8, whereas E320A is unable to trim X5-SHL8 (fraction 39). The R725Q / Q730E and K528R / R725Q are only able to generate a small amount of K-SHL8 with no SHL8 or AIVMK-SHL8 shown (b and c). Data is a representation of three experiments.

The wild type allele derived from patient samples shows a response the same as hERAAP, with no X5-SHL8 present, further showing that this allele has functional trimming activity (figure 4.6b). K528R demonstrates a peak at K-SHL8 comparable with hERAAP, however shows a reduction in the SHL8 peak, indicating that this allele is able to trim X5-SHL8 to some extent, but consistent with the T cell activation assay, has less ability to generate SHL8 than hERAAP (figure 4.6c). Mutant and M349V / K528R show a smaller peak at K-SHL8 compared to hERAAP and are unable to generate SHL8. The peak observed at fraction 39 corresponds to X5-SHL8 and indicates that these two alleles do not efficiently trim the precursor peptide and therefore have a hypo-active trimming phenotype (figure 4.6d and e). Together these data show that wild type is able to process peptide precursors to the same ability as hERAAP. The data also shows the other alleles identified containing multiple SNPs have a defective trimming phenotype and both the T cell activation assay and HPLC

analysis indicate they may fall into two categories of activity, hypoactive and hyperactive, both resulting in a failure to generate the optimal peptide epitope.

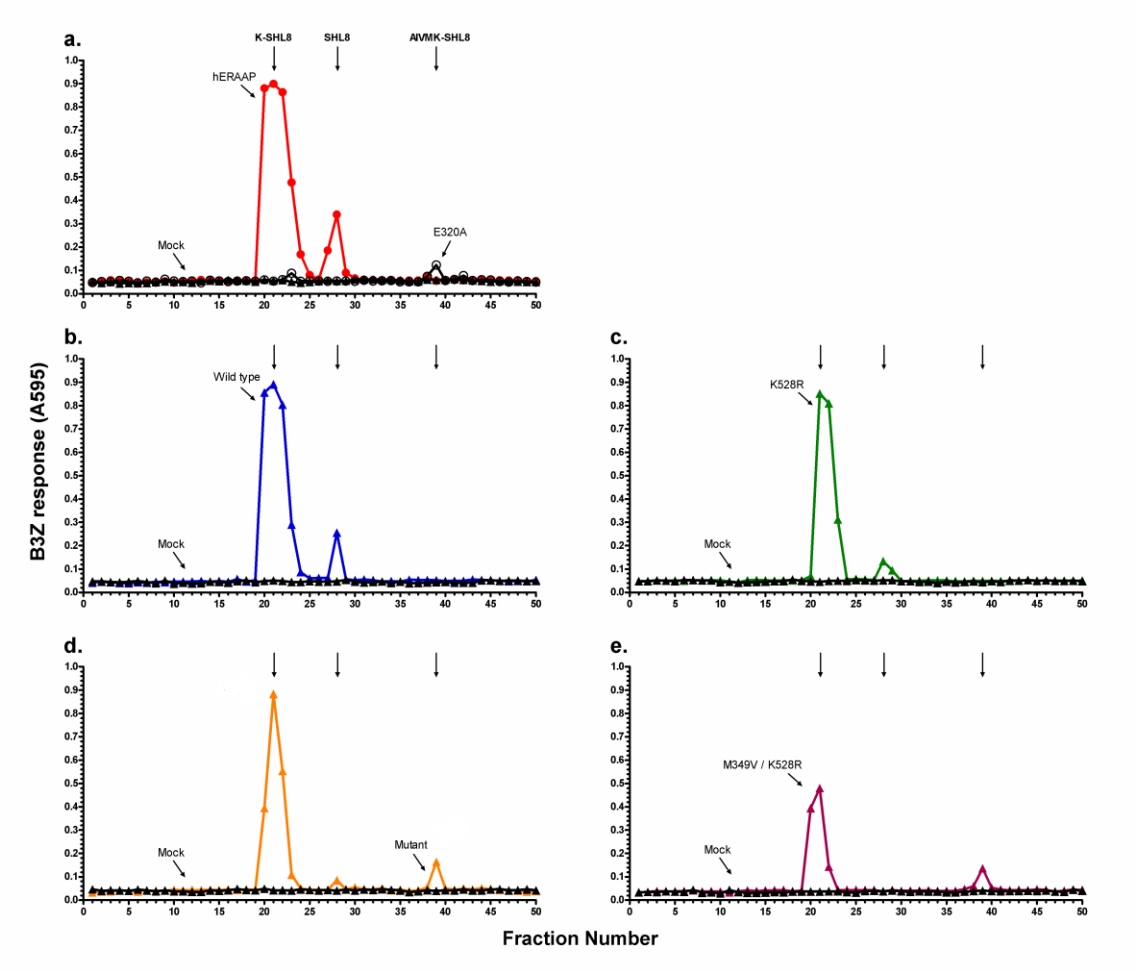

Figure 4.6. HPLC analysis of AS vs control alleles

ERAAP$^{-/-}$ cells were transfected as in figure 4.5 creating peptide extracts after 48 hours and run on RP-HPLC. Eluted peptides were treated with 50µg trypsin for 5 hours prior to incubation with K89 and B3Z. The wild type allele is able to trim peptides to the same extent as hERAAP (b), whereas K528R, mutant and M349V / K528R have a reduction in activity (c, d and e). Data is a representation of three experiments.

4.3 Functional analysis of AS associated ERAAP compound haplotypes

The identification of ERAAP alleles from both patient and control cohorts identified novel SNP combinations, and when assessed for the ability to process N-terminally extended peptides, the majority of these alleles highlighted defects in activity. The next

step was to identify the combinations of 2 alleles that were present within each patient (haplotype) and identify any common haplotypes present within AS patients vs. controls and assess their ability to generate the final peptide epitope. Through sequence analysis common haplotypes within patients and controls were identified (table 4.3). Here five different ERAAP haplotypes are identified that are present within AS patients and three that were present in control patients. Most noticeable is that the haplotypes present within the AS positive cohort are not present in the control population. The mutant allele in combination with the K528R SNP was the most common haplotype presented in the AS cohort. In addition, 90% of AS haplotypes contain at least one allele with K528R and R725Q SNP (the exception being wild type + mutant haplotype). Two of the three control haplotypes observed contain wild type hERAAP which restores trimming activity in combination with mutant (figure 3.17) and therefore may also restore trimming when expressed with K528R. In contrast, 70% of the AS ERAAP haplotypes contain two alleles that have been shown to have defective trimming activity.

Haplotype	Cases (n=10)	Controls (n=5)
Wild Type + R725Q / Q730E	**0.2**	**0.0**
Wild Type + M349V	**0.1**	**0.0**
Mutant + K528R	**0.4**	**0.0**
Mutant + K528R / R725Q	**0.2**	**0.0**
Wild Type + K528R	**0.0**	**0.4**
Mutant + K528R / Q730E	**0.0**	**0.2**
K528R + M349V / K528R	**0.1**	**0.0**
Wild Type + Mutant	**0.0**	**0.4**

Table 4.3. Patient haplotype data from cases vs. control samples

4.3.1. ERAAP haplotype peptide processing activity in AS cases vs controls

An interesting finding from the AS ERAAP haplotype data obtained was that 70% of AS haplotypes contained two defective alleles; however 80% of control samples contained functional wild type. As ability of the alleles individually to trim X5-SHL8 had been

demonstrated, identifying the role of the ERAAP haplotypes in peptide processing was important. As before ERAAP$^{-/-}$ cells were transfected with X5-SHL8 along with the identified ERAAP haplotype (two ERAAP alleles).

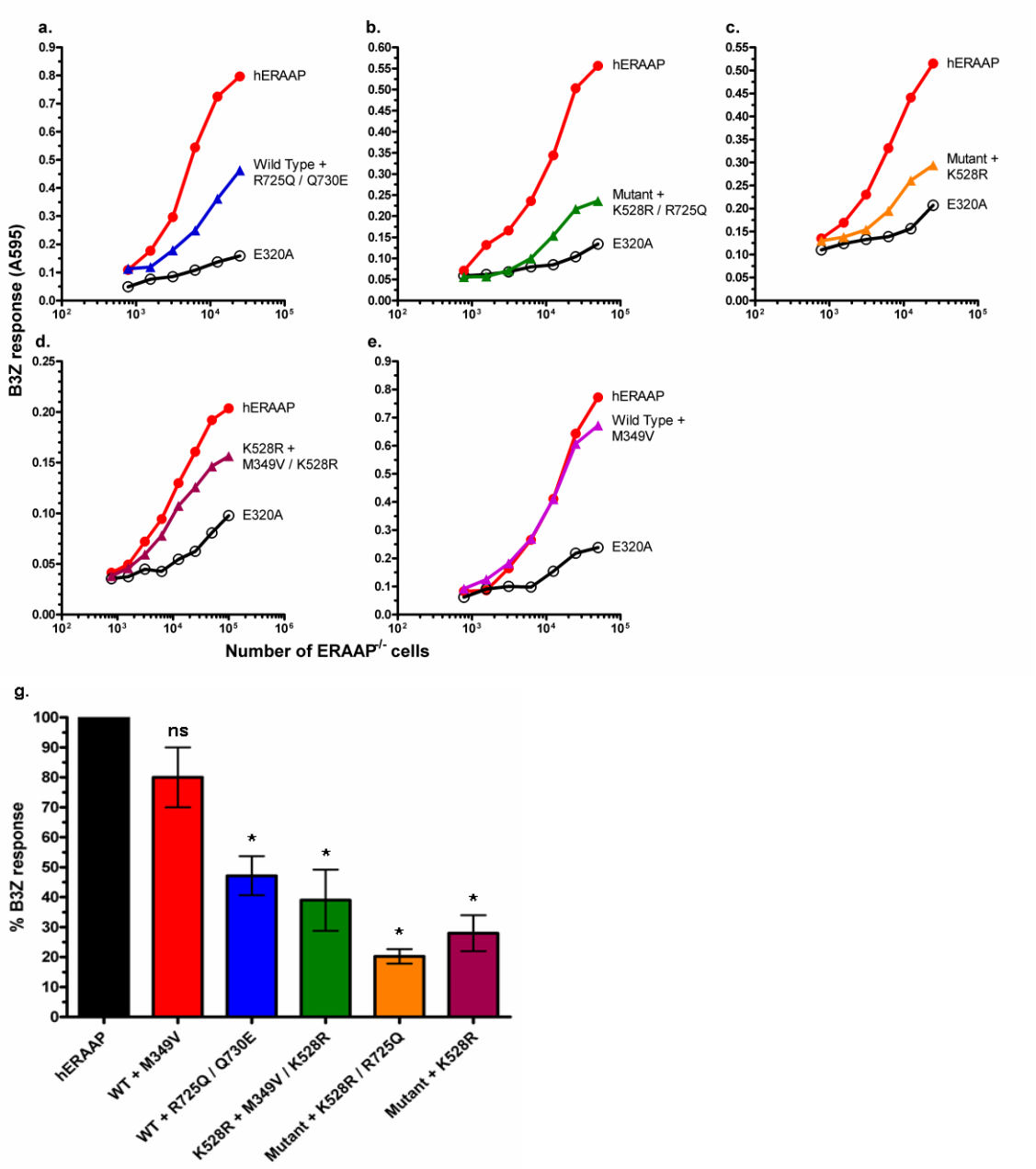

Figure 4.7. AS patient ERAAP haplotypes alter peptide processing activity

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 along with 1µg hERAAP or E320A, or 0.5µg of two ERAAP alleles giving the ERAAP haplotypes identified. All AS haplotypes demonstrate a reduction in activity except wild type + M349V (e) which is comparable to hERAAP (* *p*=<0.01). Data is a representation of 3 experiments.

Figure 4.7 shows the trimming function of AS ERAAP haplotypes and reveal that all haplotypes except for one case (wild type + M349V) generate a reduced trimming phenotype. Interestingly, the combination of wild type with R725Q / Q730E leads to enhanced trimming activity compared to R725Q / Q730E alone (figure 4.3), although not to levels seen for wild type alone. Three further haplotypes (mutant + K528R, mutant + K528R / R725Q and K528R + M349V / K528R) all show a reduction in trimming activity, consistent individual loss of function (figure 4.7b, c and d). In contrast to this, two control haplotypes (wild type + K528R and wild type + mutant) were tested and show trimming activity comparable with hERAAP (figure 4.8). Both haplotypes tested contained wild type hERAAP which is shown to restore the defective trimming activity of the second allele (either K528R or mutant) back to levels comparable with our hERAAP control.

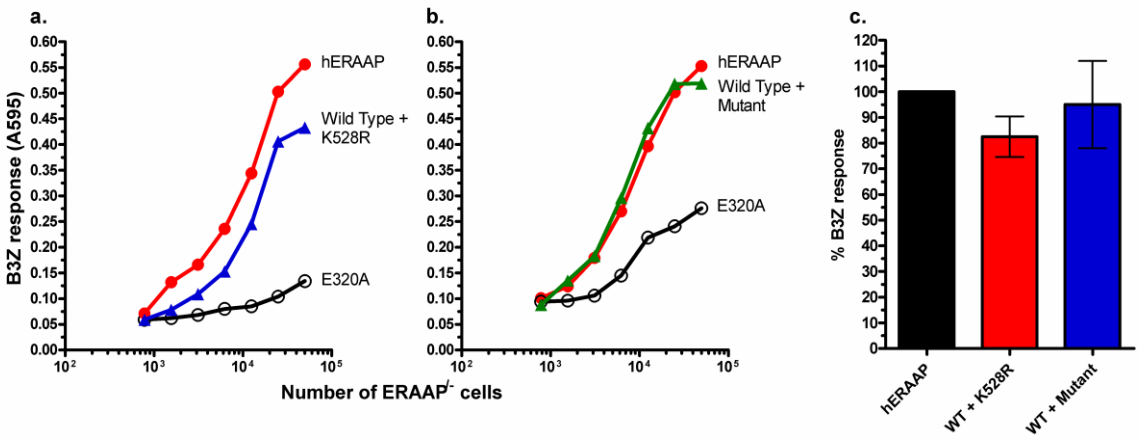

Figure 4.8 Control patient ERAAP haplotypes are similar to hERAAP activity

ERAAP$^{-/-}$ cells were transfected with X5-SHL8 along with 1µg hERAAP or E320A, or 0.5µg of two ERAAP alleles giving the control ERAAP haplotypes identified. The haplotypes demonstrate activity comparable with hERAAP in trimming of X5-SHL8. Data is a representation of 3 experiments.

To further confirm the processing activity of these haplotypes, we used RP-HPLC to fractionate peptide extracts and treated with trypsin, as in figure 4.5. So far, the processing activity of four haplotypes identified has been determined. Both wild type + R725Q / Q730E and mutant + K528R / R725Q generate a similar response to R725Q / Q730E and K528R / R725Q respectively (figure 4.9 b and c). Interestingly, wild type is

unable to restore trimming activity to normal when in combination with R725Q / Q730E. This suggests that in contrast to non-functional mutants, R725Q / Q730E may have a dominant negative function. When peptides are extracted from X5-SHL8 transfected wild type + R725Q / Q730E cells and fractionated by HPLC, only a small peak at K-SHL8 is observed, which is reduced compared to hERAAP. No SHL8 or X5-SHL8 is seen, consistent with a dominant negative phenotype arising from hyperactivity. In contrast, mutant alone generated a peak at X5-SHL8 which is not present when in combination with K528R / R725Q (figure 4.9c). When K528R is in combination with the mutant or the M349V / K528R alleles, the response shown is similar to K528R alone, with two peaks corresponding to K-SHL8 and SHL8, with the SHL8 peak being less than hERAAP, indicating some degree of trimming activity to generate SHL8 (figure 4.9d). This is consistent with the reduction in B3Z response to the K528R allele within the T cell activation assays (figure 4.3).

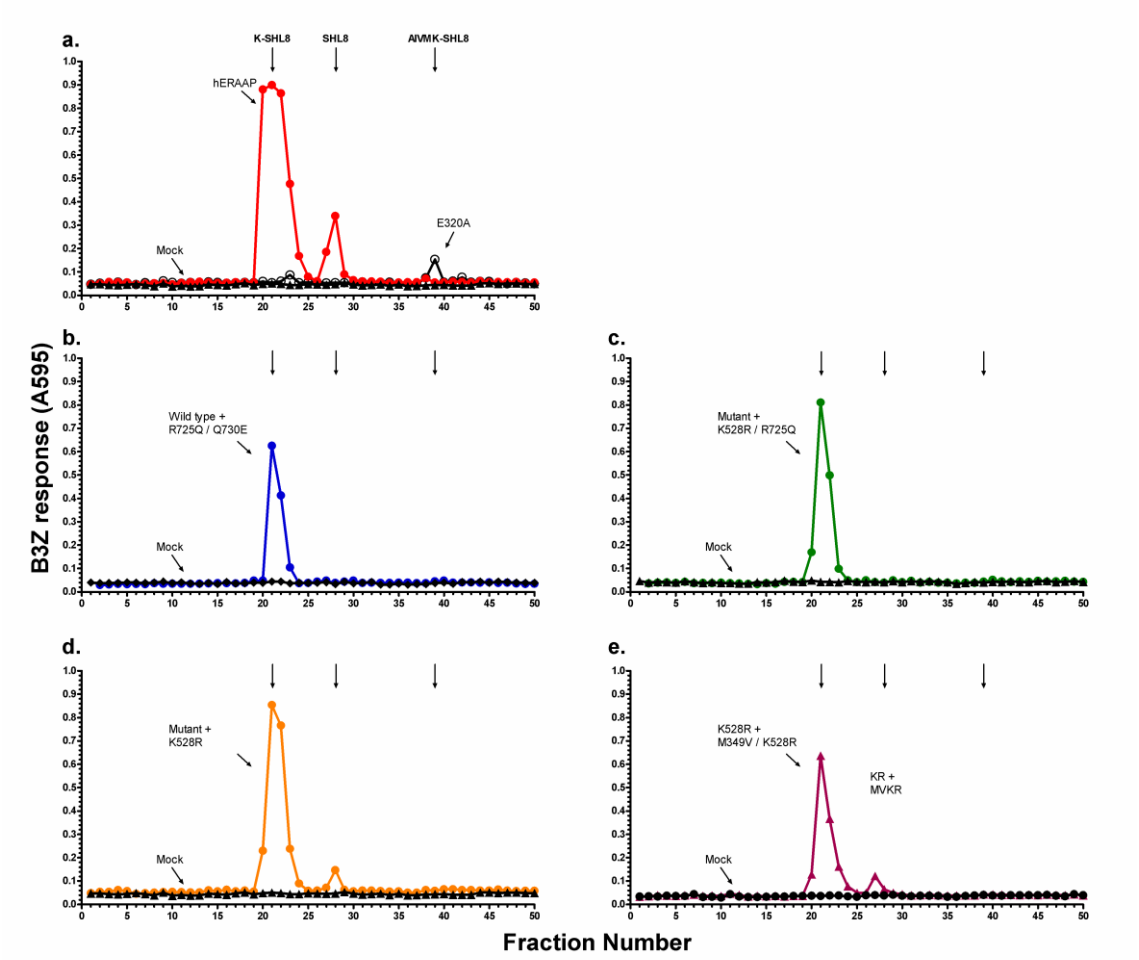

Figure 4.9. HPLC analysis of identified ERAAP haplotypes

ERAAP$^{-/-}$ cells were transfected as in figure 4.7 creating peptide extracts after 48 hours and run on RP-HPLC. Eluted peptides were treated with 50µg trypsin for 5 hours prior to incubation with K89 and B3Z. The ERAAP haplotypes show a reduction in the ability to generate final SHL8, with low levels generated with mutant + K528R and K528R + M349V / K528R (c and d). No SHL8 is seen with wild type + R725Q / Q730E and mutant + K528R / R725Q with these alleles potentially over-processing the peptide precursor (a and b). Data is a representation of three experiments.

Both the mutant and M349V / K528R alleles individually showed lack of trimming activity with a peak corresponding to X5-SHL8. However in combination with K528R this peak is not observed, indicating K528R has a dominant activity over mutant and M349V / K528R, although this allele has a reduced processing activity as shown in figure 4.3.

These data identify ERAAP haplotypes and show that AS haplotypes have a reduction in processing ability, whereas the control haplotypes are able to process peptides to a similar level as hERAAP. They identify R725Q / Q730E and K528R / R725Q to potentially have a hyperactive trimming activity and having a dominant negative function. It is also shown that mutant and M349V / K528R do not affect the trimming ability of K528R. Therefore, defective trimming activity of AS patient haplotypes compared to the functional nature of the control haplotypes may provide the link between ERAAP SNPs and AS susceptibility.

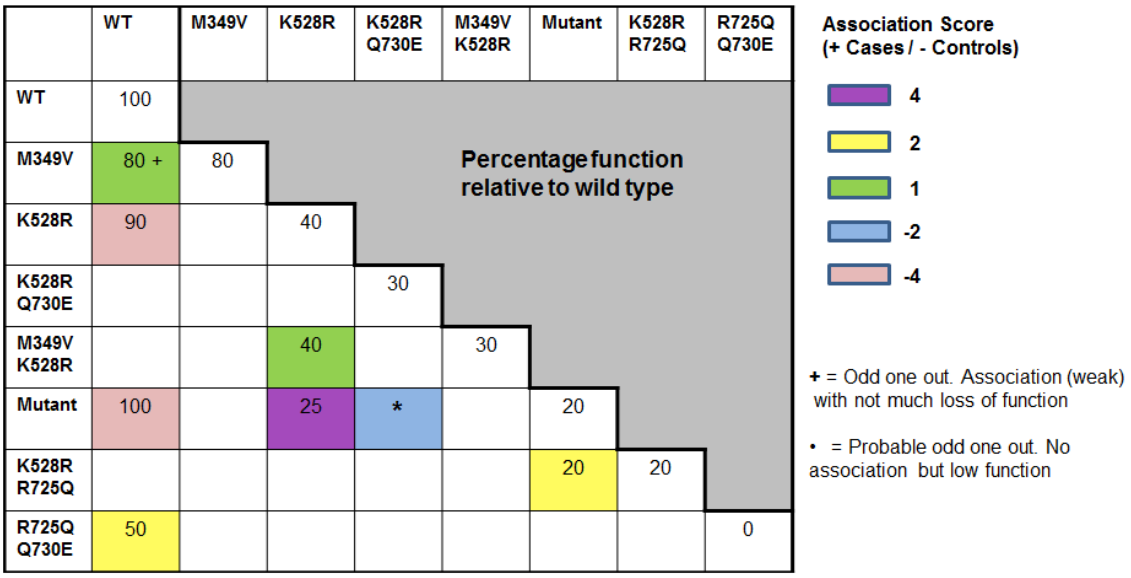

Table 4.4. Association and percentage function of identified haplotypes

4.4 Summary

Within this results chapter a number of polymorphic ERAAP alleles present within AS patients and controls have been identified. Using T cell activation assays and HPLC, the trimming function of HapMap proposed alleles and alleles identified through sequencing of patient ERAAP genes was determined. Further to this, common haplotypes were identified and the ability of two ERAAP alleles in combination was assessed for trimming ability.

1. In 2009, Harvey et al used HapMap data to identify two ERAAP SNP alleles, a susceptible allele present in more AS cases and a protective allele present in more controls (Harvey et al., 2009). Both alleles were identified to have multiple SNPs within the ERAAP, with the susceptible allele having K528R and Q730E and the protective allele containing M349V, D575N and R725Q. Upon testing these two ERAAP alleles for their functional activity in peptide processing, the susceptible allele was unable to trim a peptide precursor, whereas the protective allele was.

2. Surprisingly, when these susceptible and protective alleles were tested with the X6-SHL8, containing an extra N-terminal amino acid and a charged N-terminal extension, the susceptible allele was able to restore the trimming of this precursor, whereas the protective allele was defective. Further to previous experiments with this altered substrate (chapter 3) this result highlights the ability to trim N-terminal extensions by the mutated ERAAP molecules depends on the amino acid properties.

3. Through sequence analysis of ERAAP sequences from AS and control patient samples, a small number of frequently occurring alleles were identified within this cohort. A number of new alleles that have not previously been described from the HapMap data were also shown.

4. The cohort of case and control samples showed susceptible and protective alleles were only present in control patients within this study so far. Most surprising was the frequency of alleles containing more than one SNP (63%), with M349V and K528R being the only individual SNP alleles identified.

5. Furthermore, the presence of a mutant allele (containing six SNPs associated with AS), observed previously in the original cloning experiments, was highlighted in 44% AS cases and 28% controls and from previous investigations have demonstrated that multiple SNP combinations, including the mutant, have a cumulative deleterious effect on trimming function compared to individual SNPs alone.

6. Also evident was the high proportion of SNP combinations in alleles containing either K528R or R725Q, previously shown to reduce trimming activity. Using T cell activation assay and RP-HPLC, the processing ability of each of these alleles was identified. Here it was demonstrated that only M349V, M349V / D575N / R725Q and wild type alleles had trimming activity comparable to hERAAP, with the other allele defective in their trimming activity.

7. RP-HPLC identified R725Q / Q730E and K528R / R725Q, only present in AS patients, as having a potential hyperactive trimming phenotype.

8. In contrast to this, RP-HPLC analysis highlighted mutant and M349V / K528R as potentially having hypoactive trimming phenotypes. The other alleles show a reduction in trimming activity to generate the final SHL8 from its precursor.

9. Only AS haplotype observed to have activity comparable to hERAAP was wild type + M349V, however as yet there is no RP-HPLC data to confirm this.

10. The presence of two alleles that potentially over-trim the peptide precursor are shown, resulting in a hyperactive trimming phenotype (R725Q / Q730E and K528R / R725Q). This was shown through RP-HPLC analysis failing to generate both an AIVMK-SHL8 and SHL8 peak, with a minimal amount of K-SHL8 shown. In combination with wild type (wild type + R725Q / Q730E) or mutant (mutant + K528R / R725Q), these alleles appear to be dominant negative and therefore generate a reduction in trimming activity.

Chapter 5: The role of ERAAP in the facilitation of cytokine receptors

ERAAP has been shown to play a role in the shedding of pro-inflammatory cytokine receptors IL-6R, TNFRSF1A and IL-1 decoy receptor. The use of imunoprecipitation experiments revealed the association of ERAAP with these receptors. In addition, a correlation between increased ERAAP expression and a decrease in membrane bound receptor resulting in an increase in soluble protein was observed (Cui et al., 2002; Cui et al., 2003; Cui et al., 2003). ERAAP is a type II integral protein containing a transmembrane domain, which is lacking in mouse ERAAP and so it is possible that ERAAP is able to reside at the cell surface for cytokine receptor shedding (Kanaseki et al., 2006; Cui et al., 2002). As yet, the exact mechanism by which ERAAP facilitates receptor shedding has not been identified but one proposal is that IL-6, TNFα and IL-1 cytokines are responsible for mediating inflammatory responses and in turn receptors for these cytokines play a vital role in mediating these responses. IL-6 bioactivity can be controlled by soluble IL-6R (sIL-6R) and sIL-6R/IL-6 complexes which are able to directly activate cells through the ubiquitously expressed membrane-bound glycoprotein gp130. Thus sIL-6R/IL-6 complex can act as an agonist for cell types that do not express the membrane bound IL-6R and are normally non-responsive to IL-6 through expression of gp130. Therefore when investigating the inflammatory potential of IL-6 it is important to consider both the membrane bound IL-6R and sIL-6R. sIL-6R is generated by two distinct mechanisms, ectodomain cleavage and alternative mRNA splicing. Soluble protein is present in the plasma of healthy individuals at approximately 25-35ng/ml, however elevated levels of sIL-6R have been detected in numerous disease states, such as rheumatoid arthritis and inflammatory bowel disease (Mitsuyama et al., 1995; Robak et al., 1998). A disruption in cytokine receptor shedding, generating sIL-6R, may alter inflammatory responses suggesting a role for defective receptor shedding in inflammatory disorders. One such disorder, AS, has a strong genetic linkage with the HLA-B27 molecule as well as polymorphisms within the ERAAP gene (Burton et al., 2007; Kollnberger et al., 2002). It is therefore conceivable that a disruption in cell surface cytokine receptor shedding through changes in activity of ERAAP containing SNPs, alters the sIL-6R plasma concentration in patients. This may have detrimental effects on IL-6 downstream signaling, potentially resulting in AS disease pathology (Burton et al., 2007).

For this reason, it was important to assess the activity of wild type hERAAP and previously cloned hERAAP variants on cytokine receptor shedding to determine if they play a part in the disruption of cytokine mediated responses. This could highlight potential regions or domains within ERAAP that are important for its role in facilitating

cytokine receptor shedding and lead towards a better understanding of the mechanism of action

5.1. Human ERAAP alters IL-6R surface expression in ERAAP knock-out mouse fibroblasts

In order to determine the role of ERAAP in facilitating cytokine receptor shedding, murine ERAAP$^{-/-}$ fibroblasts were reconstituted with hERAAP cloned from CEM cells. As ERAAP$^{-/-}$ cells do not express IL-6R at the cell surface, human IL-6R was cloned from U937 cells into the pcDNA3 expression vector, allowing assessment of the ability of ERAAP to facilitate IL-6R cleavage following transfection of both cloned IL-6R and hERAAP into ERAAP$^{-/-}$ cells. To overcome the problem of poor transfection efficiency, pcDNA3-GFP was transfected into the cells along with IL-6R and hERAAP. Flow cytometry was utilised to identify positively transfected cells by the expression of GFP and assess the surface IL-6R expression on these cells (figure 5.1a and b). Transfection efficiency of ERAAP$^{-/-}$ cells, determined by the level of GFP positive cells, was between 30-40% 48 hours after transfection. Upon reconstitution with wild type hERAAP, the percentage of surface IL-6R expression was reduced compared to cells receiving IL-6R but no hERAAP (figure 5.1). Reconstituting ERAAP$^{-/-}$ cells with mutant and hERAAP tr, as well as an active site mutant, E320A, showed a reduction in ability to facilitate IL-6R cleavage (figure 5.1). hERAAP has the greatest ability to reduce surface expression (30% reduction) consistent with its known role in the facilitation of receptor cleavage (figure 5.1c and). The E320A does not have any aminopeptidase activity and there is no significant difference in the expression of cell surface IL-6R in the presence of this variant. The addition of the two other variants, either mutant or hERAAPtr, results in a slight decrease in surface IL-6R expression (~15%), however is not to the same level as wild type and show a response more consistent with E320A ERAAP (figure 5.1). The reduction demonstrated may be a result of poor transfection efficiency of ERAAP and IL-6R into cells. This would imply that these three ERAAP variants are unable to facilitate the cell surface cleavage of IL-6R.

Consistent expression of ERAAP mRNA from transfection of hERAAP variants into ERAAP$^{-/-}$ cells has been shown previously (figure 3.8); therefore the activity towards cytokine receptor shedding can be attributable to the variation in ERAAP sequence and not changes in expression levels of the transfected variants.

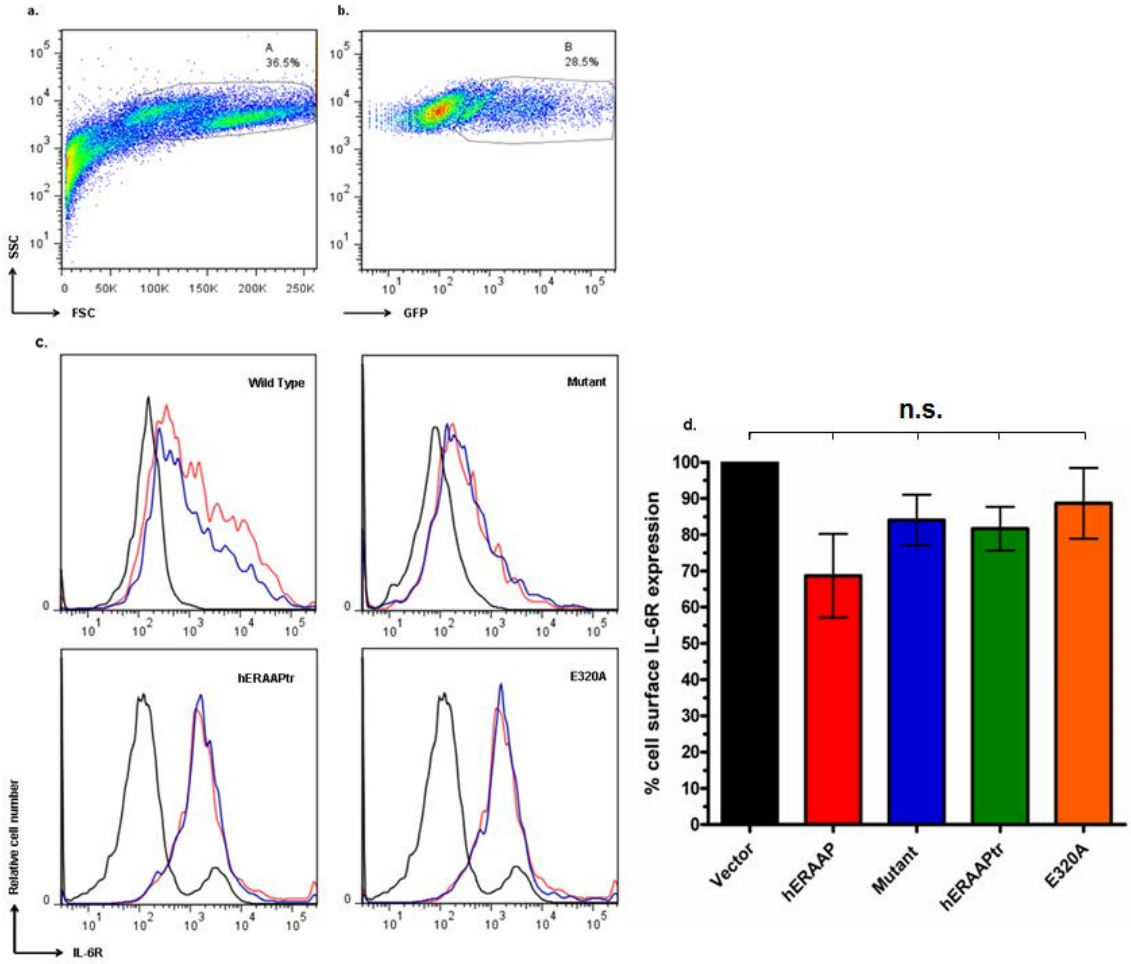

Figure 5.1. Reconstitution with human ERAAP reduces cell surface IL-6R expression

ERAAP$^{-/-}$ cells were transfected with 1µg pcDNA3-GFP and 1µg human IL-6R along with either 1µg pcDNA3 vector only, hERAAP, mutant, hERAAPtr or E320A. After 48 hours cells were harvested and analysed for cell surface IL-6R expression by flow cytometry using an IL-6R specific antibody (1:100) followed by α-mouse PE (1:100). GFP positive cells were gated (a and b) and stained with fluorescent secondary antibody alone (black lines) or for IL-6R expression of cells treated with vector only (red lines) or ERAAP variants (blue lines, c) . The addition of hERAAP reduces surface IL-6R expression, however the addition of hERAAP variants do not show the same level of activity towards IL-6R expression (d). Data is a representation of five experiments.

In 2007, a genome wide linkage analysis study (GWAS) was undertaken and revealed five polymorphisms within the ERAAP gene to have increased association with the autoimmune inflammatory disorder, AS (Burton et al., 2007). Here it has already been shown that mutant, containing six SNPs that are associated with increased risk of AS,

had a reduction in activity compared to hERAAP. In addition, these SNPs were demonstrated to alter the ability to process N-terminally extended peptides, resulting in a lack of optimal peptide for MHC I loading and expression. Therefore was important to further investigate the role of each of these SNPs individually on their activity towards IL-6R shedding.

ERAAP$^{-/-}$ cells were reconstituted with the five polymorphic hERAAP constructs associated with the development of AS and flow cytometry used to determine levels of cell surface IL-6R expression (figure 5.2). None of the individual SNPs reduced the expression to the same level as wild type. M349V and K528R show some facilitation of IL-6R cleavage, displaying a level of activity most similar to wild type, indicated by ~20% reduction in surface expression. Both D575N and R725Q have a phenotype consistent with an abrogated shedding activity, with IL-6R expression comparable to an absence of ERAAP. This highlights D575N and R725Q mutations as significant in the facilitation of IL-6R cleavage. Surprisingly, the Q730E mutation gave rise to a significant increase in surface IL-6R expression rather than no change in expression as seen with D575N and R725Q, suggesting that this mutation was not a simple loss of function (figure 5.2). The increase in IL-6R expression after reconstitution with Q730E may result from the suppression of an ERAAP independent IL-6R cleavage mechanism as proposed by Cui et al (Cui et al., 2003).

These data so far suggest that the five individual SNPs alter the function of ERAAP in facilitation of IL-6R cleavage, with polymorphisms at amino acid positions 575, 730 and 730 potentially indicating significant areas responsible for this activity of ERAAP.

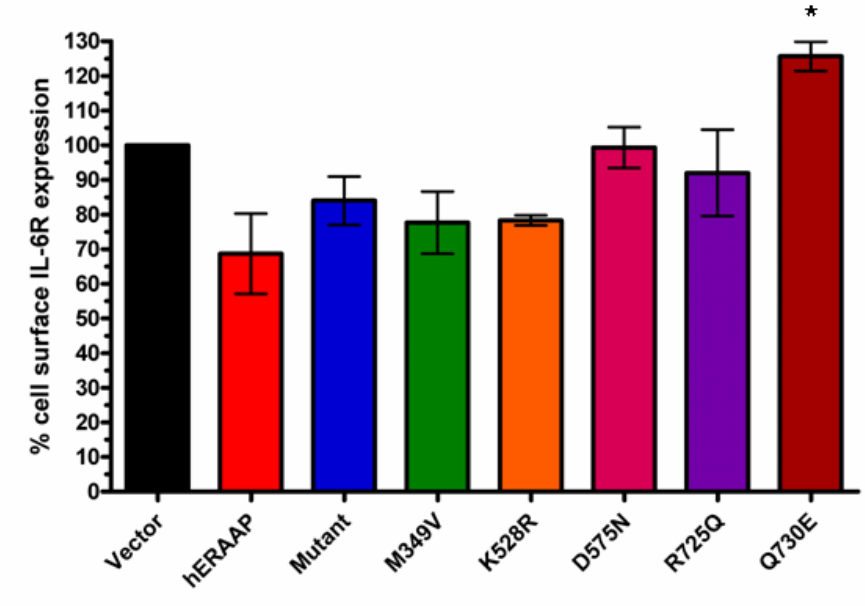

Figure 5.2. Individual SNP ERAAP alters the cell surface IL-6R expression

ERAAP$^{-/-}$ cells were transfected with 1µg pcDNA3-GFP and 1µg human IL-6R along with either 1µg pcDNA3 vector only, hERAAP, mutant or M349V, K528R, D575N, R725Q, Q730E. After 48 hours cells were harvested and analysed as before (figure 5.1). Upon reconstitution with ERAAP containing individual polymorphisms, cell surface IL-6R expression is reduced with M349V and K528R. The D575N and R725Q show little change in expression, whereas the Q730E results in a significant increase in cell surface IL-6R expression compared to wild type (*p=0.01) Data is a representation of three experiments.

The individual polymorphisms showed altered activity towards cytokine receptor shedding compared to wild type hERAAP (figure 5.2). Further assessing the activity of whether selected multiple combinations of polymorphisms would cause more pronounced phenotypes towards their ability to alter surface IL-6R expression was of interest. Three multiple SNP ERAAP mutants, K528R / Q730E, M349V / D575N / R725Q and R725Q / Q730E, were shown to increase IL-6R surface expression in a similar manner to the Q730E individually, with K528R / Q730E having the greatest increase (figure 5.3). Two of these polymorphic ERAAP molecules contain the Q730E mutation, indicating that this position is important in facilitating the cleavage of IL-6R. Surprisingly, the double SNP ERAAP D575N / R725Q showed a similar surface IL-6R expression to wild type hERAAP, even though D575N individually showed a loss of function. These data suggest that multiple SNP ERAAPs have a cumulative effect on their ability to cleave and reduce the IL-6R surface expression through facilitating receptor cleavage.

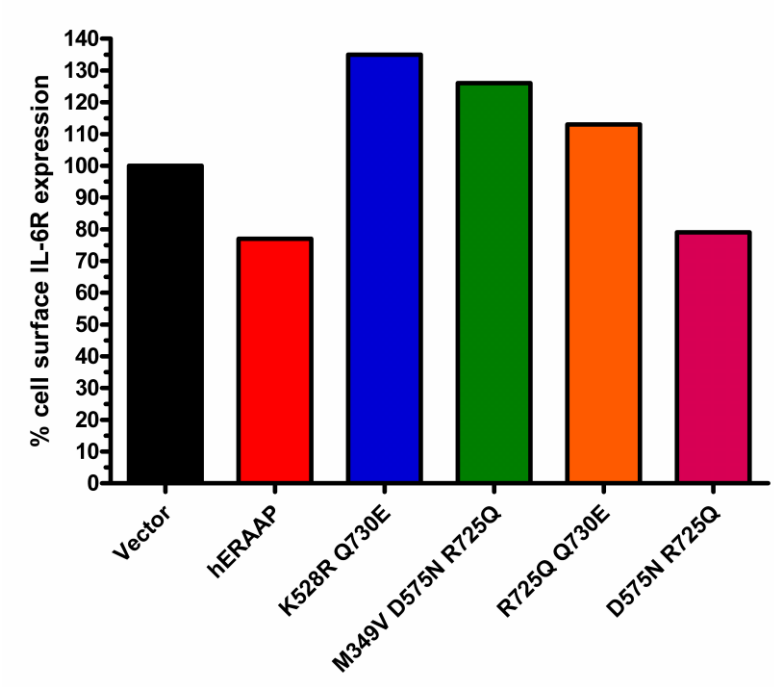

Figure 5.3. Multiple SNP ERAAP generate a more pronounced phenotype

ERAAP$^{-/-}$ cells were transfected with 1µg pcDNA3-GFP and 1µg human IL-6R along with either 1µg pcDNA3 vector, hERAAP, K528R / Q730E, M349V / D575N / R725Q, R725Q / Q730E or D575N / R725Q. After 48 hours cells were harvested and analysed as in figure 5.1. The addition of multiple SNP within ERAAP reduces the IL-6R cleavage activity.

Ectodomain cleavage of membrane bound IL-6R from the cell surface is a mechanism by which sIL-6R is generated, therefore a decrease in IL-6R cell surface expression would be expected to correlate with an increase in the concentration of sIL-6R. To determine the effect of reconstituting either wild type hERAAP or mutant on the generation of sIL-6R from the ectodomain cleavage of membrane bound IL-6R, sIL-6R concentrations in cell culture supernatants of transfected ERAAP$^{-/-}$ cells were measured at various time points over the course of 48 hours post transfection (figure 5.4). The concentration of sIL-6R increased between 11hours – 48hours after transfection in cells with and without ERAAP reconstitution. The addition of wild type hERAAP results in higher concentrations of sIL-6R after 11 hours and remains higher than vector and mutant throughout the time course. The concentration of sIL-6R increased even in the absence of hERAAP, suggesting an ERAAP independent

mechanism of sIL-6R shedding occurs. Transfection of mutant did not significantly alter the profile of receptor shedding compared to the negative control. The greatest difference in concentration of sIL-6R in the presence or absence of ERAAP is shown 30 hours after transfection. After 48 hours, the difference between the presence and absence of ERAAP is minimal, suggesting the greatest effect of transfected ERAAP can be seen between 24 and 36 hours post transfection.

Collectively, these data suggest a role for ERAAP in facilitation of cytokine receptor cleavage. The levels of cell surface expression represent a steady state involving receptor synthesis and shedding. Therefore a change in cell surface expression observed seems to be governed by the rate of receptor shedding. Restoration of ERAAP in deficient cells suggests that hERAAP can act to facilitate IL-6R shedding, with polymorphisms in the hERAAP sequence resulting in a reduction in activity compared to hERAAP. The exception is the activity of Q730E, which increases IL-6R expression, suggesting a role for this SNP in inhibiting ERAAP independent IL-6R shedding.

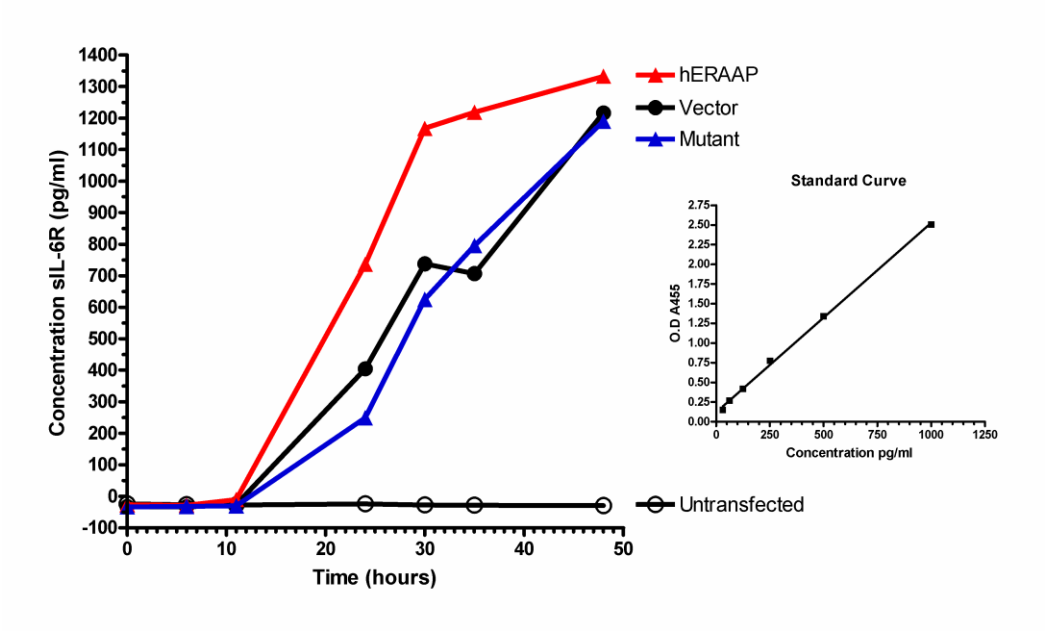

Figure 5.4. Addition of wild type ERAAP and mutant alters the cell surface and soluble IL-6R expression

ERAAP$^{-/-}$ cells were transfected with 1µg pcDNA3-GFP and 1µg human IL-6R along with either 1µg pcDNA3 vector only, hERAAP or mutant. At time points 0, 6, 11, 24, 30, 36 and 48 hours post transfection supernatant from transfected cells were taken and analysed for soluble IL-6R by ELISA. Restoration of wild type hERAAP results in an increase in sIL-6R, whereas mutant results in little change of sIL-6R expression in comparison to no ERAAP expression.

5.2 The reduction of ERAAP expression results in an increase in cell surface IL-6R expression

Transfection of ERAAP$^{-/-}$ cells with hERAAP constructs proved to be highly variable. This together with the uncertainty of the level at which cloned human IL-6R transfected into ERAAP$^{-/-}$ would associate with mouse gp130 accessory molecule essential for IL-6R signalling, indicated the use of human cells as a better model for assessing ERAAP dependent cytokine receptor cleavage. Therefore U937 cells which are a human lymphoma cell line and express ERAAP, IL-6R and TNFRSF1A were used. The cell surface expression of IL-6R and TNFRSF1A in normal U937 cultured cells was confirmed using flow cytometry (figure 5.5).

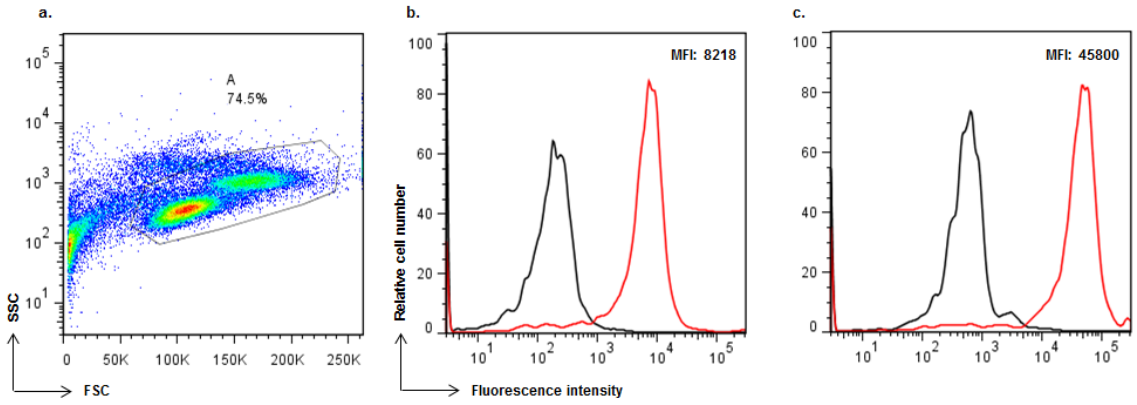

Figure 5.5. U937 cells express both IL-6R and TNFRSF1A

U937 cultured cells were stained using IL-6R (b) or TNFRSF1A (c) specific antibodies (1:100) followed by α-mouse PE or α-rabbit FITC (1:100) respectively and analysed for cell surface expression by flow cytometry. U937 cells express both IL-6R and TNFRSF1A under normal conditions.

Since hERAAP resulted in a reduction of surface IL-6R expression in ERAAP$^{-/-}$ fibroblasts, it was necessary to confirm ERAAP was responsible for the facilitation of IL-6R shedding. U937 cells were transfected with ERAAP specific siRNA oligonucleotides to reduce the endogenous ERAAP expression (figure 5.6b). This reduction of ERAAP expression demonstrated a dramatic increase in the levels of surface IL-6R in comparison to normal cells treated with control Lamin B1 specific siRNA (figure 5.6a). Alongside this, U937 cells were treated with leucinethiol, a potent inhibitor of aminopeptidase activity, for 6 hours (Serwold et al., 2001). The inhibition of ERAAP activity resulted in an increase in surface expression of both IL-6R and TNFRSF1A (figure 5.6c) Therefore, this data further confirmed the requirement for ERAAP activity in the facilitation of IL-6R shedding.

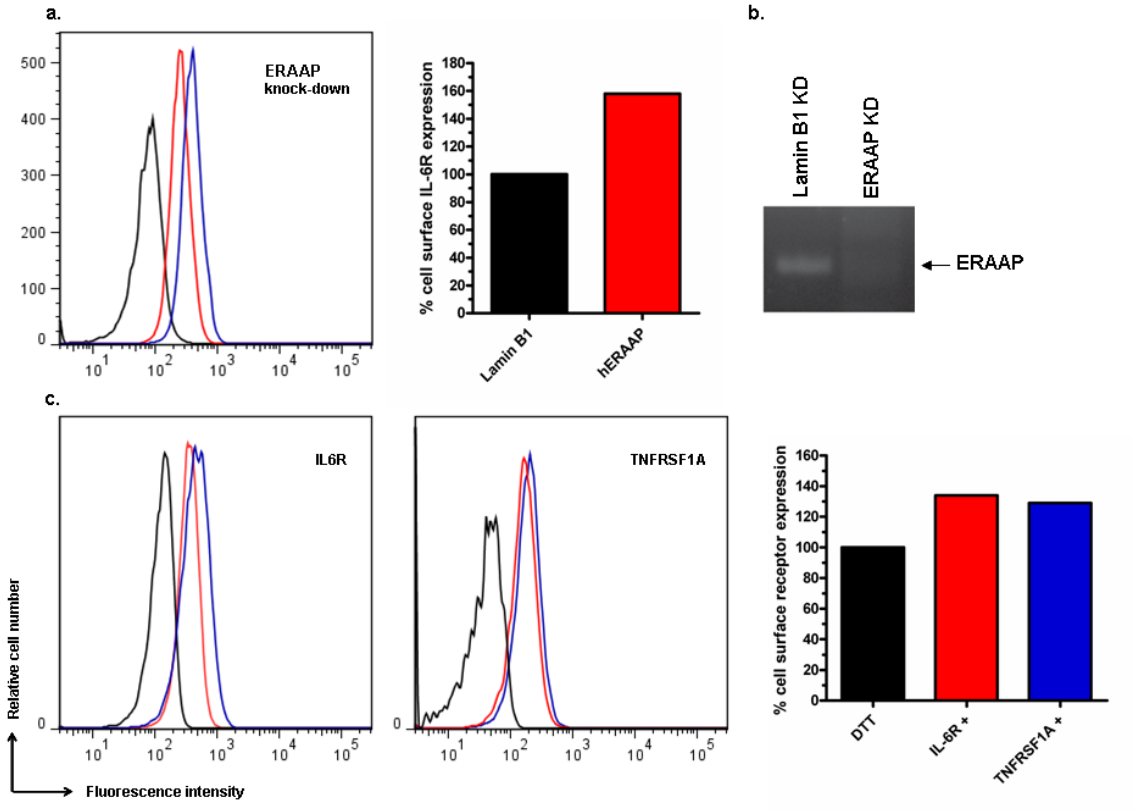

Figure 5.6 Reduced ERAAP expression and activity results in an increase in surface receptor expression

U937 cells were treated with siRNA oligonucleotides specific for Lamin B1 (control) or ERAAP (a). After 48 hours, cells were analysed by flow cytometry for cell surface IL-6R expression, using α-mouse PE only (black line) or IL-6R specific antibody (1:100) followed by α-mouse PE for cells treated with siRNA for Lamin B1 (red line) or ERAAP (blue line). Knock-down of ERAAP was determined through RT-PCR for ERAAP mRNA expression (b). U937 cells were treated with 0.5mM DTT only (red line) or in combination with 30μM leucinethiol (blue line, c) and analysed after 6 hours using flow cytometry as in a. Reduced expression or activity of ERAAP results in an increase of both IL-6R and TNFRSF1A surface expression.

5.3 Overexpression of hERAAP in human cells reduces IL-6R surface expression

To further evaluate the role of hERAAP in cytokine receptor cleavage, additional hERAAP was transfected into U937 cells. Since U937 cells already express ERAAP, it was possible that by introducing additional ERAAP into these cells, the changes in levels of surface IL-6R expression in response to the ERAAP variants could be

determined. Any changes in expression with the increased ERAAP would allow further deduction into the role ERAAP has in facilitating cytokine receptor cleavage. As shown by reconstituting wild type ERAAP into ERAAP$^{-/-}$ cells, the addition of ERAAP into U937 cells resulted in a reduction in IL-6R surface expression compared to the normal IL-6R expression pattern (figure 5.7). This supports the role for ERAAP in facilitating IL-6R shedding. The addition of either mutant or hERAAPtr demonstrated a varying response in levels of IL-6R expression within six repetitive experiments. Taken together, these experiments demonstrated a slight increase in surface expression of IL-6R with mutant, whereas hERAAPtr showed little change in expression. This indicates that both hERAAPtr and the SNPs within mutant cause a loss of function in facilitating IL-6R cleavage. However, it is important to note that in some experiments, hERAAPtr caused a large reduction in expression of surface IL-6R. In comparison to experiments undertaken in ERAAP$^{-/-}$, these data show a less pronounced change in IL-6R expression.

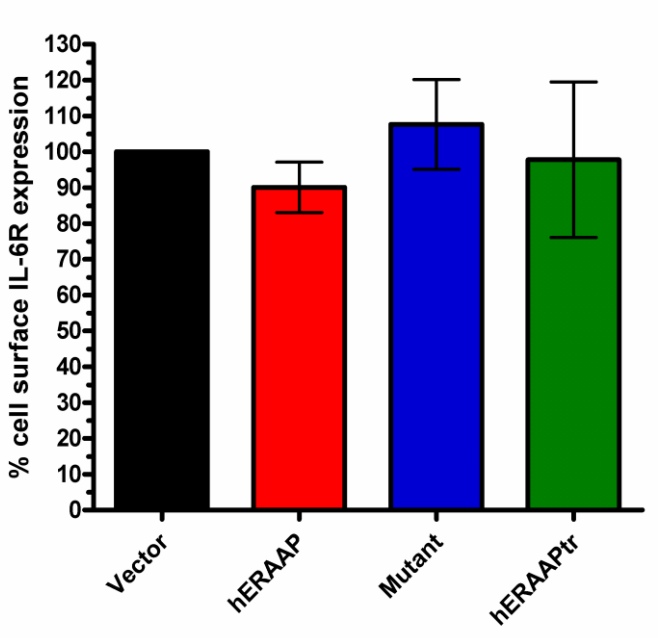

Figure 5.7 ERAAP expression alters surface IL-6R expression

U937 cells transfected with 1µg either pcDNA3 vector, hERAAP, mutant or hERAAPtr After 48 hours cells were harvested and analysed for IL-6R cell surface expression as before. The addition of hERAAP variants alters IL-6R surface expression. Data is representative of 6 experiments.

ERAAP has been shown to associate with a 55kDa IL-6R, consistent with the soluble form of IL-6R, in NCI-H292 human pulmonary mucoepidermoid carcinoma cells (Cui et al., 2003). To determine whether ERAAP associates with IL-6R in U937 cells, co-immunoprecipitation (IP) for ERAAP or IL-6R was undertaken on U937 cell lysates, followed by immunoblotting for either IL-6R or ERAAP respectively (figure 5.8) Like other components of the antigen processing machinery, ERAAP is known to be up regulated by IFN-γ treatment. In addition, many cytokines and in turn cytokine receptors are also up regulated in response to IFN-γ stimulation. To maximise the ability to detect ERAAP-IL-6R associations, U937 cells were treated with 160 units/ml IFN-γ for 48 hours prior to IP. This showed approximately 5-fold increase in ERAAP induction compared to the expression in the absence of ERAAP (figure 5.8b). As shown in figure 5.8a, IP for IL-6R in the presence and absence of IFN-γ failed to pull down the 106kDa ERAAP. However the 106kDa ERAAP was seen in the discarded supernatants after IL-6R IP, indicating that IL-6R does not associate with ERAAP. A non-specific band was also seen in mouse IgG control IP just below 100kDa, slightly lower than expected for ERAAP. In the reciprocal experiments, ERAAP IP also failed to pull down either an 80kDa or 55kDa IL-6R, consistent with membrane or soluble IL-6R respectively, further suggesting that ERAAP does not associate directly with IL-6R (figure 5.8c).

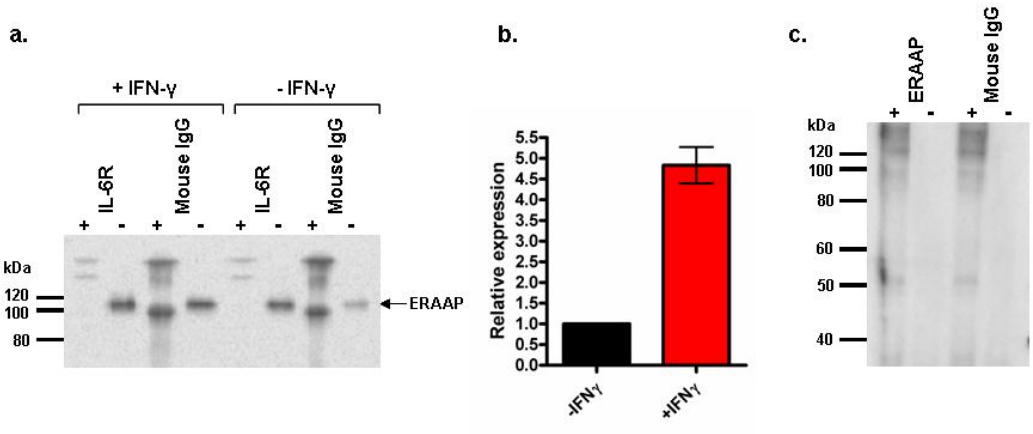

Figure 5.8 ERAAP does not associate with IL-6R in U937 cells

Co-imunoprecipitation (IP) experiments were performed using 10µg IL-6R specific antibody or mouse IgG isotype control in the presence or absence of IFN-γ stimulation (a) and Positive IP samples (+) or discarded lysate supernatants (-) were immunoblotted for ERAAP (a). Relative expression of ERAAP in the presence and absence of IFN-γ is shown in b. Reciprocal IP experiments were performed using 10µg ERAAP specific antibody and isotype control. Once again positive IP samples (+) and discarded supernatant (-) were immunoblotted for IL-6R (c).

5.4 Summary

The results shown in this chapter, through the use of both a mouse and human model system, identify that ERAAP is required to facilitate the cleavage of IL-6R from the cell surface. The requirement for ERAAP in facilitation of IL-6R shedding was first identified by Cui et al, who demonstrated an association between ERAAP and the 55kDa soluble IL-6R, revealing an inverse correlation between ERAAP expression and IL-6R shedding (Cui et al., 2003). To further investigate the proposed role of ERAAP in the facilitation of cytokine receptor cleavage, a number of studies we undertaken using flow cytometry, ELISA and imunoprecipitation, to further determine the role of hERAAP and its sequence variants on the facilitation of IL-6R cleavage.

1 Evaluation of cell surface IL-6R expression in the presence as well as in the absence of hERAAP activity demonstrated that restoring wild type hERAAP in mouse fibroblasts that lack functional ERAAP expression or alternatively increasing levels of wild type ERAAP in human cells expressing endogenous ERAAP decreased cell surface IL-6R expression.

2 Correlating with the decrease in cell surface IL-6R was an increase in sIL-6R in cell culture supernatants from ERAAP$^{-/-}$ cells reconstituted with both wild type hERAAP and IL-6R. This demonstrates that a decrease in cell surface IL-6R results in an increase in soluble IL-6R, consistent with ectodomain shedding of IL-6R releasing soluble protein.

3 Furthermore, gene silencing of ERAAP expression correlated with an increase in cell surface IL-6R, consistent with a reduction in ectodomain cleavage of membrane bound IL-6R. In addition to this, inhibiting ERAAP activity with leucinethiol, a potent aminopeptidase inhibitor that has previously shown to inhibit ERAAP activity also increased cell surface IL-6R expression, suggesting IL-6R cleavage is reduced in the absence of ERAAP activity or expression (Hammer et al., 2006). Together these data suggest a role of wild type hERAAP in the cleavage of IL-6R from the cell surface; however the mechanism of ERAAP action towards IL-6R ectodomain cleavage is still unknown.

4 Using NCI-H292 human pulmonary mucoepidermoid carcinoma cell line, Cui et al demonstrated a direct association between hERAAP and the 55kDa soluble IL-6R (Cui et al., 2003). In contrast to this, no association between IL-6R (both full length

or soluble) was observed in this study when undertaking imunoprecipitation experiments for either IL-6R or ERAAP in U937 cells in the presence and absence of IFN-γ. It is also important to note that this association between ERAAP and IL-6R shown by Cui et al has not been independently demonstrated since.

5 Two variants of hERAAP, a truncated splice variant (hERAAPtr) and an ERAAP containing all six SNPs that are linked with increased AS susceptibility (mutant) demonstrated an abrogated ability to facilitate IL-6R cleavage.

6 Altering the sequence of the GAMEN active site region (E320A) resulted in an abrogation of facilitation of IL-6R shedding, consistent with the requirement of an intact active site region for this function (Kanaseki et al., 2006; Cui et al., 2002).

7 SNPs within ERAAP altered the expression of cell surface IL-6R, having reduced activity compared to wild type hERAAP. M349V and K528R show some facilitation of cleavage activity, whereas R725Q and D575N demonstrated an abrogation in the facilitation of IL-6R shedding consistent with a loss-of-function in activity.

8 Most surprisingly was that Q730E acts to increase surface IL-6R expression which may be a result of this SNP inhibiting an ERAAP independent receptor cleavage mechanism (Cui et al., 2003). Preliminary data with multiple SNP mutants suggest a more pronounced effect on levels of surface IL-6R, with two double SNPs containing Q730E, along with a triple SNP ERAAP (M349V / D575N / R725Q), having a phenotype similar to Q730E individually. This implies that the additional SNP is not able to rescue the defective nature of the Q730E SNP. However, the mutant also contains this SNP but does not display the same phenotype which may be a result of the five other SNPs acting to overcome the Q730E. Therefore a combination of certain polymorphisms may lead to detrimental effects on the ability to facilitate cytokine receptor cleavage depending on their position within the molecule.

9 Alterations in the sequence of hERAAP results in a disruption in the ability to facilitate surface IL-6R cleavage which may suggest a link between SNPs within ERAAP and the onset of inflammatory diseases through a disruption in the role of ERAAP to mediate IL-6 cytokine signalling by regulating levels of surface cytokine receptor.

Chapter 6: Discussion

ERAAP is important in two major immune system processes: generation of antigenic peptides and cleavage of cytokine receptors. Antigen processing is important for presenting peptides derived from proteins within the cell to CTL which will recognise the peptide as 'self' or 'non-self', eliciting an immune response where appropriate. Changes in the cells peptide processing ability may impact on the capacity to resolve infection, through the inability to generate pathogen specific peptides and the lack of expression of stable MHC I at the cell surface. Cytokine receptor cleavage is an important mechanism for regulation of cytokine mediated signalling events involved in the immune response and mediating inflammation. Defects in cytokine receptor cleavage prevents normal functioning of the subsequent signalling cascade, either resulting in an increased or decreased inflammatory response.

Disease associated polymorphisms have been identified within ERAAP, however it was unknown how these polymorphisms affected ERAAP function and how this might be linked to these diseases, in particular AS (Burton et al., 2007; Mehta et al., 2007; Yammamoto et al., 2002). It was proposed that these polymorphisms would alter the ability of ERAAP to trim N-terminally extended peptide precursors to optimal length for loading on to the MHC I, and have an effect on the cleavage of cytokine receptors. Here these polymorphisms have been studied both individually and collectively to determine their effect on ERAAP activity.

6.1. ERAAP acts to trim N-terminally extended precursors

In this study an ERAAP deficient mouse model system was used to determine the role of ERAAP in antigen processing. Processing of N-terminally extended peptide precursors plays a crucial role in the generation of optimal peptides for presentation (Hammer et al., 2007). In the cytosol, peptides targeted for degradation are cleaved by the immunoproteasome, resulting in peptides with the correct hydrophobic C-terminus for assembly onto MHC I. However, the N-terminal of the peptide generated is frequently sub-optimal for stable loading onto MHC I and normally requires further processing before loading and subsequent presentation at the cell surface (Cascio et al., 2001). ERAAP is the only aminopeptidase known to reside in the ER that has the ability to trim these N-terminal extensions, preferentially cleaving peptides longer than 8 residues in length (Kanaseki et al., 2006; Saric et al., 2002; Serwold et al., 2002). Here it is demonstrated that efficient trimming of a 13 residue precursor of SIINFEHL, a mutated form of SIINFEKL, was trimmed to SHL8 peptide in the presence of hERAAP. It was previously described that the reduction in expression of ERAAP in mouse cells

correlated with an almost complete abrogation of SHL8 generation from a 15 residue precursor (Serwold et al., 2002). Here it was observed that reduced ERAAP expression in mouse cells inhibited the generation of SHL8 from the N-terminally extended 13 residue precursor X5-SHL8. By contrast, the ability to present SHL8 from a minigene requiring no trimming was unaffected by the absence of ERAAP providing evidence for the requirement of hERAAP in trimming of N-terminally extended peptide precursors in the ER.

6.2. ERAAP sequence variants alter the trimming of N-terminally extended precursors

Whilst wild type hERAAP can trim peptide precursors, incorporating the SNPs identified by the GWAS study affected the ability of hERAAP to successfully carry out its role in peptide processing. These SNPs within ERAAP have recently been linked with the development of a number of diseases including the increased susceptibility to AS (Burton et al., 2007;Mehta et al., 2007;Yamamoto et al., 2004). Using T cell activation assays, the ability of hERAAP and sequence variants to generate the final SHL8 peptide from the peptide precursor, X5-SHL8, was assessed. A reduction in processing activity in a number of hERAAP variants, including individual SNP ERAAPs (M349V, K528R, R725Q and Q730E) as well as the truncated splice variant, hERAAPtr, and mutant ERAAP, was observed. This reduction in processing was shown through a reduction in B3Z stimulation due to the lack of presentation of SHL8/H2-K^b at the cell surface. This decrease in the generation of final peptide antigen from its precursor may be explained by a number of mechanisms discussed below. The transfection efficiency of the hERAAP molecules into $ERAAP^{-/-}$ cells was assessed by both RT-PCR and immunoblotting to determine the transcription and translation of these molecules within these cells. The mRNA expression (determined by RT-PCR) as well as protein levels (immunoblotting) identified the successful transfection of these cells, resulting in equivalent protein expression between transfected cells. Therefore, the observed reduction in response was not due to a lack of ERAAP expression caused by poor transfection efficiency. It is possible however, that the addition of hERAAP is over-saturating in comparison to the amount of X5-SHL8 within these cells, resulting in an over-trimming activity of hERAAP. To address this, the optimal concentration of wild type hERAAP expression that was able to generate the maximal processing activity was identified (1µg). The addition of varying concentrations into the $ERAAP^{-/-}$ cells resulted in a reduction in response, either through over-saturation of hERAAP within the cell (1.5µg), or as a result of a reduction in the amount of ERAAP (0.5µg).

Here the cloned hERAAP variants, hERAAPtr, and mutant (ERAAP containing six SNPs identified to be associated with AS), were identified to have total abrogation of B3Z response. Assessment of single SNPs identified both the K528R and R725Q as having a decrease in trimming activity, however importantly, a complete reduction was not observed. Alongside this, the M349V and Q730E SNPs revealed an inability to trim N-terminal extensions depending on the properties of the amino acids they contain charged (M349V) or hydrophobic (Q730E). Interestingly, ERAAP deficient cells (siRNA treated or genetic knockout) transfected with X5-SHL8 did not show a complete reduction in B3Z stimulation. Although, in siRNA treated cells ERAAP knock-down was not complete, resulting in a low level of trimming activity, this response was also observed in ERAAP$^{-/-}$ cells. Therefore a more likely explanation would be that ER independent trimming of the X5-SHL8 precursor was occurring. In these circumstances, it is plausible that X5-SHL8 is able to exit the ER, possibly through transporters such as Sec61, and targeted for trimming within the cytosol by other aminopeptidases such as TPPII, PSA and LAP (Geier et al., 1999; Reits et al., 2004; Beninga et al., 1998; Stoltze et al., 2000). The final SHL8 epitope is then transported back to the ER via TAP, assembled onto the MHC I and presented at the cell surface. The observed abrogation of a B3Z response to untrimmed peptide precursors in TAP$^{-/-}$ ERAAP$^{-/-}$ double knockout cells correlates with this hypothesis (Kanaseki et al., 2006). Surprisingly, hERAAP variants (hERAAPtr and mutant) generated a response lower than X5-SHL8 alone. This indicates that these variants alter the capacity for ER independent processing to occur, identifying possible mechanisms of action that result in the phenotypes demonstrated. Firstly, the hERAAP variant may over-trim the peptide substrate, failing to cease activity at the optimal 8 amino acid peptide, resulting in the destruction of B3Z stimulating SHL8 peptide. A second possible mechanism of action is the reduction, but not total loss, of ERAAP activity. In this situation, ERAAP has some processing activity, but fails to trim all five amino acids in the N-terminal extension due to a change in amino acid specificity, ceasing activity before the final SHL8 is generated. These intermediates would no longer be substrate for ER independent trimming. The proposed mechanisms to explain the reduction in hERAAP activity by the variants are consistent with failure to generate the SHL8/H2-K^{b} complex. The use of B3Z cells within the T cell activation assay allowed identification of any hERAAP molecules that altered the presentation of SHL8/K^{b} in comparison to that shown by wild type hERAAP. However, the assay was unable to distinguish between the proposed mechanisms by which these variants act to reduce the response and fail to generate the final optimal peptide. To elucidate the mechanism of action, RP-HPLC analysis of

peptide extracts from cells expressing the defective hERAAP variants was employed. This allowed distinction of molecules with a hypoactive trimming phenotype compared to those with a hyperactive phenotype. Fractionation of peptide extracts identified an untrimmed precursor in both mutant and hERAAPtr transfected alleles consistent a loss of function phenotype. Further to this, peptides were extracted from cells and treated with trypsin before incubation with APCs and B3Z. This identified the presence of untrimmed peptide within these cells, consistent with these variants acting to sequester the peptide, remaining unprocessed through loss of function. Distinction between hypoactive and hyperactive nature of ERAAP molecules could be determined through RP-HPLC alone, however if an ERAAP molecule with a hyperactive activity was demonstrated, future experiments using RP-HPLC combined with mass spectrometry would be required to determine the level of over-trimming activity and to confirm the presence of further truncated peptides.

Within this study a number of ERAAP alleles from AS patients and a control cohort were identified. Through analysis of T cell activation, a large proportion of these alleles were shown to be defective in the generation of the final optimal peptide. Half of the alleles in control patients were defective whereas 85% of those in AS cases were defective. Of the identified alleles, the greatest reduction in B3Z response was observed for mutant, K528R / R725Q and R725Q / Q730E ERAAP. These are all present in case samples but only the mutant is present in controls. Further analysis through RP-HPLC identified hypoactive, hyperactive and functional alleles, and interestingly, hyperactive alleles were only found in AS case samples. From the identification of ERAAP alleles we were able to elucidate individual ERAAP haplotypes (consisting of two ERAAP alleles) from samples. As yet there is no common haplotype shared between cases and controls. AS patient haplotypes demonstrated a reduction in peptide processing activity, with the exception of the M349V + wild type. In contrast, in the two control haplotypes assessed, normal trimming activity was observed. Through the use of RP-HPLC I was able to identify the trimming phenotypes of the alleles and dissect their effect on the overall trimming phenotype. The combination of a hypoactive allele with a normal allele, as demonstrated with both the control haplotypes (wild type in combination with either mutant or K528R), results in the rescue of peptide trimming and shows similar activity to the functional allele alone suggesting a dominant nature as expected. However, in contrast to this, when a hyperactive allele is present together with a functional allele (R725Q / Q730E with wild type), restoration of trimming activity does not occur. This is consistent with the R725Q / Q730E having an

overtrimming phenotype. The failure to restore trimming activity is also demonstrated when a hyperactive allele is combined with a hypoactive one (K528R / R725Q with mutant), indicating the dominant negative function of the hyperactive alleles. Interestingly, both haplotypes containing hyperactive alleles show a low level of B3Z stimulation, consistent with the ability of some SHL8 peptide to bind to H-2K^b before being destroyed. Within the control cohort a hypoactive allele in combination with a functional allele results in a haplotype with restored trimming activity. Surprisingly and in contrast to this, AS case haplotypes are often comprised of two hypoactive alleles, or hyperactive allele in combination with either a functional or a hypoactive allele, all of which demonstrate a reduced ability to generate final peptide precursor. The obvious exception to this is the wild type + M349V haplotype identified within AS patients, which shows a B3Z response comparable with wild type only. At present, no HPLC analysis has been carried out on this haplotype, however in the presence of X5-SHL8 this haplotype would be expected to have functional processing activity. The effect of the M349V allele on peptide trimming may not be revealed using X5-SHL8 as a substrate and is only shown when X6-SHL8 is used since it was unable to trim this charged precursor. This would suggest that in some AS patients the in/ability to trim peptides for presentation at the cell surface may not affect the entire peptide repertoire of ERAAP dependent epitopes.

6.3. SNPs in ERAAP alter substrate specificity

Wild type hERAAP was shown here to trim a model peptide precursor with a hydrophobic N-terminal extension. Upon testing the ability of hERAAP to trim a more charged and less hydrophobic precursor, with the addition of an extra amino acid, hERAAP was able to trim this precursor to the same extent as the hydrophobic precursor. In addition, the abrogated trimming activity of hERAAPtr and mutant toward X5-SHL8 was also seen when X6-SHL8 was used. Surprisingly, however, two SNPs (Q730E and M349V) had an altered activity towards the hydrophobic precursor compared to the longer, more charged precursor. The K528R and R725Q SNPs had reduced activity towards both substrates and the D575N SNP having normal trimming function to both precursors. In the presence of the hydrophobic precursor, M349V had the ability to trim the N-terminal extension, whereas the Q730E was unable to. This activity was reversed in the presence of a longer, charged precursor, where M349V had reduced activity but Q730E could trim this extension. This suggested that the amino acid positions 349 and 730 were important for ERAAP activity depending on the properties of the peptide substrate. These findings were further reinforced when

assessing the processing activity of the susceptible (K528R / Q730E) and protective (M349V / D575N / R725Q) alleles identified by HapMap analysis. The activity of these alleles was shown to be altered in the presence of different substrates. The susceptible allele was unable to trim the hydrophobic precursor but could successfully process a more charged extension. In contrast, the protective allele could trim a hydrophobic extension but was unable to trim the charged, longer extension. As both K528R and R725Q were shown to be unable to trim X5- or X6-SHL8, this activity is likely to be due to the other SNPs present in these alleles. Therefore, it is shown here that the SNPs M349V and Q730E have altered trimming preferences, which may have important implications in the ability of patient alleles to generate final peptides within the ER for MHC I loading as discussed above. The ability to alter ERAAP substrate specificity has been observed previously. Substitution of glutamine at 181 to glutamic acid resulted in an ERAAP molecule with a preference for trimming of basic amino acids (Goto et al., 2006). It is therefore plausible that the changes in amino acids at these positions (349 and 730) alter the ability of ERAAP to interact with the side chains of the peptide substrate requiring trimming, resulting in a loss of activity towards specific substrates. This indicates that the phenotype observed in patient haplotype M349V + wild type may only be applicable to trimming of hydrophobic amino acids with the ability to trim charged amino acids compromised.

6.4. K528R and R725Q SNPs reduce the processing activity

When assessing the individual SNPs for their ability to generate the final optimal peptide, both K528R and R725Q had a reduction in B3Z response when expressed with both X5-SHL8 and X6-SHL8. When analysed for its activity by RP-HPLC, the K528R demonstrated a hypoactive trimming phenotype. The reduction in activity of K528R demonstrated here is consistent with previous findings using ERAAP molecules containing this SNP which showed a decrease in enzymatic activity towards the synthetic fluorogenic substrate, Leu-NA (Goto et al., 2006). The K528R SNP is associated with increased risk of hypertension and was shown to be unable to trim angiotensin II and Kallidin to angiotensin III and bradykinin respectively, having ~70% reduction in enzymatic activity towards these substrates (Goto et al., 2006). In addition, Evnouchidou et al demonstrated K528R had a reduced activity towards the hydrolysis of L-AMC substrate in vitro. When tested for activity of L-AMC hydrolyisis in the presence of a non-substrate peptide (SIINFEKL), K528R was shown to be activated the most in comparison to wild type and the Q730E variant, however still had the most reduction in L-AMC hydrolysis (Evnouchidou et al., 2011). K528R also resulted in a

reduction in cell surface HLA-B27 when introduced into HeLa-K^b/HLA-B27/ICP47 cells (Evnouchidou et al., 2011). These findings are consistent with the reduction of K528R in generating the final SHL8 for presentation at the cell surface shown here. Conversely, the decrease in activity of R725Q ERAAP has not yet been documented within the literature.

Further to this, other SNPs in combination with either K528R or R728Q showed a further reduction in processing activity, displaying an additional effect. However when double SNP generated ERAAPs without either of these SNPs were assessed, the activity was comparable to wild type hERAAP, further highlighting the importance of K528 and R725 in normal ERAAP function. Interestingly, the patient AS alleles previously discussed contain either/both K528R or R725Q in at least one allele within their haplotype. This together with the observation that double SNP ERAAPs contain K528R or R725Q exhibit a reduction in activity, suggests a cumulative dysfunctional role of SNP alleles containing these two SNPs.

6.5. Positioning of SNPs within the ERAAP protein

Recently, the crystal structure of ERAAP has been elucidated identifying ERAAP as a four domain protein that changes from an open to closed conformation in order to carry out its aminopeptidase activity (Nguyen et al., 2011). It was proposed that in the open conformation, the ERAAP molecule is inactive but peptide receptive and once peptide is bound, ERAAP is able to close around the peptide, activating its enzymatic function. It was also proposed that binding of substrates to regulatory sites within the protein promotes the change in conformation from an open to a closed state. In this closed conformation, the C-terminal region (domains III and IV) closes off the catalytic active site, and so is no longer accessible from the exterior of the protein (Nguyen et al., 2011; Kochan et al., 2011). This change in conformation in order to successfully carry out its activity is consistent with the structural changes shown with other M1 metalloprotease family members, such as LTA4H and TIFF3 (Nguyen et al., 2011). It is suggested that the substrate binds to a large cavity formed by the interface of the active site within domain II and the C-terminal concave surface within domain IV. The region closest to the active site is thought to be narrow and accommodates the N-terminal region of the peptides, with the C-terminal end of the protein occupying the larger part of the cavity formed by domain IV. In the open conformation, domain IV extends away from the active site forming a larger cavity than demonstrated with other M1 family members. The increase in size of this cavity is consistent with the role of

ERAAP in trimming N-terminal extensions, cleaving bioactive peptide hormones or kinases in hypertension and angiogenesis (Nguyen et al., 2011; Kochan et al., 2011; Saric et al., 2002; Serwold et al., 2002; Yamazaki et al., 2004).

The six SNPs identified to have an association with increased AS susceptibility are located throughout the proposed four domains of the ERAAP protein (Nguyen et al., 2011). The K528R resides at the junction between domains II and III which serves as a hinge allowing the conformational change of ERAAP from an open to a closed state upon peptide binding (Nguyen et al., 2011). This role was suggested by Goto et al when they observed that the change in activity of 528 was different to that seen when amino acids at 320 and 357 (active site) were changed (Evnouchidou et al., 2011, Goto et al., 2006). It is therefore possible that altering this amino acid alters the ability for ERAAP to close following peptide binding; thus changes in conformation within the active site upon closing cannot occur, reducing the enzymatic activity. R725Q is exposed on the inner surface of the C-terminal cavity within domain IV and may prove vital for substrate specificity through binding of the side chains of the substrate. This amino acid position is likely to reside within the proposed regulatory domain which interacts with the C-terminal region of the peptide substrate. Altering the amino acid at this position may result in local conformational changes in turn affecting the interactions of this region with peptide substrate. Also, if peptide fails to occupy the regulatory site, the activation of the conformational change is likely to be inhibited, resulting in lack of enzymatic activity.

Interestingly, M349V and Q730E revealed a change in substrate specificity with these differences affecting their ability to generate optimal peptide epitopes. M349V is located within the active site region of domain II and this position may act to maintain the structure of the catalytic pocket by interacting with other amino acids as well as interacting with the N-terminal region of the peptide. It is possible that altering this amino acid will result in a local conformational change which restricts peptide specificity through changing interactions with the peptide. The Q730E is situated five amino acids from the R725Q SNP, residing on the inner surface of domain IV in the regulatory domain. Substrate specificity is likely to result from altered interactions with the C-terminal region of the peptide due to conformational changes by altering the amino acid at this position. It is therefore not surprising that the greatest reduction in the ability to process N-terminally extended peptides is demonstrated by the R725Q / Q730E, which are in such close proximity to each other within the regulatory domain, thought to be

important for ERAAP function. These SNPs in combination demonstrate an additional effect on function, generating a hyperactive trimming phenotype. How the changes affect N-terminal trimming is not clear however, it is possible that changing the amino acids at these positions results in conformational changes which alters the ability to interact with the peptide substrate, or change the ability of ERAAP to undergo structural change in order to exert its enzymatic activity. The overall consequence of these changes are a failure to engage the substrate properly resulting in the ability to trim peptides of 8-9 amino acids in length destroying antigenic epitopes.

6.6. ERAAP is involved in cytokine receptor shedding

In addition to the role in peptide processing, the role of hERAAP in the facilitation of IL-6R shedding from the cell surface, resulting in the soluble form of this receptor, has been demonstrated here (Cui et al., 2003). The requirement for ERAAP in the facilitation of IL-6R shedding was first identified by Cui et al, known in this context as aminopeptidase regulator TNFR shedding 1 (ARTS1) who showed that ERAAP could associate with the 55kDa soluble IL-6R, revealing a correlation between ERAAP expression and IL-6R shedding. Here both human and mouse cell systems were used to determine the facilitation of IL-6R shedding by the restoration or addition of hERAAP into these cells. A reduction in the levels of cell surface IL-6R in the presence of hERAAP was observed, consistent with the increase in sIL-6R in cell culture supernatants when tested using ELISA. However, using U937 cells, no physical association between IL-6R and hERAAP was detected when undertaking co-immunoprecipitation experiments. These results would suggest that ERAAP activity alters IL-6R expression but does not act to directly cleave IL-6R. However, there could be a number of experimental reasons for this conflicting data. In the experiments here, U937 cells known to express ERAAP and IL-6R were used; however Cui et al used NCI-H292 human pulmonary mucoepidermoid carcinoma. IP was undertaken in the 2003 study using membrane fractions in triton X lysis buffer, however whole cell lysates created in NP40 were used here. Further to this, an antibody specific for both membrane and soluble IL-6R was used here, whereas Cui et al used an antibody specific for the ectodomain region of IL-6R, suggesting a more specific antibody may be required to observe any IL-6R/ERAAP associations. The data suggest ERAAP as having an indirect mechanism of facilitating cytokine receptor cleavage; this is more consistent with the literature which suggests ERAAP serves to allow the recruitment of other enzymes that act as endopeptidases or sheddases (Adamik et al., 2008; Yamazaki et al., 2004; Cui et al., 2003). This is a more favourable mechanism due to

the exopeptidase nature of ERAAP, which has been shown to trim N-terminal amino acids from proteins in a number of biological processes (Saric et al., 2002; Serwold et al., 2002; Goto et al., 2006; Yamazaki et al., 2004).

Consistent with the findings that hERAAP variants reduce the ability to process N-terminally extended peptides, it is demonstrated here that these variants reduce the ability of ERAAP to facilitate IL-6R shedding. E320A, mutant and hERAAPtr showed an abrogation of facilitation of IL-6R shedding, with levels of IL-6R within the individual SNP variants comparable to that with no ERAAP expression. The R725Q showed a loss of function consistent with peptide processing observations and once again it is likely that the positioning of this SNP alters the ability to interact with the substrate. Interestingly, however, the D575N SNP, shown to have normal peptide processing activity, demonstrates a loss of function in the facilitation of IL-6R shedding. This SNP may therefore be involved in the interaction and association with other proteins involved specifically within this process. The most noticeable activity was shown with the Q730E SNP, which increased the IL-6R surface expression. As discussed above, this SNP may alter the ability to bind to substrate similarly to R725Q, however in this case it blocks ERAAP independent cytokine shedding mechanisms. Combinations of SNPs (K528R / Q730E, R727Q / Q730E, M349V / D575N / R725Q) show a cumulative effect, having a mechanism similar to Q730E individually. The ability for the SNP variant ERAAP molecules to alter IL-6R ectodomain cleavage is still unclear. IL-6R ectodomain cleavage occurs between Gln357-Asp358 in the transmembrane and ligand binding domains, therefore requiring proteolytic cleavage by an endopeptidase (Althoff et al., 2000; Mullberg et al., 1994). A more likely scenario for the role of ERAAP in IL-6R shedding is that ERAAP is able to recruit other enzymes in their pro-active state which subsequently require N-terminal amino acid removal by ERAAP to activate the sheddases. It is possible that ERAAP can form complexes with these enzymes as well as IL-6R. These sheddases will target the IL-6R by ectodomain cleavage, releasing soluble protein. A similar mechanism is demonstrated in angiogenesis where ERAAP indirectly activates S6K through the removal of 9 N-terminal amino acids from PDK1 which block the S6K binding site. Once trimmed, S6K can bind to PDK1 and become activated, resulting in downstream gene transcription promoting endothelial cell growth and migration (Biondi et al., 2001; Akada et al., 2002; Yamazaki et al., 2004). Two other cytokine receptors, IL-1RII and TNFRSF1A, were identified by the same group to have association with ERAAP and they correlated expression of ERAAP with cytokine receptor cleavage (Cui et al., 2002; Cui et al., 2003b). Consistent with

the findings here that ERAAP does not associate directly with IL-6R, TNFRSF1A was shown to associate with NUCB2, a calcium binding protein, and RBMX, a member of the spliceosome, correlating with receptor shedding (Adamik et al., 2008;Islam et al., 2006). The structural changes of ERAAP following substrate binding are consistent with an exopeptidase rather than endopeptidase activity. ERAAP is therefore likely to function as an extracellular regulatory molecule that is able to recruit and bind proteins which when activated act as sheddases to cleave the membrane bound cytokine receptors, releasing their soluble forms.

Ectodomain shedding of over 100 transmembrane proteins is a result of proteases or sheddase acting to cleave the extracellular domain of these membrane tethered proteins to release soluble forms. The facilitation of IL-6R shedding by the activity of ERAAP is not the only mechanism for the generation of sIL-6R through ectodomain cleavage, as suggested by Cui et al (2003a). This is also consistent with the role of other sheddases such as the ADAM family of metalloproteases that are known to cleave the TNFRSF1A and IL-1RII ectodomains (Reddy et al., 2000). Treatment of cells with a metalloprotease inhibitor, TNFα protease inhibitor (TAPI), blocked the shedding of both IL-6R and p60 TNFR, implicating a metalloprotease or family of related metalloproteases in the shedding of IL-6R (Mullberg et al., 1995). It was later shown that like TNFRSF1A, IL-6R is a substrate for cleavage by both ADAM17 and ADAM10, with ADAM17 responsible for PMA induced ectodomain shedding and ADAM10 responsible for constitutive ectodomain shedding of IL-6R (Matthews et al., 2003); Althoff et al., 2000). When identifying the role of ERAAP in TNFRSF1A shedding, Cui et al (2002) demonstrated that the ability of ERAAP to enhance shedding was not mediated by an increase in ADAM17 expression and maturation. This suggests that ERAAP and both ADAM17 and ADAM10 act independently to cleave the IL-6R ectodomain rather than ADAM metalloproteases being substrates for the indirect ability of ERAAP to facilitate shedding.

The biological significance of these data is that SNPs within ERAAP cause a significant disruption in the regulation of cytokine receptor shedding and in turn inflammatory responses. The IL-6 cytokine is important in a number of inflammatory responses, with a reduction in cleavage of cytokine receptors from the cell surface resulting in an increase in the presence of receptors for binding IL-6 and in turn stimulating intracellular signalling events. This increases the transcription and translation of

inflammatory mediators, causing disruption of the highly regulated system and an increase in localised inflammation from cells expressing IL-6R.

6.7. Implications for disease

AS is an autoimmune inflammatory condition strongly linked to the HLA-B27 molecule (Kollnberger et al., 2002). Although it provides a strong genetic link with AS development, the presence of the HLA-B27 molecule is not the single causative factor in the development of AS. SNPs within ERAAP were shown to be associated with the susceptibility of AS and recent advances have identified this association between ERAAP SNPs and AS to be restricted to HLA-B27 positive individuals only (Evans et al., 2011). This observation points towards the altered ability of ERAAP SNPs in the generation of final peptide epitopes, in turn affecting its folding in the ER and stabilisation of HLA-B27 cell surface expression. These MHC I molecules confer unusual cell biology by having slower folding kinetics and β_2m association compared to other MHC I molecules. These kinetics highlight a tendency to bind lower affinity peptides which have a faster dissociation rate when expressed at the cell surface (Peh et al., 1998). In addition, the unusual B pocket characteristics and presence of an unpaired cys67 in the α-chain causes in an increase in incorrect disulphide bond formation within the ER, leading to the prolonged ER retention of HC homodimers and an increase in misfolding and aggregation of HC (Dangoria et al., 2002). The correct folding of HC requires optimal peptide supply for the formation of stable MHC I complexes. Here it is shown that polymorphisms within ERAAP affect the generation of the correct epitope for presentation on MHC I. Further to this, AS ERAAP alleles show a defective trimming phenotype, consistent with a reduction in the generation of final optimal peptides, therefore altering the peptide repertoire presented at the cell surface. Consequently these SNPs may affect the generation of peptide epitopes for loading on to the HLA-B27 molecule for their stabilisation and exit to the cell surface (Burton et al., 2007). In addition, HLA-B27 is known to form heavy chain homodimers both within the ER and at the cell surface (Antoniou et al., 2004). This incorrect peptide generation may, through defective ERAAP activity, aid the misfolding of HLA-B27 and trigger dimerisation of these molecules within the ER. Under normal circumstances, ERAD is able to eliminate misfolded heavy chains. However, if optimal peptides for stable HLA-B27 loading are not generated, through a defective ERAAP, an increase in misfolding and aggregation is likely. This subsequently activates the UPR, leading to the expression of inflammatory mediated genes and the up regulation of pro-inflammatory cytokines (Tak and Firestein., 2001; Zhang et al., 2006).

If unstable or sub-optimal peptides are bound, these peptides will have a fast dissociation rate from the HLA-B27 at the cell surface. This dissociation of unstable peptides, resulting in empty HLA-B27 molecules may facilitate the formation of homodimers at the cell surface (Bird et al., 2003). Patients with AS have an increased HLA-B27 homodimer expression level at the cell surface and it is the recognition of these homodimers by specific receptors on NK cells and lymphocytes that may be responsible for the onset of AS (Kollnberger et al., 2002; Bird et al., 2003). HLA-B27 homodimers are ligands for both KIR3DL1 and KIR3DL2, which are significantly upregulated in patients with SpAs, specifically HLA-B27 positive patients (Chan et al., 2005). KIR interactions with MHC I can promote T cell survival, therefore interactions with HLA-B27 homodimers and KIR3DL2 may promote the survival of self-reactive T cells. In addition, loss of recognition of inhibitory KIRs or an up regulation in recognition of stimulatory KIRs, a result of unstable heterotrimeric HLA-B27 complexes at the cell surface or an increase in the expression of HLA-B27 homodimers, may lead to NKT/T cell activation.

ERAAP may also be associated with AS through inadequate cytokine receptor cleavage. Here individual SNPs reduce IL-6R cleavage and if SNP mutated ERAAP have the same reduced ability to cleave TNFRSF1A and IL-1RII this would be important causing the generation of a prolonged inflammatory response. Binding of cytokines to their receptors initiates a signalling cascade within the cell. IL-6 is able to bind to IL-6R, which in turn, activates Janus Kinase (JAK) through the signalling transducer gp130 (Devin et al., 2000; Guschin et al., 1995). This phosphorylates the signal transducer and activator of transcription (STAT) which dimerises and translocates to the nucleus where it is able to initiate transcription of many factors involved in cell regulation, growth and inflammation. TNF-α binds to its receptor, TNFRSF1A, which recruits the TNFR1-associated death domain proteins (TRADD, (Hsu et al., 1995). TRADD binds to the receptor interacting protein (RIP) and TNF-receptor association factor-2 (TRAF-2) to mediate IκK activation, initiating the NF-κB signalling pathway (Devin et al., 2000). Through the NF-κB pathway, gene transcription of IL-1 and other cytokines important in inflammation along with genes involved in angiogenesis are promoted (Legler et al., 2003; Micheau and Tschopp, 2003). Regulation of these responses by reduction of the amount of signalling through binding of cytokines to their receptors occurs through the cleavage of cytokine receptors at the cell surface. The soluble cytokine receptors generated are also important for mediating responses induced by cytokines and are able to increase the half-life of the cytokine.

Along with this, the soluble form can bind and sequester cytokines away from cells, reducing the amount of cytokine able to bind and activate signalling within the cell (Arend et al., 1994). A defect in cytokine receptor cleavage would therefore alter the level of soluble receptor and in turn will the amount of cytokine able to bind to its cell surface receptor to initiate a response.

Reduction in the expression or function of APM components, in relation to HPV infection, and also down-regulation of ERAAP, have been linked to many cervical carcinoma cases (Georgopoulos et al., 2000; Mehta et al., 2007; Mehta et al., 2009; Seliger et al., 2006). As cervical carcinoma is associated with HPV, polymorphisms within ERAAP may cause incorrect HPV-specific peptide generation for MHC loading. Unlike AS, it is possible polymorphisms within ERAAP reduce the ability to generate peptides and therefore no HPV specific epitopes are presented. This would result in an inability to eliminate HPV infected cells by $CD8^+$ CTL responses. ERAAP activity is also important in hypertension and angiogenesis through its ability to trim bioactive peptide hormones. It is possible that the presence of SNPs within ERAAP may cause a decrease in activity therefore reducing the conversion of angiotensin II into angiotensin III and IV, increasing blood pressure as a result. Polymorphisms within ERAAP have been shown to be associated with increased risk of hypertension, of which the K528R polymorphism has been shown to reduce ERAAP functional activity due to a decrease in peptide hormone affinity (Goto et al., 2006; Yamamoto et al., 2002). It is therefore likely that this polymorphism will have a significant effect on ERAAP activity leading to dysregulation of blood pressure and angiogenesis.

The two main functions of ERAAP discussed here are shown to be extremely relevant in the onset of these diseases. Incorrect ERAAP activity as a result of the presence of SNPs may lead to the onset or progression of these diseases and are likely to have an increased role in these diseases when in combination with other factors, such as the presence of HLA-B27 molecules.

6.8. Conclusions

This study aimed to identify the functional role of polymorphisms within ERAAP on the ability to a) generate the final peptide antigen for stable MHC I loading and b) facilitate IL-6R cleavage from the cell surface.

Here ERAAP was shown to be required for both these immune processes and polymorphisms within ERAAP affected the ability to generate the final epitope from its precursor for presentation on MHC I. Specifically, individual SNPs showed less reduction in activity compared to multiple SNP combinations, suggesting a cumulative effect. Two SNPs, K528R and R725Q consistently resulted in a loss of function either individually or in combination with other SNPs, suggesting K528 and R725 are important regions within the protein. When considering the positioning of these amino acids within the protein they were shown to reside at the domain junction between domain II and III (K528R) and within the regulatory site on the surface of the C-terminal cavity (R725Q), suggesting these regions are essential for processing activity. In addition, two novel hERAAP variants were identified, revealing the presence of a naturally occurring mutant allele (containing six SNPs) which was further reinforced by the presence of this hERAAP variant within both AS cases and normal control populations. The second hERAAP variant, a truncated splice variant was not observed in AS cases or controls, and the functional relevance of this variant remains unknown. Assessment of processing of mutated ERAAP revealed three functional phenotypes; normal, hypoactive and hyperactive trimming activity. Distinction between normal and dysfunctional (hypoactive and hyperactive) ERAAP molecules was apparent from T cell activation assays. To distinguish between hypo and hyperactive ERAAP I used RP-HPLC. This revealed definite differences between hypo and hyperactive alleles, however if an ERAAP molecule with a hyperactive activity was observed, future experiments using RP-HPLC and mass spectrometry would be required to determine the level of over-trimming activity and to confirm the presence of further truncated peptides.

Within this study, a number of ERAAP alleles from AS patients and a control cohort were identified and revealed that the majority of AS ERAAP alleles and all the haplotypes have a defective trimming phenotype, consistent with a reduction in the generation of final optimal peptides. A high frequency of alleles identified contained more than one SNP, consistent with the findings that multiple SNPs have a cumulative effect. This has strong implications for the peptide repertoire presented at the cell surface. As a result, these defective ERAAP alleles may affect the generation of

optimal peptides for stable loading onto HLA-B27 and exit to the cell surface. If sub-optimal peptides are loaded an increase in the formation of cell surface homodimers would be observed; these are a target for KIR on NK cells. Therefore further investigation into the changes in the expression of cell surface homodimers in the presence of different alleles or haplotypes will provide an insight into the role of these defective alleles/haplotypes on the peptide generation and stabilisation of HLA-B27. If observed, it will be important to assess whether this increase in HLA-B27 homodimers alters the ability to engage/activate NK cells. The reduction in optimal peptide generation by defective ERAAP alleles may also result in an increase in misfolding and aggregation of HLA-B27 within the ER having implications for the induction of ER stress. The ability of ERAAP haplotypes to increase ER stress can be determined through detection of UPR activation; identifying the role of these ERAAP in HLA-B27 stabilisation. Further to the altered ability of mutated hERAAP to process peptides, two SNPs, M349V and Q730E demonstrated altered substrate specificity. This has important consequences on the specificity of alleles/haplotypes identified within AS patients and their ability to generate final peptides. This should be further investigated by using altered substrates with different N-terminal extensions to determine the level of substrate specificity.

In conclusion, a number of polymorphic hERAAP variants have been identified in this study that reduce the ability to trim N-terminally extended peptides, a process essential for antigen presentation. This has important implications for the role of these SNPs within disease and should be further investigated to elucidate the exact mechanism by which these defective alleles result in disease.

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