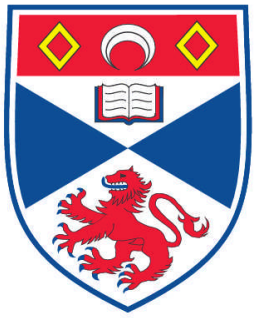
THE ROLE OF HLA-B27 IN INFLAMMATORY ARTHRITIS

Sarah Janice Lynch

A Thesis Submitted for the Degree of PhD
at the
University of St. Andrews



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The Role of HLA-B27 in Inflammatory Arthritis

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Thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

University of St Andrews

School of Medicine

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Abstract

The MHC class I allele, HLA-B27, is strongly associated with a group of inflammatory arthritic conditions collectively known as spondyloarthropathies (SpA). Ankylosing spondylitis (AS) shows the strongest association with 90-95 % of patients being HLA-B27 positive. The relationship between HLA-B27 and SpA has been known for over 30 years, however despite ongoing research, the reason for this association has not yet been elucidated. In more recent years, research has focused on intrinsic properties of the HLA-B27 allele, in particular its propensity to misfold, forming homodimers. It has been proposed that these homodimers could be associated with the disease process through the activation of an ER stress response known as the unfolded protein response (UPR), or through aberrant recognition at the cell surface.

We have investigated whether the expression of HLA-B27 is associated with the activation of the UPR. We have studied the expression of BiP, and the cleavage of XBP1 and ATF6 using stable and transiently expressing cell lines. We have also investigated the formation of non-B27 homodimers using a human cell line stably expressing HLA-B8, and finally we have studied the expression of homodimers in exosomes, small immunomodulatory vesicles released from numerous cell types. The results presented here lead us to conclude that *in vitro* studies of the UPR are complicated, prone to a number of technical issues, and may therefore not be appropriate for gaining information that would be of significant use when comparing to the real disease scenario. Our data suggest that non-B27 dimers may be strongly influenced by both the overexpression of MHC class I heavy chains and also the redox environment within the cell.

We have isolated a novel fully folded, β_2m-associated, MHC class I homodimer in exosomes and have detected a novel HLA-A and HLA-B mixed heavy chain dimer. Our results suggest that these dimers form through interactions between the cysteine residues in the cytoplasmic tail and that these dimers form in exosomes because they contain lower levels of the important antioxidant glutathione when compared to whole cells. Together, these results define a new MHC class I structure present on exosomes at

significant levels, which could potentially influence immune recognition by both antigen-specific T cell receptors and NK family receptors. The data also poses questions about whether these novel structures, when they involve HLA-B27, could influence the pathogenesis of spondyloarthropathies.

Abbreviations

ABC	ATP-binding cassette
AICD	Activation-induced cell death
AIDS	Acquired Immune Deficiency Syndrome
APCs	Antigen presenting cells
APS	Ammonium persulfate
ARTS1/ERAP1	Aminopeptidase reugulator of TNFR1 shedding/endoplasmic reticulum aminopeptidase 1
AS	Ankylosing spondylitis
ASK1	Apoptosis signal-regulating kinase 1
ATCC	American type culture collection
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
β_2m	β-2-microglobulin
BCR	B cell receptor
BiP	Immunological binding protein
BMDCs	Bone marrow-derived dendritic cells
BSA	Bovine serum albumin
CHOP	CCAAT/enhancer-binding proteins (C/EBP) homologous protein
CIA	Collagen-induced arthritis
CLIP	Class II-associated invariant chain peptide
CNX	Calnexin
CRT	Calreticulin
CYP2D6	Cytochrome P450 gene debrisoquine 4-hydroxylase
Cys	Cysteine
DAPI	4’6-diamidino-2-phenylindole
DCs	Dendritic cells
DEL-1	Developmental endothelial locus-1
DMARDs	Disease modifying anti-rheumatic drugs
DMEM	Dulbecco’s Modified Eagle’s Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphates
DRM	Detergent resistant membranes
DTNB	5,5’-dithiobis(2-nitrobenzoic acid)
DTT	Dithiothreitol
EBV	Epstein barr virus
ECACC	European collection of cell cultures
ECL	Enhanced chemi-luminescence
EDTA	Ethylene diamine tetraacetic acid
eIF2α	Eukaryotic translation initiation factor-2α
Endo H	Endoglycosidase H
ER	Endoplasmic reticulum
ERAD	ER-associated degradation

ERAP	ER-associated aminopeptidase
ERAP	ER-luminal aminopeptidase
ESCRT	Endosomal sorting complex required for transport
FBS	Foetal Bovine Serum
FDCs	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
GAA	Glacial acetic acid
GADD34	Growth arrest and DNA damage-inducible gene 34
GC	Germinal centre
GILT	γ-interferon inducible lysosomal thio reductase
GM-CSF	Granulocyte macrophage colony-stimulating factor
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidised)
HC	Heavy chain
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horse radish peroxidase
IDDM	Insulin-dependent diabetes mellitus
IEF	Isoelectric focusing
IFN	Interferon
Ig	Immunoglobulin
Ii	Invariant chain
IL	Interleukin
IL-1RN	Interleukin-1 receptor N
IL-23R	Interleukin 23 receptor
ILT	Immunoglobulin-like transcript
IMDM	Iscove's Modified Dulbecco's Medium
IP	Immunoprecipitation
IRE1	Inositol-requiring enzyme-1
JNK	c-jun N-terminal kinase
KIR	Killer immunoglobulin-like receptor
LB	Luria broth
LILR	Leukocyte immunoglobulin-like receptor
LPS	Lipopolysaccharide
MBC	Methyl-β-cyclodextrin
MHC	Major histocompatibility complex
MIC	MHC class I chain related proteins
MIIC	MHC class II compartment
MVB	Multivesicular bodies
NADPH	Nicotinimide adenine dinucleotide phosphate-oxidase
NBD	Nucleotide binding domain
NEM	N-ethylmaleimide
NF-κB	Nuclear factor- κB
NK cells	Natural killer cells
NP40	Nonidet P-40
NRSB	Non-reducing sample buffer

NSAIDs	Non-steroidal anti-inflammatory drugs
NZF	Np14 zinc finger
PAGE	Polyacrylamide gel electrophoresis
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween 20
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PERK	PKR-like ER kinase
PFN	Phosphate buffered saline + foetal bovine serum + sodium azide
PHA	Phytohaemaglutinin
PI(3)P	Phosphatidylinositol-3-phosphate
PLC	Peptide loading complex
PMA	Phorbol myristate acetate
PMSF	Phenylmethylsulfonyl fluoride
PrPc	Cellular prion protein
PrPscr	Prion protein scrapie
PRR	Pathogen recognition receptors
pVIPR	Vasoactive peptide type 1 receptor peptide
RAMP4	Ribosome-associated membrane protein 4
ReA	Reactive arthritis
RNA	Ribonucleic acid
RPMI	RPMI (Roswell Park Memorial Institute)-1640
RSB	Reducing sample buffer
RT-PCR	Reverse transcriptase-polymerase chain reaction
S1P	Site-1-protease
S2P	Site-2-protease
SDS	Sodium dodecyl sulfate
SpA	Spondyloarthropathies
TAE	Tris-acetate-EDTA buffer
TAP	Transporter associated with antigen processing
TBE	Tris-borate-EDTA
TCR	T cell receptor
TEMED	Tetramethylethylenediamine
TGF	Transforming growth factor
TNB	5-thio-2-nitrobenzoic acid
TNF	Tumour necrosis factor
TPN	Tapasin
TRAF2	TNF receptor-associated factor 2
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
UIM	Ubiquitin-interacting motif
UPR	Unfolded protein response
UV	Ultraviolet
XBP1	X-box binding protein 1

Amino Acids

AMINO ACID	THREE-LETTER CODE	ONE-LETTER CODE
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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Chapter I: Introduction

The Human Immune System – An Overview

The human immune system is a complex arrangement of cells, tissues and organs that function together to protect the body from harm. A fully functioning immune system can protect against numerous types of potential pathogens including viruses, bacteria, parasites and fungi, and it can even destroy cancer cells as they develop. The complexities of such a system are often explained by dividing a system that normally functions as a whole entity into separate sections, or in the case of the immune system, into lines of defence.

The first line of defence, the non-specific or innate immune response, occurs immediately or within a few hours after the detection of an infection. The cells involved are designed not to detect a single specific antigen but, instead, to detect conserved structures present on many microorganisms.

The second line of defence, the specific or adaptive immune response, can take several days to develop. It is a response to a specific antigen that can result in long term protection, in addition to enhanced detection and removal if the same antigen is encountered again. Adaptive immunity is further sub-divided into two sections – humoral immunity and cell-mediated immunity.

Humoral immunity involves the production of antibody and is mediated by B-lymphocytes, or B cells. Antibodies circulate as soluble proteins in the blood plasma and the lymph. Humoral immunity is most effective against bacteria, bacterial toxins, and viruses prior to these agents entering cells, and for this reason the majority of vaccines aim to generate effective antibody responses.

Cell-mediated immunity involves mostly actions performed by T-lymphocytes, or T cells. It is initiated by the interaction of T cell receptors (TCRs), present on T-lymphocytes, with antigenic peptides presented by MHC molecules on the surface of infected cells. This results in the activation of cells from both the innate and adaptive immune systems that act together to clear the infection or destroy the infected cells. Cell mediated immunity allows the immune system to recognise virally infected cells, cells with intracellular bacteria and cancer cells that display tumour antigens.

Some lymphocytes that encounter antigen develop into memory cells that continue to circulate around the body after the initial infection has been cleared. The development of immunological memory is one of the most important properties of the immune system. The more immediate proliferation and differentiation of antigen-specific memory cells into effector cells allow a secondary response to the same antigen to be more rapid and more effective than that to a primary infection.

Ultimately, all aspects of the immune system function together towards the same goal of preventing infection within the body and clearing it as quickly as possible when an

infection does occur. When the immune system is compromised opportunistic diseases that would normally be easily cleared from the body can take hold. The dramatic effects of a compromised immune system can be seen in such diseases as HIV and AIDS. The downside to the evolution of such a powerful immune system is the risk of it failing to maintain the distinction between self and non-self, in which instance autoimmunity may develop.

Cells of the Immune System

The cellular component of the immune system develops from multipotent heamatopoietic stem cells. These cells are the pre-cursors for two cell lineages involved in the immune response: the myeloid lineage and lymphoid lineage.

The Myeloid Lineage

The myeloid lineage originates from a common myeloid progenitor. This progenitor differentiates to form erythrocytes, megakaryocytes or polymorphonuclear leukocytes of which there are two main types; granulocytes and monocytes. Monocytes can further differentiate to form macrophages or dendritic cells. Mast cells also develop from a common myeloid progenitor but these cells complete their maturation in tissues.

All granulocytes have multi-lobed nuclei and contain cytoplasmic granules. There are three types of granulocyte; basophils, neutrophils and eosinophils. Basophils are the smallest circulating granulocyte, they store inflammatory mediators in their cytoplasmic granules and de-granulation can be induced through physical damage, or chemical toxins and cationic proteins released from eosinophils and neutrophils.

Eosinophils are attracted to cells coated with complement, where they release major basic protein, cationic proteins, perforins and oxygen metabolites mediating inflammation. Neutrophils, the most abundant leukocyte in the blood, migrate to extravascular sites of inflammation through a process of rolling, adhesion and diapedesis. They are primarily responsible for the phagocytosis of opsonised particles. Monocytes and macrophages also have important roles in phagocytosis. Monocytes are found circulating within the blood, but chemotactic stimulation results in the migration of monocytes into the tissue where they undergo further differentiation to become multifunctional macrophages.

Mast cells are large cells found in connective tissue throughout the body, most abundantly in the submucosal tissues and the dermis, and usually do not circulate in the blood stream. They also contain specialised granules that contain mediators of inflammation, including the vasoactive amine, histamine. Antigen binding to mast cells triggers degranulation and activation, producing a local or systemic reaction.

Dendritic cells (DCs) are a key component of innate and adaptive immunity and are extremely efficient in the endocytosis of extracellular antigens. Once activated, they

migrate to local draining lymph nodes where they behave as professional antigen presenting cells. In particular DCs are efficient at activating, or priming, naïve T-lymphocytes. DCs are the most potent stimulators of T-lymphocyte responses.

The Lymphoid Lineage

The lymphoid lineage develops from a common lymphoid progenitor and leads to the production of T-lymphocytes, B-lymphocytes and Natural Killer cells.

B-lymphocytes are the effector cells of humoral immunity, they develop and mature in the bone marrow before being released into the periphery. Mature B cells express B cell receptors (BCRs) on their surface and, once activated, undergo further differentiation to form plasma cells. Plasma cells secrete antibodies that target antigens for destruction.

T-lymphocytes play a central role in the adaptive immune response both directly and indirectly. They develop in the bone marrow but migrate to the thymus to undergo maturation. Mature T-lymphocytes express T cell receptors (TCR) on their surface. These receptors recognise antigen in the context of MHC molecules, which can result in the destruction of infected cells or the activation of B-lymphocytes.

Natural killer (NK) cells develop in the bone marrow and represent approximately 10-15% of circulating lymphocytes in the blood. They are competent killing cells that express both activatory and inhibitory receptors. Whether an NK cell initiates killing of a

target cell depends on the balance of inhibitory and activatory signals that are received (reviewed in Bryceson and Long, 2008). NK cells receive inhibitory signals from surface MHC (major histocompatibility complex) molecules and therefore play an important role in the detection of 'missing-self'. NK cells have a multitude of functions in the immune system, many of which are still not fully understood.

Innate Immunity

Innate immunity is the immunity you are born with. It is the body's first level of defence and includes the physical barriers that bar the entry of potential pathogens into the body. The response is immediate or within several hours of a detected infection (reviewed in Tosi, 2005).

The innate immune response consists of cellular components and soluble factors. Cells involved in innate immunity recognise pathogen-associated molecular patterns (PAMPs) through special pattern-recognition receptors (PRR), including Toll-like receptors and Nod receptors. Stimulation of PRR by PAMPs activate downstream signaling pathways that induce cytokine production, which attracts other cells involved in innate immunity but also stimulates the activation of the adaptive immune response (reviewed in Bourhis and Werts, 2007, and Arancibia et al., 2007).

Phagocytic cells (neutrophils, monocytes, macrophages and dendritic cells) express a broad spectrum of receptors that participate in pathogen recognition. Receptor

stimulation can trigger phagocytosis or can simply bind the pathogen to the phagocyte to enhance internalization. DCs engulf pathogens and display pathogen-derived peptides in the context of MHC molecules on their cell surface. Antigen presentation by DCs forms one of the bridges that connect the innate and adaptive immune systems together. Complement proteins in the serum can enhance phagocytosis by binding to invading pathogens in a process known as opsonisation. Complement proteins are recognised by a specific set of receptors on the phagocyte stimulating internalisation. Pathogen internalisation by phagocytes is usually accompanied by pro-inflammatory signals and the activation of antimicrobial mechanisms (reviewed in Underhill and Ozinsky, 2002).

Upon stimulation, basophils, mast cells and eosinophils release molecules such as complement proteins, acute phase proteins and cytokines, which promote inflammation and can enhance phagocytosis. Natural killer cells also have an important function in the innate immune response. However, instead of recognizing pathogen directly, NK cells recognize and destroy cells that are expressing reduced levels of MHC molecules on their surface, an indication that they are infected, as described in the 'missing-self hypothesis' (reviewed in Karre, 2002).

The non-discriminating nature of innate immunity is compensated for by the adaptive immune system, which adds specificity to the immune response and enhances the mechanisms involved in innate immunity. Most cells involved in an innate immune response also have extremely important roles in adaptive immunity, and although they do

not themselves respond specifically, without them a specific response would not be possible.

Adaptive Immunity

Adaptive immunity continues to develop throughout life and consists of two major branches: humoral immunity and cell-mediated immunity.

Humoral Immunity

The main function of the humoral immune response is to destroy extracellular pathogens and prevent the spread of intracellular infections. B-lymphocytes, the effector cells of humoral immunity develop in the bone marrow before being released into the circulation.

B-lymphocyte stimulation can occur directly through antigen stimulation of the BCR but usually requires co-ordinated signalling from a subset of helper T-lymphocytes (Th), resulting in clonal expansion of the B cell. B-lymphocytes migrate to lymphoid organs where they under go BCR receptor education and selection, known as affinity maturation, in specialised regions known as germinal centres (GC). This results in the production of high-affinity antibody-secreting plasma cells and also memory cells (reviewed in Allen et al., 2007, and Tarlinton et al., 2008).

Plasma cells produce large quantities of antibodies that circulate around the body, coating infecting organisms in a process known as antibody-mediated opsonisation, targeting them for destruction through phagocytosis. Antibodies bound to the surface of pathogens can activate the complement system. Complement proteins also bind to the surface of pathogens, targeting them for degradation through phagocytosis, other complement molecules recruit phagocytic cells to the site of infection and some can directly lyse pathogens by punching holes in their membranes.

Multiple subsets of memory B-lymphocytes emerge from the GC reaction, including long-lived plasma cells that remain terminally differentiated continually producing small amounts of high affinity antibody that is released into the circulation. Other memory B-lymphocytes that are produced express a greater proliferative capacity, or have a greater propensity for plasma cell formation, if the same antigen is detected again. The formation of these memory cells mean that detection and removal of the same antigen will be much more rapid and efficient (reviewed in McHeyzer-Williams et al., 2006).

Antibodies

The specificity and diversity of antibodies is achieved through sequential gene rearrangements. Each type of antibody that is produced can be expressed as a soluble molecule or in a membrane bound form on the surface of circulating B cells where they function as the BCR.

Antibodies consist of four polypeptide chains, two heavy (H) chains and two light (L) chains. Each H chain and L chain consists of constant (C) and variable (V) regions. The variable regions determine the antigen-binding specificity and therefore diversity of antibodies, this region is called the Fab region and is generated through sequential gene rearrangements. The constant region of the H chain, termed the Fc region, determines the functional class of the antibody, of which there are five, each with a different function, denoted IgM, IgD, IgE, IgA and IgG.

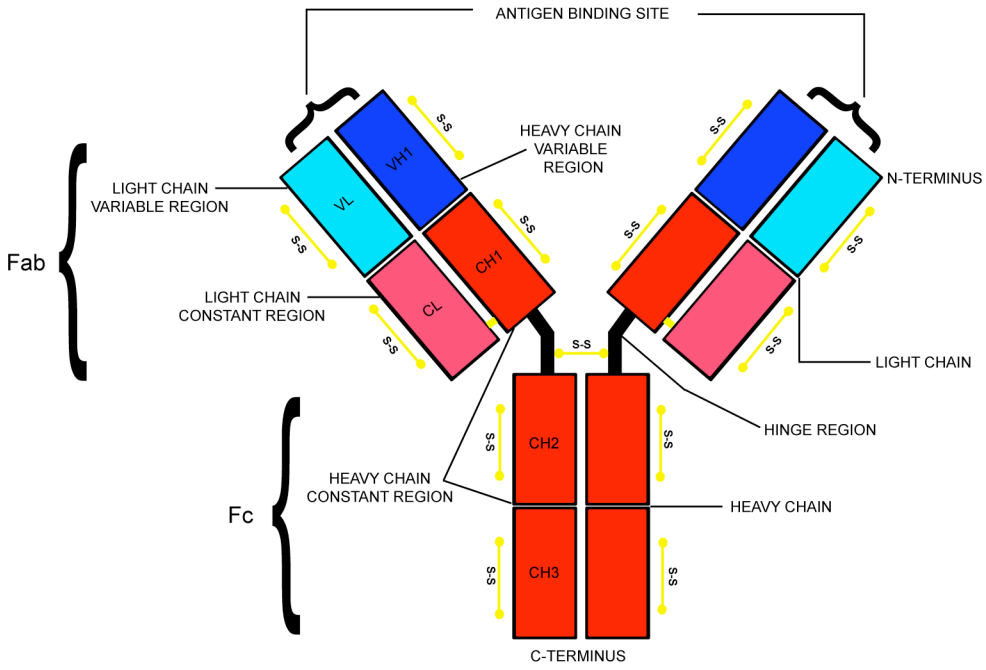


Figure 1. 1: Schematic representation of an antibody molecule. The basic structure of a mammalian antibody consists of two light chains (each ~ 25 kDa) and two heavy chains (each ~ 50 kDa), connected through disulphide bonds. Constant regions are shown in red and variable regions are shown in blue. The hinge region allows flexibility between the two Fab fragments.

Cell-Mediated Immunity

T-lymphocytes are the effector cells involved in cell-mediated immunity. T cells, like B cells, are derived from multipotent stem cells in the bone marrow but at an early stage migrate to the thymus where they undergo differentiation. Cell-mediated immunity allows the immune system to monitor both the extracellular and the intracellular environment. A sophisticated arrangement of molecular machinery allows the continual presentation of intracellular and extracellular antigens at the cell surface. These antigens are presented in the context of MHC molecules and are available for immunosurveillance by T-lymphocytes.

The TCR is responsible for the unique specificity of an individual T cell and a large number of different TCRs are generated from a series of gene rearrangements. TCRs are heterodimers, composed of either an α- and β-chain or a γ- and δ-chain. The $\alpha\beta$ T cells and $\gamma\delta$ T cells are different lineages of T cells that have different anatomical locations, specificities and functions.

The $\alpha\beta$ T cells recognise foreign antigen in the context of classical MHC molecules through the TCR. Whether a T cell functions as a helper T cell or a cytotoxic T cell is determined by the expression of the co-stimulatory receptors CD4 and CD8. $CD4^+$ T cells recognise peptide antigen bound to MHC class II molecules and function as helper T cells, recruiting, and facilitating the activation of B-lymphocytes. Expression of the CD8 co-receptor ($CD8^+$) facilitates the interaction between the TCR and peptide bound to

MHC class I molecules. $CD8^+$ T cells are cytotoxic, signalling the destruction of infected cells.

γδ T cells account for 1 -10 % of circulating T cells but can comprise a much higher proportion in some regions such as the epithelia of the small intestine. These cells do not recognise conventional ligands but instead recognise diverse non-peptide ligands (Morita et al., 1999) including lipids (reviewed in Cui et al., 2005). Some reports suggest that these T cells also recognise the MHC heavy chain related molecules MICA and MICB (Groh et al., 1998). Once stimulated γδ T cells function in a similar way to αβ T cells displaying broad cytotoxicity and secreting cytokines and chemokines.

One of the most important features of the adaptive immune response is its ability to develop memory. Memory B- and T- lymphocytes result from infections that activate the adaptive immune response. The mechanisms that lead to the generation of memory T cells have not been fully determined. The classical pathway of memory T cell differentiation suggests that naïve T cells, once stimulated develop into effector T cells, most of which eventually die, but those that survive go on to develop into memory T cells, however there is also evidence that memory T cells can develop directly from naïve T cells (reviewed in Moulton and Farber, 2006). The development of memory T cells allows a second infection by the same organism to be detected and removed much faster than in the primary response.

The MHC

The human major histocompatibility complex genomic region is located on the short arm of chromosome 6. The entire 3.6 Mb genomic sequence of the MHC was first reported in 1999 by the Human Major Histocompatability Complex Sequencing Consortium (The-MHC-Sequencing-Consortium, 1999). In 2004, the gene information for this region was reviewed and updated (Shiina et al., 2004). A total of 239 gene loci have been identified. 130 of these genes code for expressed genes, 17 for gene candidates, 4 for non-coding genes and 88 for pseudogenes.

The MHC is traditionally divided into three sub-regions, denoted class I, class II and class III. The class III region is located between the class I and class II regions and is often referred to as the central MHC.

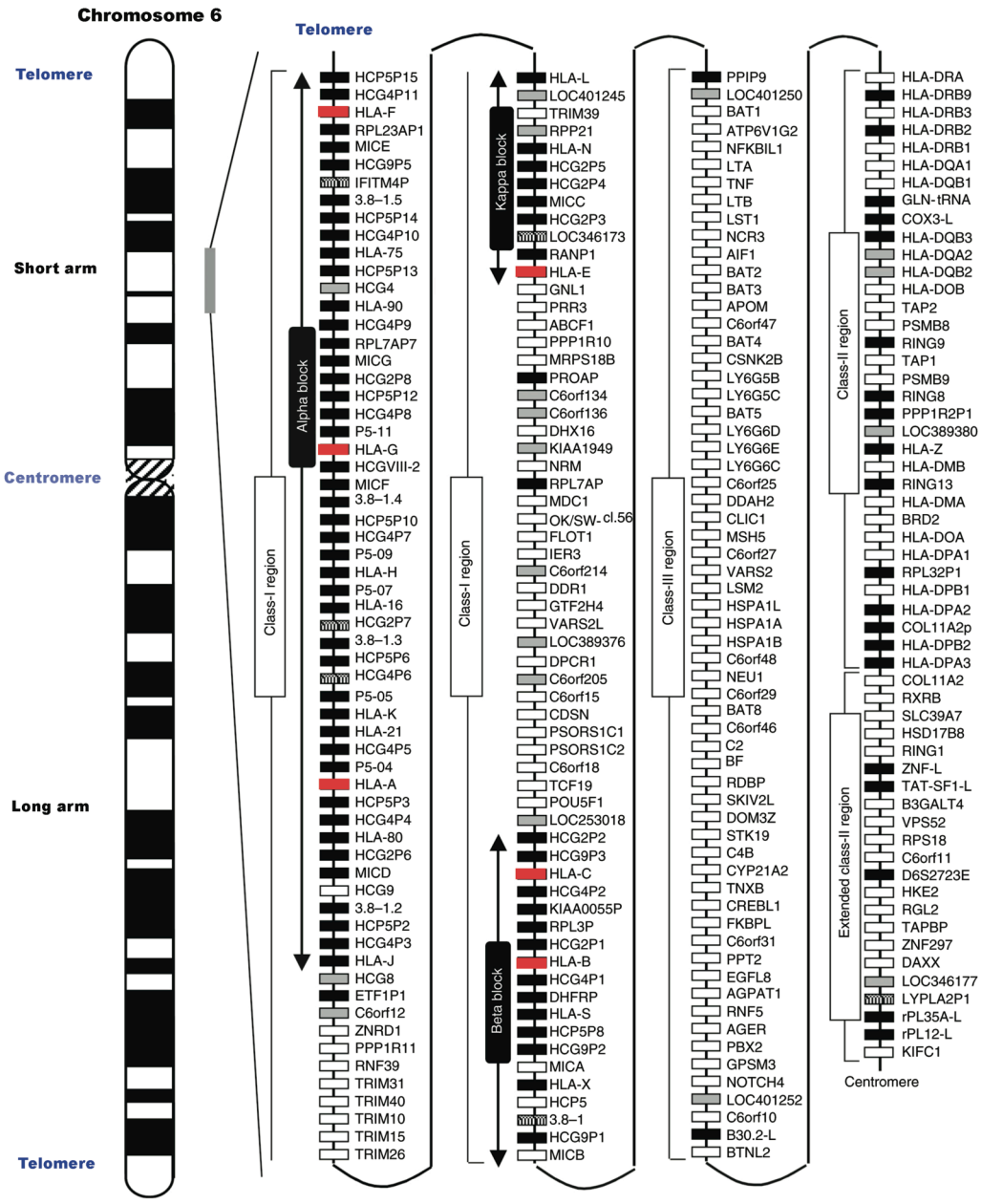


Figure 1.2: Gene map of the MHC on chromosome 6. White, grey, striped and black boxes show expressed genes, gene candidates, non-coding genes and pseudogenes, respectively. The position of the coded classical and non-classical MHC class I genes are highlighted in red. Diagram published in Tissue Antigens, Shiina et al 2004 (Shiina et al., 2004).

Class III Region

The central region of the MHC is one of the most gene dense regions of the human genome, coding for more than 60 genes in approximately 900 kb of genomic sequence. This region contains a more heterogeneous set of genes than the MHC class I and class II regions. These genes include those involved in the activation of complement cascades, inflammation and cell stress, hormonal synthesis, heat shock proteins, extracellular matrix organisation as well as members of the immunoglobulin superfamily. Other genes in the class III region encode for proteins whose functions have no direct connection to the immune system (reviewed in Yung Yu et al., 2000, and Hauptmann and Bahram, 2004).

Class II Region

The class II region spans approximately 700 kb of DNA and contains the classical MHC class II α- and β-chain genes, HLA-DP, -DQ and -DR and the non-classical HLA-DM and -DO genes. Almost all of the genes encoded in the class II region have an immunological function. Notably the genes for TAP1 and TAP2, involved in MHC class I antigen presentation, are also encoded in this region (discussed below) (Shiina et al., 2004).

Classical MHC Class II

The classical MHC class II molecules are constitutively expressed on immune cells such as B-lymphocytes, macrophages/monocytes and dendritic cells, where they present exogenous antigen to $CD4^+$ T cells. All of the class II molecules consist of non-covalently associated α- and β-chains that are both coded for in the class II region of the MHC. The α- and β-chains of all the classical MHC class II molecules have the same conformation, two extracellular domains and a membrane spanning tail region. The membrane-distal parts of each chain come together to form a peptide-binding groove that is capable of binding a wide array of peptides.

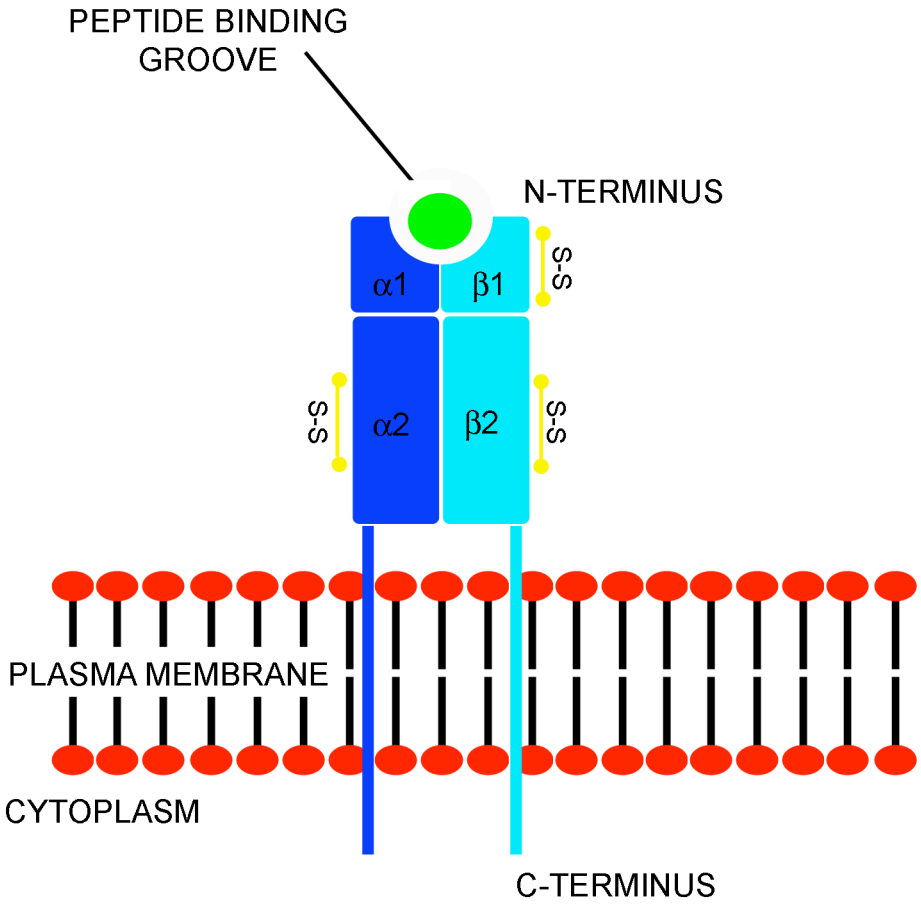


Figure 1.3: Schematic diagram of classical MHC class II molecules. MHC class II molecules consist of two non-covalently associated non-identical polypeptide chains, the α-chain and the β-chain. The most membrane distal portions of each chain, α1 and β1, come together to form the peptide-binding domain that is capable of binding a wide array of peptides. All domains are stabilised by disulphide bonds with the exception of the α1 domain.

Non-Classical MHC Class II

There are two non-classical MHC class II molecules encoded for within the MHC, HLA-DM and HLA-DO. HLA-DM is expressed in all classical MHC class II expressing cells, however it is not expressed at the cell surface, but is instead retained intracellularly within the MHC class II compartments (MIIC). The conformation of its α- and β-chains result in its peptide-binding groove being almost completely closed off. As its structure implies, HLA-DM does not have a role in antigen presentation but instead facilitates the release of CLIP (part of the invariant chain) (discussed below) from the peptide-binding groove of classical MHC class II molecules, whilst at the same time, maintaining their peptide binding site in an open and peptide-receptive confirmation (reviewed in Denzin et al., 2005, and Sadegh-Nasseri et al., 2008).

The peptide loading of classical MHC class II molecules is modified in B cells, DCs and thymic epithelial cells because of the expression of HLA-DO. It is suggested that the expression of HLA-DO inhibits the function of HLA-DM although the *in vivo* relevance of this is not yet fully understood. It is speculated that in resting cells, HLA-DO downmodulates steady-state class II antigen processing and, when a B cell or DC requires active antigen presentation, HLA-DO levels are reduced. The net effect being that in steady-state conditions presentation of self-peptides is reduced and during infection class II antigen presentation is enhanced (reviewed in Denzin et al., 2005).

Class I Region

The 1.8 Mb class I region contains the three classical MHC class I genes, HLA-A, -B and -C, and the three non-classical MHC class I genes, HLA-E, -F and -G. This region also codes for two expressed MHC class I chain related (MICA and MICB) genes that are HLA class I-like molecules and a large group of non-HLA genes.

MIC proteins, although similar to MHC class I, neither bind to β-2-microglobulin (β_2m) nor exhibit classical class I peptide binding and are not expressed on normal circulating lymphocytes. MIC proteins are transcribed in keritinocytes, endothelial cells, fibroblasts and seemingly most epithelial tissue. MIC proteins are expressed at the cell surface in response to cell stress where they can engage the activating NK-receptor, NKG2D (reviewed in Stephens, 2001).

Classical MHC Class I

Classical MHC class I molecules, referred to as MHC class Ia molecules, are expressed on almost all nucleated cells. Functional class Ia molecules are heterotrimeric complexes consisting of the class I heavy chain (coded for in the MHC) non-covalently associated with a β_2m molecule (coded for on chromosome 15) and an approximately 8 - 10 amino acid peptide. MHC class Ia molecules present antigen derived from endogenous proteins for surveillance by cytotoxic $CD8^+$ T cells (reviewed in Zinkernagel and Doherty, 1997).

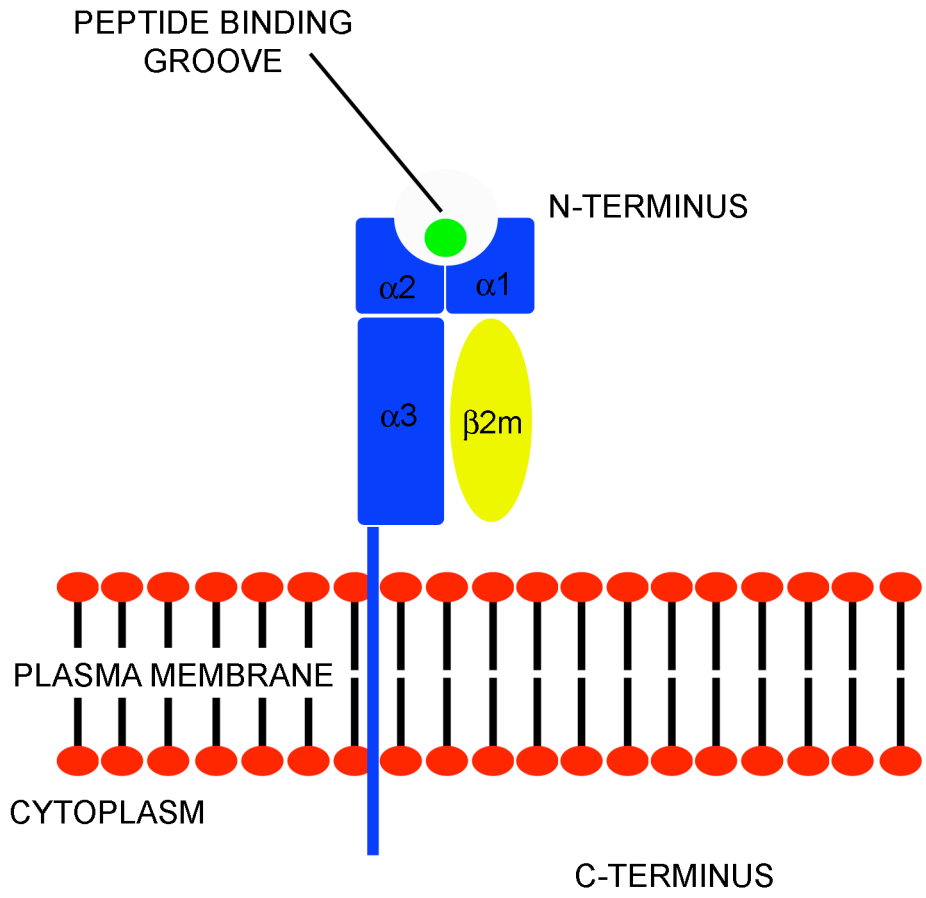


Figure 1. 4: Schematic diagram of a classical MHC class I molecule. MHC class I molecules are heterotrimers consisting of an MHC class I heavy chain, non-covalently associated with β_2m, and an approximately 8-10 amino acid peptide. The heavy chain folds to form 3 domains α1, α2, and α3. The α1 and α2 domains come together to form the peptide-binding domain, which is capable of binding a wide array of peptides. The α3 domain interacts with β_2m and also helps to support the peptide-binding region.

3-dimensional structures of MHC class I molecules have been determined by x-ray crystallography (Bjorkman et al., 1987, Garrett et al., 1989, Madden et al., 1991). Class I heavy chains consist of three extracellular domains known as α1, α2 and α3, with the α1

and α2 domains forming the peptide-binding groove. The α3 domain interacts with β_2m and also helps to support the peptide-binding region.

Figure 1. 5: **Ribbon diagram of the MHC class I crystal structure.** The α1 and α2 domains are shown in purple and the α3 domain is shown in blue. The non-covalently associated β_2m molecule is shown in grey. The conserved cysteine residues at positions 101 and 164 in the peptide-binding groove, and at 203 and 259 in the α3 domain are indicated in yellow. Also shown in this representative structure of HLA-B27 is the additional unpaired cysteine residue in the peptide groove at position 67. Diagram generated from HLA-B27 accession data, for example - PDB:1JGE, using Cn3D (www.ncbi.nlm.nih.gov).

MHC class I molecules exhibit extensive polymorphism within the HLA-A, -B and –C genes, most of which occurs within the α1 and α2 peptide-binding region. These polymorphisms alter the peptide specificity of each allele. Peptides bound to class I molecules possess conserved amino acids that form the anchor residues. The side chains of these amino acids are accommodated in six pockets (A-F) that make up the peptide-binding groove.

Non-Classical MHC class I

The non-classical MHC class I molecules are referred to as MHC class Ib molecules. These molecules share a remarkably similar structure to class Ia molecules however they only express very limited polymorphism. HLA-E, -F and -G have different functions in the immune system and are expressed in different cell types.

HLA-E is expressed by the same cells that express classical class Ia molecules. The limited polymorphisms shown by HLA-E result in a peptide-binding groove able to bind very specific peptide rather than the promiscuous nature of the class Ia binding groove (reviewed in O'Callaghan and Bell, 1998). HLA-E binds the leader peptides from the class Ia molecules resulting in the enhanced expression of HLA-E at the cell surface, here HLA-E acts as an inhibitory ligand for NK cells expressing CD94/NKG2 receptors (Braud et al., 1998b, Borrego et al., 1998, Braud et al., 1998a, Braud et al., 1997). The function of HLA-E offers another checkpoint to ensure that the MHC class Ia pathway is

intact. If classical class I molecules are not expressed, HLA-E will not be able to bind the leader peptide, resulting in lower expression of HLA-E, rendering the cell susceptible to NK lysis.

HLA-F is the least understood of the non-classical class I molecules. HLA-F associates with β_2m and has a peptide binding groove, similar to the other class I molecules, it has not yet been clearly established if this molecule can actually bind peptide (Wainwright et al., 2000, Lepin et al., 2000). HLA-F shows a restricted distribution of tissue expression, and the cellular localisation of this allele is disputed, with some reports that it is located on the cell surface and conflicting reports suggesting that the molecule remains intracellular (Wainwright et al., 2000, Lepin et al., 2000, Lee and Geraghty, 2003). There are reports showing that HLA-F is expressed in placental tissue, suggesting that HLA-F may be involved in providing a state of immuno-privilege to the growing foetus, although this is still debated (Shobu et al., 2006, Ishitani et al., 2003, Apps et al., 2008, Nagamatsu et al., 2006). The concentration of HLA-F in the golgi is enhanced compared with other class I molecules which led in a recent report to the suggestion that HLA-F may have evolved in order to monitor the golgi for infection, however this still remains to be proven (Boyle et al., 2006).

HLA-G expression is limited to a small subset of cells, principally trophoblasts of invading placental tissue (McMaster et al., 1995), where it is believed to have an important role in providing a state of immunoprivilege to the growing foetus (reviewed in Hunt et al., 2006). HLA-G transcripts are alternatively spliced, producing both

membrane-bound and soluble HLA-G proteins (Moreau et al., 2002). Soluble HLA-G molecules bind to the CD8 co-receptor resulting in the apoptosis of NK cells and T cells (Contini et al., 2003), whilst membrane-bound HLA-G has immunosuppressive effects through binding to the inhibitory receptors LILRB1/ILT-2, LILRB2/ILT-4 and KIR2DL4 on NK cells, T cells and APCs (Colonna et al., 1997, Colonna et al., 1998, Rajagopalan and Long, 1999). HLA-G is unusual amongst class I molecules for its ability to form β_2m-associated homodimers (Boyson et al., 2002, Gonen-Gross et al., 2003). Disulfide-linked HLA-G homodimers make up a significant proportion of the total HLA-G expressed by trophoblast cells (Apps et al., 2007) and it has been shown that dimeric HLA-G molecules have a higher affinity than monomers for LILRB1 and LILRB2 (Shiroishi et al., 2006, Gonen-Gross et al., 2003, Apps et al., 2007).

Antigen Processing and Presentation

Peptide binding is required to complete the folding of classical, and some non-classical, class I and class II molecules. This process is very specific and ensures only proteins of the right length and origins are able to bind to the MHC molecules and therefore be presented at the cell surface. Usually, MHC class I molecules present endogenous antigen and MHC class II molecules present exogenous antigen. However, in dendritic cells MHC class I molecules can also present exogenous antigen through a process known as cross presentation (reviewed in Rock and Shen, 2005).

The two major pathways used for antigen presentation, the MHC class I antigen processing pathway and the MHC class II antigen processing pathway, are discussed in more detail below.

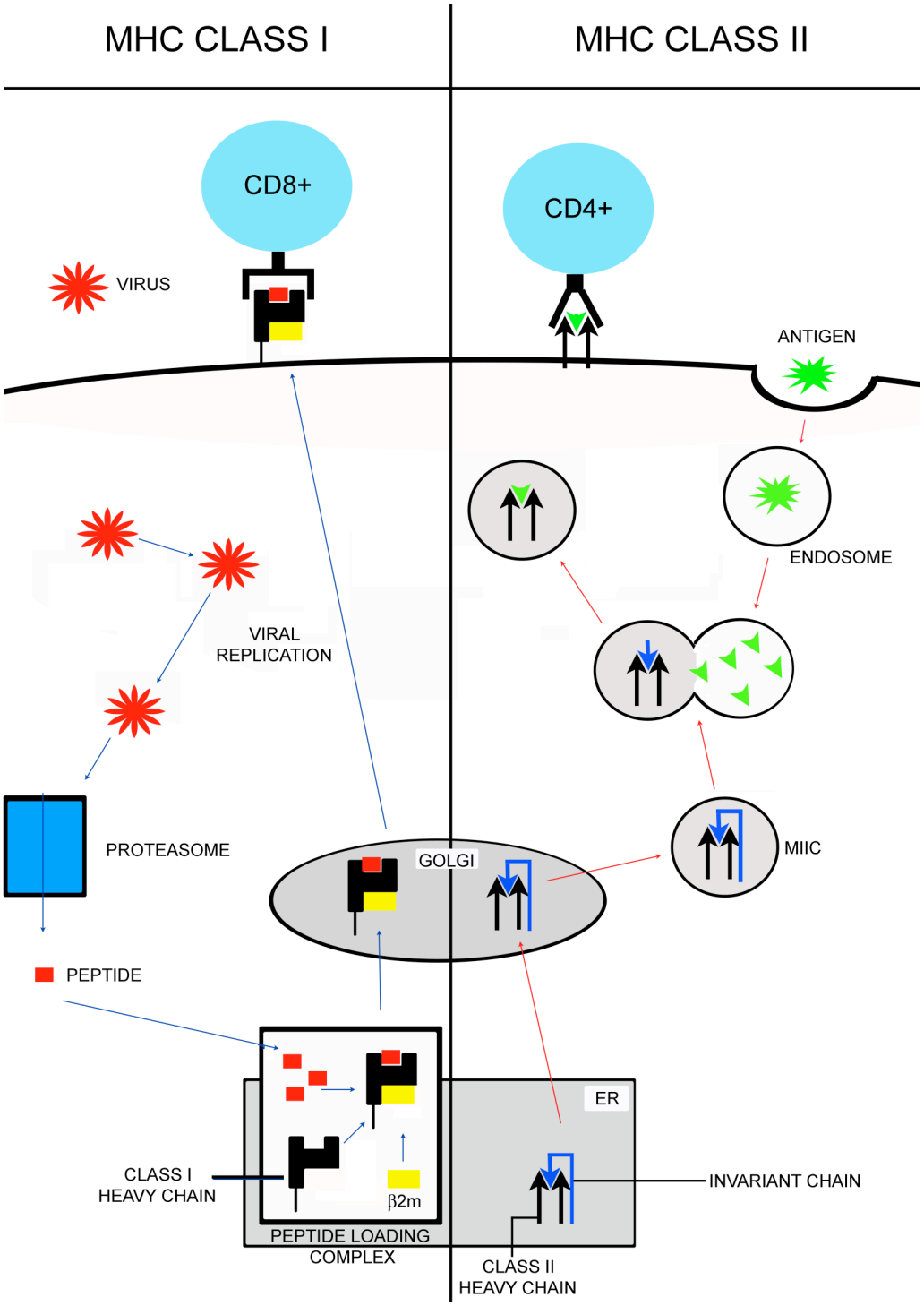


Figure 1. 6: **Schematic diagram of the classical MHC class I and class II antigen processing and presentation pathways.** MHC class I molecules assemble in the ER with the help of the peptide loading complex. MHC class I molecules present endogenous peptide antigen at the cell surface for surveillance by $CD8^+$ T lymphocytes. MHC class II molecules present exogenous antigen at the cell surface for surveillance by $CD4^+$ T cells.

MHC Class II Antigen Processing and Presentation

MHC class II α- and β-chains fold in the ER and associate with the invariant chain (Ii). A region of the Ii chain, known as CLIP (class II associated Ii peptide), binds to the MHC class II peptide binding domain, stabilising the class II molecule and preventing peptides in the ER from binding (Anderson and Miller, 1992, Romagnoli and Germain, 1994, Roche and Cresswell, 1990). The Ii chain also contains an endosomal targeting motif that directs the α- and β-chain/Ii complex to the endosomal pathway (Pieters et al., 1993).

The regions of the endosomal/lysosomal pathway where MHC class II molecules accumulate are generally referred to as MHC class II compartments (MIIC). Different morphologies of MIIC have been reported and it is suggested that these reflect different maturation stages in the MHC class II folding pathway (Kleijmeer et al., 1996, Kleijmeer et al., 1997, reviewed in Stern et al., 2006).

MHC class II molecules bind to, and present at the cell surface, peptides derived from exogenous antigens. Exogenous antigen may be ingested through phagocytosis, receptor-mediated endocytosis or macropinocytosis (reviewed in Vyas et al., 2008, and Watts, 1997). Professional APCs use phagocytosis as an important mechanism for the uptake of antigen. Internalised phagosomes eventually fuse with the endosomal/lysosomal compartment (Desjardins et al., 1994) delivering peptide antigen to where MHC class II molecules are located.

In the MIIC the Ii chain is degraded by endosomal/lysosomal proteases known as cathepsins, until only the CLIP region remains bound to the peptide-binding groove (Riese et al., 1996). The non-classical MHC class II molecule, HLA-DM, functions as a unique chaperone, catalysing the dissociation of CLIP, stabilising peptide-empty class II molecules and facilitating peptide exchange, enabling high-affinity peptide/class II complexes to form (Denzin and Cresswell, 1995, Denzin et al., 1996).

The final step in antigen processing and presentation is the delivery of the fully-folded peptide-bound MHC class II molecule to the cell surface, a process that has not been fully defined. It has been demonstrated that the late MIIC form tubular structures that are directed towards the plasma membrane which can deliver the class II molecules to the cell surface although the mechanism behind this process is not yet fully understood (Vyas et al., 2007, Boes et al., 2003). Once at the cell surface, peptide loaded MHC class II molecules are recognised by $CD4^+$ T cells.

MHC Class I Antigen Processing and Presentation

Endogenous proteins are constantly degraded within the cytosol by the proteasome. Most of the peptides that are generated are destroyed shortly after their production but a small fraction is further processed and is subsequently transported into the ER lumen by the transporter associated with antigen processing (TAP) (Stoltze et al., 2000, Reits et al., 2003, Reits et al., 2004).

TAP is a heterodimeric transporter, consisting of TAP1 and TAP2, which is part of the ATP-binding cassette (ABC) transporter family. ABC transporters have a four-domain structure consisting of two hydrophobic transmembrane domains and two hydrophilic nucleotide binding domains (NBD) (reviewed in Higgins, 1992). The transmembrane domain of the two subunits of TAP form the translocation pore within the ER membrane (Vos et al., 2000, Koch et al., 2004), and the cytosolic NBD region energisers peptide translocation across the membrane by ATP hydrolysis (reviewed in Abele and Tampe, 2004). The N-terminal domains have been shown to bind to tapasin, an important component of the peptide loading complex (discussed below) (Koch et al., 2004). TAP most efficiently transports peptides of 8 – 10 amino acids in length into the ER lumen, but can transport longer proteins up to 40 amino acids in length (Androlewicz and Cresswell, 1994, Koopmann et al., 1996). Within the ER, peptides can be further trimmed by the ER-luminal aminopeptidase 1 and 2 (ERAP1/2), to produce peptides that bind efficiently to MHC class I molecules (Saric et al., 2002, Saveanu et al., 2005b, Serwold et al., 2002).

Peptides transported into the ER are loaded onto peptide receptive MHC class I molecules through the help of a macromolecular complex known as the peptide-loading complex (PLC). This complex consists of TAP, tapasin (TPN), calreticulin (CRT), ERp57, protein disulfide isomerase (PDI) and MHC class I molecules (Hughes and Cresswell, 1998, Morrice and Powis, 1998, Degen and Williams, 1991, Nossner and Parham, 1995, Sadasivan et al., 1996, Ortmann et al., 1997, Park et al., 2006).

Nascent MHC class I heavy chains are targeted to the ER where they rapidly associate with the chaperone calnexin (CNX) (Sugita and Brenner, 1994, Nossner and Parham, 1995). However, the role played by CNX is not essential for class I folding as cell surface expression still occurs in calnexin-deficient cell lines (Scott and Dawson, 1995). Subsequent binding of the MHC class I HC to β_2m disfavours binding to CNX and the HC/β_2m complex binds instead to the related chaperone CRT.

TPN stabilises the class I molecule and bridges the source of the peptides (TAP) to the peptide-receptive class I heavy chain (Sadasivan et al., 1996, Ortmann et al., 1997, Grandea et al., 1997). The N-terminal 50 residues of TPN have been shown to mediate the interaction between TPN and MHC class I molecules whereas the C terminus seems necessary for binding to TAP (Bangia et al., 1999, Lehner et al., 1998). Tapasin also appears to have a role in increasing peptide supply into the ER (Lehner et al., 1998, Li et al., 2000). Work carried out on the TPN deficient cell line 721.220, and TPN knock-out mice, have confirmed the importance of TPN for correct MHC class I assembly (Greenwood et al., 1994, Peh et al., 1998, Garbi et al., 2000, Grandea et al., 2000).

ERp57 and PDI are thiol oxidoreductases involved in assuring correct disulfide bond formation. Disulphide-bonded intermediates between MHC class I heavy chain and both ERp57 and PDI have been reported (Lindquist et al., 2001, Park et al., 2006, Antoniou et al., 2002a). ERp57 can bind both CNX and CRT (Oliver et al., 1999), and we have recently demonstrated a direct interaction between ERp57 and MHC class I molecules (Santos et al., 2007). ERp57 predominantly associates with cysteine residues in the

peptide binding domain, indicating that ERp57 has direct access to the peptide-binding groove during class I assembly (Antoniou et al., 2007). Recent data suggest that PDI can control the oxidation of the peptide binding groove disulfide bond between Cys^{101} and Cys^{164} of MHC class I molecules, thereby also regulating peptide optimisation (Park et al., 2006).

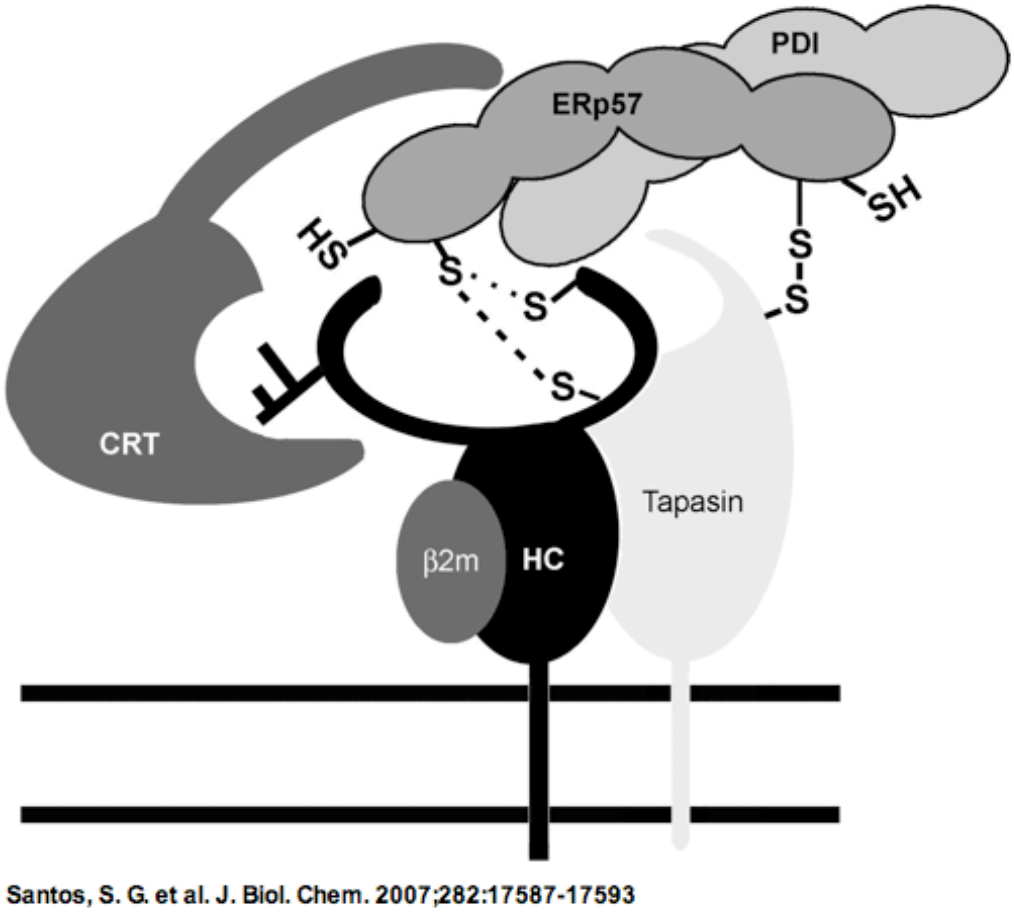


Figure 1. 7: Schematic diagram of the peptide loading complex. The peptide loading complex facilitates the binding of peptides to MHC class I molecules. The TAP transporter (not indicated on the diagram) also forms an important part of the PLC and is linked to the other components through an interaction with tapasin.

The PLC functions together to ensure that the nascent class I heavy chains are folded correctly and supplied with peptide. Fully folded class I molecules associated with high affinity peptide exit the ER and translocate through the golgi apparatus to the cell surface, where they can then be recognised by $CD8^+$ T cells.

Tolerance to Self

The ability of the immune system to detect foreign antigens but to ignore self-antigens is central to retaining a healthy body. Tolerance of both B- and T-lymphocytes is maintained by several mechanisms.

B Cell Tolerance

During development BCR diversity is generated by random rearrangement of immunoglobulin genes, a process that inevitably results in the genesis of receptors that recognise self-antigens. A rescue mechanism, known as receptor editing, allows the specificity of a BCR that recognises self-antigen to be changed (Gay et al., 1993, Tiegs et al., 1993). However if this process is unsuccessful, these lymphocytes are deleted before they can develop into fully immunocompetent cells in a process known as clonal deletion (Nemazee and Burki, 1989).

Some self-reactive B cells may escape clonal deletion in the bone marrow and instead be released into the circulation. Self-reactive B cells released into the periphery are silenced through a process known as anergy (reviewed in Cambier et al., 2007). B cell tolerance in the periphery is also maintained by helper T cells. Most B cells require additional signals from helper T cells in order to become activated, if helper T cells are tolerant to self-antigens the auto-reactive B cell will not differentiate to form an antibody secreting plasma cell.

T Cell Tolerance

T cell tolerance is mainly achieved in the thymus during T cell development through positive and negative selection. Some auto-reactive T cells can escape this process and enter the periphery further mechanisms are active here to ensure tolerance to self is maintained

Central Tolerance through Positive and Negative Selection

During T cell development, CD4 and CD8 receptors are temporarily expressed at the same time. These cells, known as double positive thymocytes, reside in the cortex of the thymus. At this time, thymocytes with TCRs that engage either MHC class I or MHC class II molecules with low affinity receive a survival signal, this is known as positive selection. Whether the thymocyte will develop to become only $CD4^+$ or $CD8^+$ is

determined by which MHC molecule the TCR was able to engage. Those cells neglected by the MHC molecules will undergo apoptosis (reviewed in Takahama, 2006, Robey and Fowlkes, 1994, and Jameson et al., 1995). Thymocytes that bind peptide/MHC complexes with high affinity are deleted, removing potentially self-reactive cells in a process known as negative selection (reviewed in Siggs et al., 2006). The thymocytes that survive both positive and negative selection, complete maturation and are released to the periphery. Further regulatory mechanisms function in the periphery to control autoreactive T cells.

Peripheral Tolerance

Peripheral tolerance is maintained in several ways. The primary regulators of peripheral tolerance are regulatory T cells (Tregs). Tregs are a sub-population of $CD4^+$ T cells that express high levels of IL-10 and TGFβ. These cytokines suppress T cell function in order to prevent chronic activation and auto-reactivity in the periphery (reviewed in Vignali et al., 2008).

T cell inactivation, or anergy, can also occur. Those T cells that engage an MHC/peptide complex in the absence of co-stimulatory receptors are induced into an anergic state. Similarly, interaction with a tolerogenic DC (usually an immature DC expressing low levels of co-stimulatory molecules) will induce anergy in the T cell. During an infection the levels of co-stimulatory molecules present on APCs are increased, therefore T cell

anergy prevents the activation of T cells in the absence of infection (reviewed in Macian et al., 2004).

Following an immune response activated T cells must be removed in order to maintain self-tolerance and prevent chronic inflammation. Some activated T-cells undergo autonomous cell death whilst others receive signals to die through repeated stimulation of their TCR. This second pathway, known as activation-induced cell death (AICD) may be responsible for the deletion of some auto-reactive T cells in the periphery (reviewed in Zhang et al., 2004, Brenner et al., 2008, and Green et al., 2003).

Autoimmunity

When an organism becomes unable to distinguish self-antigens from those of an invading organism, an immune response will be launched in a process known as autoimmunity. Autoimmune responses are usually sustained and persistent resulting in long-term tissue destruction. Autoimmune diseases can be the result of either B cells (antibodies), as in Goodpasture's syndrome, or T cells as in insulin-dependent diabetes mellitus (IDDM).

In Goodpasture's syndrome, antibodies are produced that recognise collagen in the basement membrane of renal glomeruli. Destruction of this tissue leads to kidney dysfunction and failure, and can be rapidly fatal (reviewed in Bergs, 2005). IDDM is a chronic disease, caused by both $CD4^+$ and $CD8^+$ autoreactive T cells. These cells destroy

the insulin-producing β cells found in the pancreatic islets, resulting in an inability of the patient to control their blood glucose levels (reviewed in Knip and Siljander, 2008).

It is not known exactly what triggers an autoimmune response although both environmental and genetic factors, particularly MHC genotype, influence susceptibility.

HLA and Disease

The classical MHC class I and class II molecules have been associated with more than 100 diseases, however the molecular mechanisms for most of these disease associations remain poorly understood. Diseases associated with the HLA region can be of a wide variety of classes, including immune, neurodegenerative, metabolic and infectious.

The strongest HLA-linked disease association that is known is between the MHC class I allele HLA-B27 and a specific group of arthritic diseases known as Spondyloarthropathies.

HLA-B27 and Spondyloarthropathies

The Arthritis Research Campaign (www.arc.org.uk) estimates that up to 9 million people each year in the UK will visit their doctor because of arthritis and related musculoskeletal conditions. The most commonly occurring disorder is osteoarthritis, with around 2 million people receiving this diagnosis. Rheumatoid arthritis affects approximately 350,000 individuals, and ankylosing spondylitis affects between 100,000 to 150,000. http://www.arc.org.uk/arthinfo/patpubs/6020/6020.asp.

Ankylosing spondylitis (AS) is a major form of spondyloarthropathy (SpA), the name given to a group of arthritic diseases that are associated with HLA-B27 expression and predominantly affect the joints of the spine. Spondyloarthropathies encompasses a number of different disorders including: ankylosing spondylitis, reactive arthritis or Reiter's syndrome (ReA), psoriatic arthritis, arthritis-associated inflammatory bowel disease, enthesitis related juvenile arthritis and undifferentiated SpA.

The archetypal SpA, ankylosing spondylitis, shows the greatest association with HLA-B27, with between 90 and 95 % of patients expressing this allele. The association of HLA-B27 with AS has been known for over 35 years (Brewerton et al., 1973, Schlosstein et al., 1973) but despite ongoing research the reason for this association still remains a mystery.

Ankylosing Spondylitis

AS is a chronic systemic inflammatory disease affecting primarily the sacroiliac joint and the axial skeleton, but it can also affect the peripheral joints and entheses, as well as having extra-articular manifestations (Mansour et al., 2007). AS shows significant inherited susceptibility and affects men more than women, usually with a ratio of 2:1. Onset generally occurs in late adolescence or early adulthood, most frequently between 20 and 30 years of age.

AS patients typically present with low back pain, characterised by stiffness and pain that is worse in the morning or after prolonged periods of inactivity, which is improved with exercise, but not through rest. As the disease progresses the involvement of the cervical spine increases, usually presenting as neck pain and stiffness (Mansour et al., 2007). In severe cases the cervical bones may fuse causing the head to protrude forward, a compensatory flexion of the knee results in the classic stooped posture of the patient with advanced AS (Hoh et al., 2008).

Treatments

Non-steroidal anti-inflammatory drugs (NSAIDs) are the first line of treatment in AS, and are used to control pain and reduce inflammation. Their full effect is rapid and can usually be observed 42-78 hours after treatment. However, they are also associated with

significant side effects through prolonged use, including effects on the cardiovascular system, gastrointestinal tract and renal function. A review discussing the potential benefits and risks of NSAIDs in AS treatment has recently been published (Song et al., 2008).

Whether NSAIDs function as 'disease modifying drugs', having long-term effects on disease progression has not yet been fully determined. A study in 2005 implicated the continuous use of NSAIDs (compared with on demand use) in the reduction of radiographic progression of disease (Wanders et al., 2005) although more data is needed before a conclusive answer to this question can be given.

Patients can also be prescribed disease-modifying anti-rheumatic drugs (DMARDs) such as methotrexate and sulfsalazine. However the efficacy of these drugs is debatable. Sulfsalazine is the most widely studied DMARD for the treatment of AS and large randomised control trials have shown that the effects of this drug is limited, reducing inflammation in the periphery in some patients but having limited effects on spinal inflammation (Clegg et al., 1996, Kirwan et al., 1993).

Some antimalarials, such as hydroxychloroquine, can be used in conjunction with DMARDs to treat spondyloarthropathies and it has been suggested that the use of this combination of drugs is more effective than DMARD monotherapy (Calguneri et al., 2004). Hydroxychloroquine is a lysomotropic agent, and functions primarily by entering the lysosomal compartment and raising the pH. How exactly this treatment affects the

disease process in spondyloarthritis has not been determined, although its effects on antigen processing, for MHC class II presentation especially, have been exploited by immunologists for many years.

More recently, TNFα inhibitors have been shown to offer good improvement to AS patients and three TNFα inhibitors are currently available for clinical use, these are Infliximab®, Etanercept® and Adalimumab®. These agents block the effects of the immune system by binding to TNFα and preventing its downstream effects, thus reducing inflammation. Since cytokine blockade therapies are relatively recent introductions to the AS treatment regime, there must remain some concerns about the effects of their long-term use, both in terms of patient compliance and tolerance, and also relating to the immune-suppressive action of such anti-TNF agents, which may include increased rates of serious infections, and also, potentially, of malignancies (Dixon et al., 2006, Listing et al., 2005, Bongartz et al., 2006).

Surgery can be performed under some circumstances to restore mobility to some damaged joints. Prolonged and severe disease results in the ossification of the vertebrae resulting in a condition known as 'bamboo spine'. Fusion of the cervical vertebrae pushes the head forward in a 'chin-on-chest' position and tends to result in a compensatory flexion of the knees. Surgical correction is available but is technically demanding and carries the serious risk of permanent neurological injury (Hoh et al., 2008).

HLA-B27

HLA-B27 is a classical MHC class I molecule expressed by approximately 1 in 10 of the general population in the UK. It is important to note that the majority of people who express HLA-B27 will not go on to develop any symptoms of SpA. However, the association of HLA-B27 with this group of diseases is one of the strongest MHC-disease associations that is known.

It is also of great importance to note that HLA-B27 functions perfectly well as a typical MHC class I molecule. Antigen presentation by HLA-B27 is extremely efficient, with HLA-B27 positive individuals showing increased clearance of hepatitis C compared with other alleles (McKiernan et al., 2004) and slower progression from HIV to AIDS (reviewed in Carrington and O'Brien, 2003).

Currently 42 subtypes of HLA-B27 have been identified, denoted HLA-B*2701-B*2743 (with the discrepancy in numbering due to HLA-B*2722 having been removed), and as with most MHC class I alleles, the differences between them tend to be in the α1 and α2 domains. It is thought that most HLA-B27 alleles evolved from the most frequent subtype, B*2705, and it is this founder allele that is most strongly associated with SpA in Caucasians. Intriguingly, alleles B*2706 and B*2709, found in Sardinia and Southeast Asia respectively, are not associated, or only very weakly associated, with disease. B*2706 differs from B*2705 by five residues (positions 74, 114, 116, 152 and 211)

whereas B*2709 differs at only one residue (position 116), potentially implicating these residues directly in the disease process.

HLA-B27 contains 4 conserved structural cysteine residues at positions 101, 164, 203 and 259, which it shares with all MHC class I alleles (Figure 1.8). These cysteine residues form intra-chain disulfide bridges early in the class I molecules folding, and are essential to the stability of MHC class I molecules (Antoniou et al., 2004). The HLA-B27 allele also possess three unpaired cysteine residues; one at the relatively rare position 67, located on an α-helix close to the peptide binding site, and two other cysteine residues at positions 308 and 325 located within the transmembrane domain and cytoplasmic tail, respectively. Importantly, none of these unpaired residues is unique to HLA-B27 and thus is unlikely to be the sole causative factor in AS. All HLA-B alleles share the cysteine at position 308, just at the border of the transmembrane and cytoplasmic domains, whilst in contrast no HLA-A alleles have a cysteine at this location and the expression of a cysteine residue at position 325 is variable amongst HLA-B alleles. The function of these cysteine residues is unknown, though we propose a new and novel function in the later part of this thesis. The cysteine at position 67 (Cys^{67}) is a feature shared by a cohort of other class I alleles including subtypes of HLA-B07, -B14, -B15, -B35, -B38, B-39, -B40, -B51, -B73 and -B95.

Figure 1. 8: Ribbon diagram of the MHC class I allele HLA-B27. The α1 and α2 domains are shown in purple and the α3 domain is shown in blue. The non-covalently associated β_2m molecule is shown in grey. The cysteine residues are indicated in yellow. Diagram generated from HLA-B27 accession data, for example - PDB:1JGE, using Cn3D (www.ncbi.nlm.nih.gov).

Detailed structural analysis of HLA-B27, and peptide elution studies, have enabled a HLA-B27 peptide-binding motif to be determined as well as probable peptide interactions with the TCR. Peptides bound to HLA-B27 are anchored at four positions, P2, P3, P7 and P9 allowing the side chains of residues at P1, P4, P5, P6 and P8 to interact with the TCR (Madden et al., 1991, Madden et al., 1992). It was also demonstrated that HLA-B27 shows an almost absolute restriction for arginine at position 2 of the bound peptides (Jardetzky et al., 1991, Madden et al., 1991, Madden et al., 1992). There are, however, some cases in which arginine is not found at position 2, and it is proposed that the differences observed for P2 residues might be specific to the origin of the cell-type used (Stodulkova et al., 2006).

A small proportion of HLA-B27 molecules react with the monoclonal antibody MARB4 (Urban et al., 1994). Although class I molecules tend to bind a nonomeric peptides, it has been shown that the HLA-B27 molecules that react with this antibody can be bound to unusually long peptides, up to 33 amino acids in length (Urban et al., 1994). However, peptides longer than the standard length of nine amino acids (although not as long as 33 amino acids) have been reported previously both for HLA-B27 and the non-disease associated allele HLA-A2. Thus the length of bound peptide is also unlikely to be a sole driver of AS disease (Parker et al., 1992, Robbins et al., 1989).

HLA-B*2705 exhibits the unusual characteristic of being partially TAP and TPN independent (Peh et al., 1998). This can lead to the binding and presentation of low affinity peptides in complexes that are less stable on the cell surface, resulting in an

increased expression of HC10-reactive free heavy-chains on tapasin deficient cell lines (Peh et al., 1998). Further investigation has shown that the non-disease associated alleles B*2706 and B*2709 are equally TPN independent with B*2706 showing the highest level of HC10 reactive material on the cell surface (Goodall et al., 2006). In normal TPN sufficient cells however, B*2705 alleles appear to utilise the TPN-dependent pathway (Peh et al., 1998) suggesting tapasin-independent folding may only occur when infection results in perturbed tapasin function (Park et al., 2004).

A key step forward in AS research occurred with the development of transgenic rats expressing HLA-B27. The expression of HLA-B27 in several transgenic rat lines leads to the development of an arthritic condition that closely resembles many of the features of AS, whilst a control transgenic expressing the non-disease associated HLA-B7 allele remains healthy, thus providing strong evidence that HLA-B27 has a direct role in disease pathogenesis (Hammer et al., 1990, Khare et al., 1995, Taurog et al., 1999).

Theories for HLA-B27 Associated Disease

Several hypotheses have been put forward to explain the association of B27 with this group of diseases. It was suggested by Edwards *et al*. that a viable hypothesis for the role of HLA-B27 in SpA must explain five observations: 1) the predilection of spondyloarthropathies for specific tissues and young adult males; 2) the strong, but not absolute, link with B27; 3) the association of peripheral arthritis with intracellular bacterial infection; 4) the genesis of similar diseases in B27-transgenic rodents; and 5)

the ability of B27 to modulate the expression of other diseases (Edwards et al., 2000). As yet, no such unifying hypothesis is available, but each hypothesis discussed below has found some support in the literature.

Arthritogenic Peptide Hypothesis and Molecular Mimicry

It would be logical to assume that the pathological role of HLA-B27 is related to its antigen presentation function. The classical 'Arthritogenic Peptide Hypothesis' is based upon just that, the normal physiological functions of an MHC class I molecule. This theory, proposed in 1990, suggests that normal tolerance to HLA-B27, acquired through thymic selection, is somehow broken resulting in auto-immunity and on-set of disease (Benjamin and Parham, 1990).

MHC class I molecules constitutively present a large peptide repertoire on the cell surface. Most of these peptides arise endogenously, and are then presented to $CD8^+$ T cells for immunosurveillance. Presentation of an antigenic peptide by HLA-B27 would result in a B27-restricted cytotoxic T-lymphocyte response. The arthritogenic peptide hypothesis predicts that the activating antigen shows molecular mimicry to a self HLA-B27 peptide resulting in tolerance to the self-peptide being lost and an autoimmune inflammatory response being launched.

It has been established that HLA-B27 is almost distinct from other MHC class I molecules for its nearly absolute restriction for peptides with arginine at position 2

(Jardetzky et al., 1991, Madden et al., 1991, Madden et al., 1992). Further research has determined that there are differences in the peptide repertoires of disease and non-disease associated alleles, with B*2706 and B*2709 unable to bind a small proportion of the peptides that are able to bind the disease associated alleles B*2704 or B*2705 respectively (Sesma et al., 2002, Ramos et al., 2002b). These data suggest peptide specificity may be important for disease association.

The onset of Reactive Arthritis, a related SpA, has long been known to be associated with enterobacterial infections. Because of this association it has been plausible to assume a link between enterobacteria and AS. Indeed evidence from transgenic mice and rats suggests an important role for microorganisms in disease onset, because in the HLA-B27 transgenic rodents disease activity develops after transfer from a specific pathogen-free area to a more conventional animal facility (Khare et al., 1995, Khare et al., 1996, Taurog et al., 1994).

The search for an arthritogenic peptide led to the discovery that some self-ligands of HLA-B27 show similarity to antigenic peptides from gram-negative bacteria. Frauendorf *et al*. have reported the presence of $CD8^+$ T cells with specificity for a HLA-B27-binding peptide, that is derived from the HLA-B27 heavy chain itself and shows similarities to enterobacteria-derived peptides, in the peripheral blood of HLA-$B27^+$ patients with AS but not in healthy HLA-$B27^+$ controls (Frauendorf et al., 2003).

Epidemiological evidence also indicates a role for bacteria in disease onset and severity, with HLA-B27$^+$ individuals being at increased risk of prolonged or severe arthritis following gastro-intestinal infection. Several types of gram-negative bacteria have been associated with the onset of SpA including *Kleibsiella*, *Chlamydia* and *Salmonella*.

Possible molecular mimicry between HLA-B27 and *Kleibsiella pneumaniae* has been investigated and two peptide antigens from *K. pneumaniae* proteins that show similarity to sequences from HLA-B27 have been described. Firstly, the 'QTDRED' sequence found in the *K. pneumaniae* nitrogenase enzyme (Schwimmbeck et al., 1987). However it is debated whether the nitrogenase enzyme is actually produced in the human gut shedding doubt on the potential role of this protein in AS. Secondly, the 'DRDE' sequence from the *K. pneumaniae* pulD secretion protein (Fielder et al., 1995). This same study also identifies a sequence from pulA secretion protein that shows homology with sequences from types I, III and IV collagen. Antibodies against these *K. pneumoniae* proteins were increased in HLA-B27$^+$ AS patients compared with healthy controls and were shown to be autoreactive to HLA-B27 in vitro (Fielder et al., 1995). The evidence supporting a role of *K. pneumoniae* in the disease process has recently been summarised (Rashid and Ebringer, 2007).

The role of *K. pneumoniae* in AS still remains a controversial subject. In a report in 2004, Stone et al were unable to detect any differences in cellular or humoral immune responses to *K. pneumoniae* between familial AS and unaffected controls (Stone et al., 2004), and comparison of the faecal microflora of patients with AS and unaffected

controls were unable to detect any significant difference in *K. pneumoniae* load (Stebbings et al., 2002). These results, and others, mean the role of *Klebsiella pneumoniae* in AS remains undetermined.

Some studies have suggested that the expression of HLA-B27 increases the susceptibility to *Salmonella* infection whilst others have reported conflicting findings (Kapasi and Inman, 1992, Huppertz and Heesemann, 1997, Saarinen et al., 2002). The Granfors lab have shown that the elimination of intracellular infection with *Salmonella* is impaired *in vitro* in HLA-B27 expressing cells, potentially resulting in an increased bacterial load in HLA-B27^{+} individuals (Laitio et al., 1997, Virtala et al., 1997). It has also been shown that HeLa cells expressing HLA-B27 have an altered signalling response when infected with *Salmonella* bacteria compared with HLA-A1 (Ikawa et al., 1998) and, in the U937 human monocytic cell line, it has been reported that the expression of HLA-B27 enhances the activation of NF-κB in response to LPS (Penttinen et al., 2002). However, a recent report by Goodall *et al*. found that the expression of HLA-B27 did not profoundly alter the response to LPS in the U937 cell line (Goodall et al., 2006).

HLA-B27, *in vitro*, can bind a peptide derived from its own cytoplasmic region that shows significant homology with a peptide derived from *Chlamydia trachomatis* (Ramos et al., 2002a). However whether this *Chlamydia* peptide is produced in vivo is still to be determined.

The continued search for an arthritogenic peptide led to the discovery of a self-peptide derived from the vasoactive peptide type 1 receptor, known as pVIPR. It has been demonstrated that this peptide shares sequence homology to a peptide derived from the latent membrane protein of Epstein Barr virus (EBV). It was postulated that the arthritogenic peptide, may not be a presented solely by disease associated alleles but may in fact be presented by all alleles but held in different combinations. A sub-optimally loaded antigen on disease-associated alleles may enable T cells to overcome tolerance. There is evidence that the disease associated allele B*2705 does in fact bind pVIPR in a dual confirmation compared with a single conformation seen in the non-disease associated B*2709 subtype (Hulsmeyer et al., 2004). Cytotoxic T-lymphocytes directed against pVIPR have been found in healthy HLA-B27^{+} controls, but their numbers are increased in patients suffering with AS (Fiorillo et al., 2000). How an immune response targeting this protein could confer the tissue-specific nature of SpA has, however, not been determined.

CD8^{+} T cells that react with collagen-derived self-peptides have been isolated from the synovial fluid of some AS patients (Atagunduz et al., 2005) but the significance of this finding is yet to be established.

New research, and old problems, seriously challenge the ability of the arthritogenic peptide hypothesis to explain the link between HLA-B27 and SpA. Firstly, despite nearly 20 years of investigation, researchers have been unable to determine a single convincing specific arthritogenic peptide. Secondly, not all associated alleles share

peptide specificity. It was postulated that an arthritogenic peptide may possess a tyrosine residue in its C-terminus, a peptide binding feature shared by B*2705, B*2704 and B*2702 but not B*2706 or B*2709 (Fiorillo et al., 1997, Garcia et al., 1997). However it has been demonstrated that the disease associated allele B*2707 does not naturally present peptides with a tyrosine at the C-terminus (Tieng et al., 1997). Also, the allele B*1403 has been found to be associated with AS in some populations (Lopez-Larrea et al., 2002) however comparison of the B*1403 and B*2705 peptide repertoire showed limited overlap (Merino et al., 2005).

Thirdly, and perhaps most importantly, transgenic rodent studies have shown that although disease onset is T-lymphocyte-dependent, $CD8^+$ $\alpha\beta$ T cells are not absolutely required (May et al., 2003), whilst $CD4^+$ T cells are crucial. If the animal model does indeed reflect human AS, this suggests a more crucial role for cells such as helper T cells and perhaps DCs. Further studies on (the somewhat controversial) HLA-$B27^+$ human β_2m^+ transgenic mice have also indicated that TAP is not required for spontaneous disease to occur (Khare et al., 2001) and although it has been shown that HLA-B27 can be expressed at the cell surface in the absence of TAP (Peh et al., 1998), the peptide repertoire presented in the presence of TAP is likely to be different to that in its absence. Although these data do not exclude the possibility of arthritogenic peptide being presented by HLA-B27 it does make it seem increasingly unlikely.

Homodimers and other Unconventional Structures

The discovery that HLA-B27 exists in confirmations other than the traditional heterotrimeric structure of heavy chain, β_2m and peptide, has led to the development of new theories for its association with disease. These theories are based on other intrinsic properties of the B27 allele rather than its natural antigen presenting abilities.

Transgenic animal studies have directly implicated free HLA-B27 heavy chains (HCs), not associated with β_2m or peptide, in the development of disease. In the HLA-B27$^+$/hβ_2m$^+$ transgenic mouse model, the incidence of disease is decreased following *in vivo* treatment with the HLA-B heavy chain specific antibody, HC10 (Khare et al., 1996), although to the best of our knowledge, this experiment has not yet been repeated in the transgenic rat model. In AS patients, Tsai *et al*. have shown an increase in free HC surface expression in monocytes compared with normal controls (Tsai et al., 2002), although Cauli et al. were unable to detect the same differences (Cauli et al., 2002).

Some β_2m-free B27-HCs, recognised by the monoclonal antibody MARB4, have been shown to be able to remain bound to peptide at the cell surface (Malik et al., 2002) suggesting that free-HCs may perhaps play a role in antigen presentation. Indeed peptide presentation by mouse class I molecules in the absence of β_2m has previously been demonstrated (Zugel et al., 1994, Lehmann-Grube et al., 1994).

Empty HLA-B27 heterodimers, not containing a peptide, have also been shown to be present on the surface of some cells and these structures are able to bind exogenous antigen (Benjamin et al., 1991) raising the possibility that HLA-B27 presents an unconventional peptide for MHC surveillance.

A further unusual structure adopted by HLA-B27 occurs when β_2m-free HLA-B27 HCs form disulfide-bonded homodimers. These homodimeric structures have been implicated in disease through two main mechanisms: 1) recognition by immunomodulatory receptors on the cell surface and 2) accumulation in the ER resulting in the activation of a pro-inflammatory stress response.

Homodimer Formation

HLA-B27 homodimers were first identified and isolated in 1999 through *in vitro* folding studies of HLA-B27 (Allen et al., 1999). Misfolded HLA-B27, particularly homodimers, have been implicated in the pathogenesis of AS and related spondyloarthropathies, either by misfolding in the ER which may result in the activation of a proinflammatory stress response (Colbert, 2000), or through their presence at the cell surface. Cell surface homodimers may be involved in peptide presentation or be recognised themselves as neoantigens by some immune receptors (Allen et al., 1999). ER homodimers and cell surface homodimers appear to be distinct populations, with homodimers forming in the ER unable to egress to the cell surface (Dangoria et al., 2002, Antoniou et al., 2004)

ER Homodimers

In 1999 Mear et al demonstrated that HLA-B27 has slower folding kinetics than other class I alleles and has a tendancy to misfold, a characteristic attributed to residues in the B pocket of the peptide-binding groove which has been directly linked with the formation of homodimers (Mear et al., 1999, Antoniou et al., 2004). This observation formed the basis of the hypothesis proposed by Colbert et al in 2000, in which he postulated that the accumulation of these misfolded proteins could lead to the activation of an ER stress response, the aim of which is to alleviate stress and re-establish normal physiological conditions (Colbert, 2000).

The folding of proteins within the endoplasmic reticulum is tightly regulated by molecular chaperones. If misfolded proteins accumulate within the ER a stress response, known as the Unfolded Protein Response (UPR), is activated. The UPR is a signalling pathway regulated by the immunoglobulin binding protein (BiP), that aims of to alleviate stress and re-establish normal physiological conditions. Under normal conditions BiP is bound to the three transducers of the stress response, PERK, ATF6 and IRE1. When misfolded proteins accumulate, BiP releases these molecules, binding instead to the misfolded proteins. This allows the stress signal to be relayed across the ER membrane, resulting in the transcriptional induction of UPR genes, translational attenuation of global protein synthesis and enhanced ER-associated degradation (ERAD). The UPR, if not perturbed, will eventually result in apoptosis (for a concise review see Liu and Kaufman, 2003).

The formation of HLA-B27 homodimers was thought to be dependent on the unpaired cysteine at position 67 in the peptide binding groove (Allen et al., 1999, Dangoria et al., 2002). However mutational studies of the structural cysteine residues in the B27 molecule indicated that both Cys^{67} and Cys^{164} are involved in homodimer formation (Antoniou et al., 2004) and further studies have shown some dimer formation to be independent of any individual free cysteine residues present in the HLA-B27 molecule (Saleki et al., 2006).

The misfolding hypothesis can potentially explain some observations made in transgenic rodent studies. In transgenic rats disease only occurs in those animals that have a high gene copy number with HLA-B27 expression above a certain threshold (Taurog et al., 1993), and in HLA-B27 transgenic mice, spontaneous arthritis occurs in the absence of β_2m (Khare et al., 1995). In both of these situations, HLA-B27 HCs could be more likely to misfold, resulting in the activation of the UPR. In 2004 it was demonstrated in a rat cell line model that HLA-B27 heavy chains were found in association with BiP, the key regulator of the UPR (Antoniou et al., 2004). Shortly afterwards Tran et al. showed that HLA-B27 alleles are more likely to form heavy chain complexes with BiP than the disease resistant allele HLA-B7 (Tran et al., 2004).

Further support for this hypothesis came from the detection of a UPR response in bone-marrow macrophages from HLA-B27-transgenic rats with active disease, but not in those of younger, pre-morbid animals (Turner et al., 2005) and the more recent demonstration

that the magnitude of the UPR in rat macrophages correlates well with the accumulation of misfolded HLA-B27 heavy chains (Turner et al., 2007). Evidence of an IFN response in cells from rats suffering with disease was also obtained, a response that seems to exacerbate the UPR, probably through the up-regulation of HLA-B27 heavy chains (Turner et al., 2005). More recently a study on a human cell line model indicates that expression of HLA-B27 alone is enough to activate a stress response (Lemin et al., 2007). In contrast to the above, a recent study in transgenic rats has challenged the UPR hypothesis. The accumulation of misfolded HLA-B27 heavy chain and UPR activation could be reduced significantly by over-expression of human β_2m (Tran et al., 2006). These rats, despite showing reduced misfolding and UPR activation still develop arthritis, which in many respects is even more akin to human AS than the original HLA-B27 rat model. Turner *et al*. have suggested though, that the reduction in misfolding caused by the increased β_2m may not be enough to prevent the activation of the UPR and that prolonged expression of increased levels of β_2m may actually amplify any ongoing immune responses (Turner et al., 2007).

Cell Surface Homodimers

The expression of cell surface dimers was first demonstrated in cell lines with defective antigen presentation pathways (Allen et al., 1999). Cell surface homodimers can be recognised by both HC10 and W6/32, and in 2002 it was demonstrated that these represent distinct populations (Allen et al., 1999, Dangoria et al., 2002). Recognition of some dimers with W6/32 indicates that this population of dimers retain at least partial

confirmation of the peptide-binding groove (Figure 1.9). Immunoprecipitations of homodimers with W6/32 in T2 cells were unable to co-precipitate β_2m indicating these dimers exist as two free heavy chains (Allen et al., 1999).

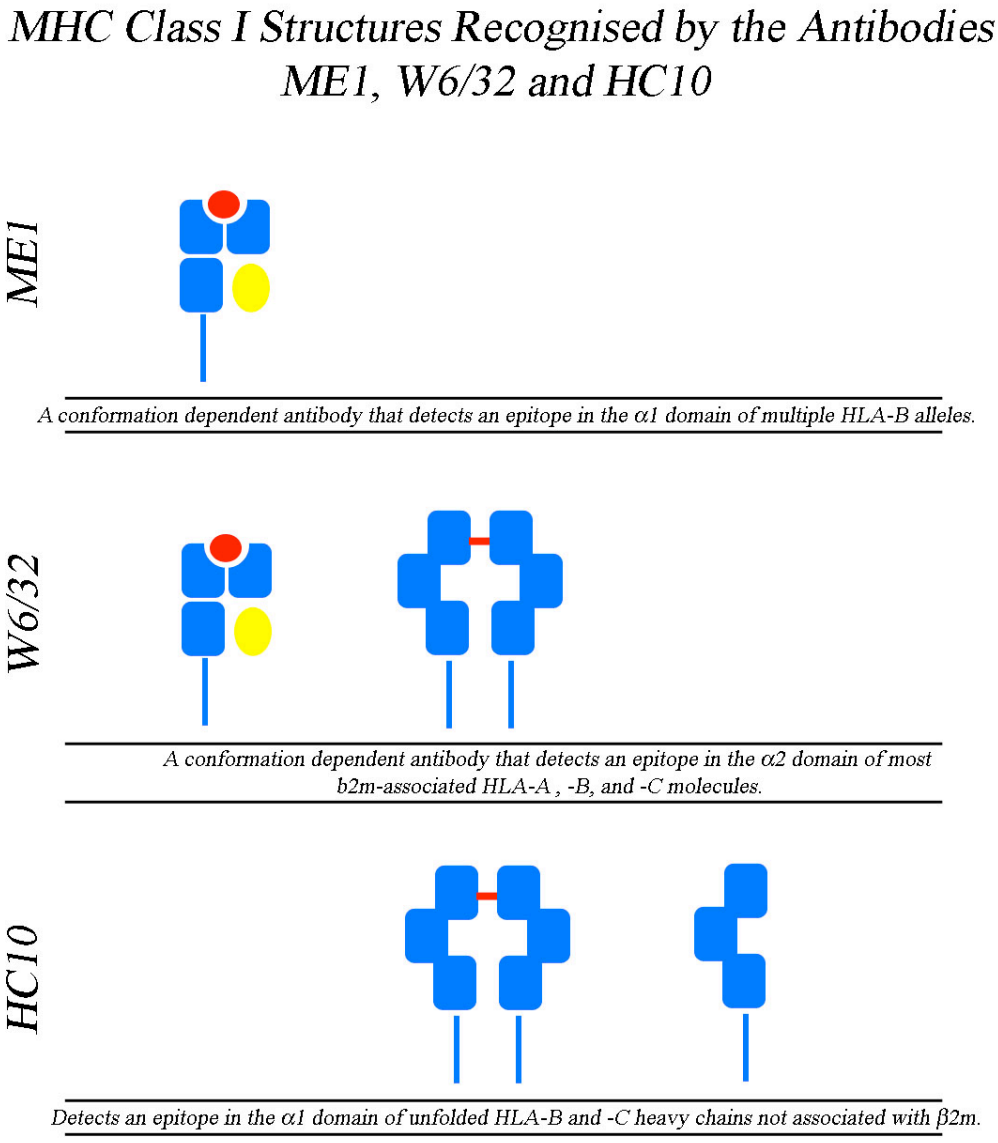


Figure 1. 9: MHC Class I structures recognised by the antibodies ME1, W6/32, and HC10. Classical MHC class I molecules are non-covalently associated with β_2m and are recognized by the antibodies ME1 and W6/32. Free-heavy chain not associated with β_2m

is recognized by HC10. The MHC class I allele HLA-B27 is also known to form disulphide bonded homodimers that are usually recognized by HC10 but a small proportion may also be recognized by the conformation dependent antibody W6/32.

W6/32 reactive and HC10 reactive dimers can be further distinguished by their formation kinetics, and their dependency on TPN, TAP, and β_2m (Dangoria et al., 2002). Dangoria *et al*. found that the formation of W6/32 reactive homodimers was dependent on TPN, TAP and β_2m, a finding that disagreed with the previous study by Allen et al (Allen et al., 1999, Dangoria et al., 2002). In pulse-chase analysis HC10 reactive dimers were determined as forming early after heavy chain synthesis suggesting they form within the ER, whereas W6/32 reactive dimers formed much later suggesting they are formed following exit from the ER (Dangoria et al., 2002). As both populations can be isolated from the cell surface, HC10 reactive dimers must also form later following exit from the ER (Dangoria et al., 2002). Consistent with this finding, Bird et al demonstrated that HC10 reactive cell surface homodimers could form from fully folded heterotrimers that are internalised and recycled back to the cell surface via an endosomal compartment (Bird et al., 2003).

HLA-B27 possess the relatively rare cysteine residue at position 67 in the extracellular α1 domain and this cysteine has been strongly implicated in the formation of homodimers (Allen et al., 1999, Dangoria et al., 2002, Bird et al., 2003). A possible structure for an HLA-B27 homodimer bound through this residue was suggested in 2002 (Figure 1.10) (McMichael and Bowness, 2002). This back-to-back model renders the

MHC class one molecules undetectable by confirmation dependent class I antibodies such as ME1.

Image unavailable owing to copyright restrictions.

Figure 1. 10: Proposed structure of an HLA-B27 homodimer. The disulphide bond between the two Cys^{67} residues is shown (yellow, centre of the image) and putative peptides are depicted as yellow tubes. This image was published in 2000, in Rheumatology by Bowness et al. (Bowness, 2002).

However, Antoniou et al, have shown that the conserved structural cysteine at position 164 is also involved in homodimer formation which would lead to a significant change in the proposed back-to-back structure (Antoniou et al., 2004). Dangoria et al also demonstrated that substitution of residues in the B pocket of HLA-B27 influenced dimer formation, and also that HLA-B7 was able to form both HC10 and W6/32-reactive dimers (Dangoria et al., 2002), suggesting that dimers are able to form in the absence of Cys^{67}.

Dimer Recognition by Immune Receptors

Since their initial discovery, cell surface HLA-B27 homodimers have subsequently been isolated in transgenic rat studies as well as on populations of peripheral blood and synovial fluid monocytes from patients with spondyloarthritis (Kollnberger et al., 2002, Tran et al., 2004, Kollnberger et al., 2004). Their proposed role in AS is different from that already discussed in the context of UPR induction.

MHC class I molecules not only interact with the TCR but also with other immune receptors including the killer immunoglobulin-like receptors (KIR) and leuckocyte immunoglobulin-like receptors (LILR, also known as ILTs) (Lanier, 1998, Colonna et al., 1998, Colonna et al., 1999a). KIR are expressed on both NK and T cell subsets and can have both activatory and inhibitory effects (Lanier, 1998, Colonna et al., 1999a). LILR show a differential expression pattern to KIR, some LILR, such as LILRB1/ILT2, are expressed on B cells, T cells, NK cells and monocytes/macrophages (Colonna et al., 1998) whilst the expression of others, such as LILRB2/ILT4, is more restricted (Lanier, 1998).

The recognition of homodimers by some immune receptors has been reported, with HLA-B27 homodimers able to interact with KIR3DL1, KIR3DL2, LILRB2/ILT4, and LILRA1/LIR6 but not LILRB1/ILT2 (Allen et al., 2001, Kollnberger et al., 2002). LILRB1 and LILRB2 usually recognise classical HLA-A and HLA-B alleles, but show an increased preference for HLA-G (Shiroishi et al., 2003). KIR3DL1 usually recognise

fully folded heterotrimeric HLA-B alleles that have the bw4 motif, including HLA-B27 (Peruzzi et al., 1996) and KIR3DL2 usually shows specificity for HLA-A alleles (Dohring et al., 1996). LILRA1 is expressed on myelomonocytic cells only (Borges and Cosman, 2000, Colonna et al., 1999b), and had no previously identified binding ligands (Allen et al., 2001).

Supporting the theory that an abnormal interaction between immune receptors and homodimers are implicated in disease, Chan et al have detected increased numbers of NK cells and $CD4^+$ T cells expressing KIR3DL2 from patients suffering with spondyloarthritis (Chan et al., 2005). These results demonstrate that HLA-B27 homodimers are recognised by a specific, although overlapping, subset of receptors compared with traditional, fully folded HLA-B27 molecules, and that at least one of these receptors is enhanced in patients suffering with spondyloarthritis.

Interaction of KIR with normal trimeric class I molecules is dependent on peptide binding (Hansasuta et al., 2004, Peruzzi et al., 1996), and it has been demonstrated that HLA-B27 free-HCs and homodimers are able to bind peptide (Allen et al., 1999, Urban et al., 1994). However Kollnberger et al. have recently shown that homodimer recognition by KIR is independent of any bound peptide (Kollnberger et al., 2007). In the transgenic rat model HLA-B27 homodimers have been detected at the cell surface of rodent leukocytes and can be recognised by paired immunoglobulin receptors (PIR) in an interaction that is ameliorated by the HC10 antibody (Kollnberger et al., 2004). However, as previously mentioned, transgenic rodent studies have also shown that rats

expressing enhanced amounts of human β_2m show reduced levels of free-HCs but still go on to develop disease (Tran et al., 2006).

Homodimers Expressed on Dendritic Cells

The role of DCs in AS has recently become an area of significant interest, and three lines of evidence support a role for DCs in AS. Firstly, $CD4^+$ T cells are required for disease to develop, and these T cells are heavily reliant on DCs for activation (Breban et al., 1996). Secondly, HLA-B27 derived bone marrow cells transfer disease (Breban et al., 1993), and thirdly, disease does not occur in the absence of commensal gut flora. The most likely cell to fulfil the above requirements; to be bone marrow derived and transfer immunological signals from the gut to the peripheral immune system, are DCs.

Furthermore, recent data has shown that DCs from HLA-B27 transgenic rats are defective in their stimulation of T cells (Hacquard-Bouder et al., 2004). The most recent study in this area has shown that expression of HLA-B27 causes this defect by interfering with the engagement of co-stimulatory molecules (Hacquard-Bouder et al., 2007). These observations could be extremely important seeing as it has been shown that co-stimulation is important for the expansion of Tregs (Lin and Hunig, 2003), leading to the suggestion that in AS and other SpA, defective activation of these cells may result in a loss of tolerance toward self antigens (Hacquard-Bouder et al., 2004).

We recently published data showing that, in both the KG1 dendritic-like cell line and in human monocyte-derived DCs from a HLA-B27^{+} individual, HLA-B27 homodimers are a transiently expressed population that are induced in response to activation (Santos et al., 2008). Homodimers usually appeared 24 hours post activation and, in the human monocyte-derived DCs, began to decrease 48-72 hours after activation. This finding is interesting considering recent *in situ* studies in the rat showing DCs from the intestine migrate to mesenteric lymph nodes within 24 to 48 hours (Kobayashi et al., 2004). This could result in the presence of enhanced numbers of HLA-B27 homodimers on the surface of DCs whilst they are in the lymph node, possibly having an effect on the initiation of any immune response.

Following activation, total levels of MHC class I are increased (Santos et al., 2008), leading to the possible scenario where increased expression of class I molecules results in the formation of dimers. It has previously been suggested that over-expression of an HLA allele alone is not enough to confer disease susceptibility as the HLA-B7 transgenic rats express -B7 to a similar level as the HLA-B27 transgenic rats express -B27 but still remain healthy (Taurog et al., 1999). However, as it has been demonstrated, HLA-B27 is more susceptible to form dimers than other alleles and therefore other alleles may need to be expressed to a higher level before significant dimer formation is seen. We, and others, have detected non-B27 dimers previously (Santos et al., 2008, Dangoria et al., 2002) although their formation (with the exception of HLA-G) has not been fully investigated.

Recognition of HLA-B27 by $CD4^+$ T cells

As discussed, evidence suggests that $CD4^+$ T cells rather than $CD8^+$ T cells are involved in the disease process. Transgenic rat studies have shown that there is a T cell requirement for disease onset but that $CD4^+$ rather than $CD8^+$ T cells are most important (Breban et al., 1996) and depletion in rats of $CD8^+$ αβ T cells had no effect on disease progression (May et al., 2003). However, Khare et al, show that in transgenic mice, MHC class II molecules are not required for disease onset (Khare et al., 1998) suggesting an unconventional role for $CD4^+$ T cells in this model.

In 2001 Boyle et al. demonstrated that $CD4^+$ T cells isolated from AS patients could recognise HLA-B27 (Boyle et al., 2001), and in a review in 2004 postulated that abnormal forms of HLA-B27 are recognised by these $CD4^+$ T cells maintaining inflammation due to this continual recognition (Boyle et al., 2004). Supporting this theory, it has been shown that cytotoxic $CD4^+CD28^-$ T-lymphocytes are enhanced in the peripheral circulation of AS patients compared with healthy controls (Duftner et al., 2003).

Auto-display of residues 169-181

In 2004 Guptasarma and Singh proposed a novel theory whereby HLA-B27 heavy chain devoid of β_2m could rearrange to auto-display residues 169-181 (Luthra-Guptasarma and

Singh, 2004). They propose that these hugely misfolded heavy chains could associate with other heavy chains to form homodimers and multimers. This idea links together two of the biggest theories surrounding HLA-B27 and its association with spondyloartropathies; heavy chain misfolding, and the arthritogenic peptide hypothesis.

A β2m Deposition Disease

A further theory has been proposed suggesting that the pathogenesis of AS occurs as a result of β_2m dissociation from peptide-complexed, surface-expressed HLA-B27 molecules (Uchanska-Ziegler and Ziegler, 2003). This hypothesis suggests that β_2m dissociates from fully folded HLA-B27 molecules at a higher rate than non-disease-associated subtypes. This β_2m can accumulate and become trapped in synovia. Here β_2m can bind to collagen, form amyloid deposits or interact with synovial fibroblasts, inducing the synthesis and secretion of proteins involved in tissue destruction. This process would result in local inflammation that in turn leads to an up-regulation of class I molecules, increasing the release of β_2m, resulting in chronic inflammation. However, Vázquez and López de Castro have shown that HLA-B27 subtypes differentially associated with AS are similar in their extent of β_2m dissociation providing evidence against this hypothesis (Vazquez and Lopez de Castro, 2005).

Non HLA-B27 Contributions to Disease

HLA-B27 is the strongest pre-disposing factor for AS but only a small fraction of HLA-B27 positive individuals actually develop disease. There is evidence supporting other genetic-susceptibility factors as the principal determinants for which HLA-B27$^+$ individuals will go on to develop AS.

To date there are four non-MHC genes that have been strongly linked to the heritable susceptibility of AS. The first gene to have replicated association with AS was CYP2D6 (cytochrome P450 gene debrisoquine 4-hydroxylase) (Beyeler et al., 1996, Brown et al., 2000). This gene, encoded on the long arm of chromosome 22, produces an enzyme involved in oxidative drug metabolism and loss of function has been associated with AS. It has been suggested that this gene may be involved in disease onset if the environmental trigger for AS is a natural toxin, its metabolism and therefore removal, maybe reduced if this enzyme is defective.

The second gene loci to be implicated in AS was the long arm of chromosome 2, a region that corresponds to the IL-1 gene cluster (Brown et al., 1998a, Laval et al., 2001). In particular, polymorphisms within the interleukin 1 receptor gene (IL-1RN) have repeatedly been shown to be associated with AS (McGarry et al., 2001, van der Paardt et al., 2002, Maksymowych et al., 2003, Chou et al., 2006, Agrawal et al., 2008). The IL-1RN gene encodes an anti-inflammatory non-signalling molecule that competes for receptor binding with IL-1α and IL-1β. A commercial interleukin 1 receptor antagonist,

Anakinra, is currently used in the treatment of rheumatoid arthritis but has been shown in some cases to improve the symptoms of AS (Tan et al., 2004, Haibel et al., 2005)

More recently two other genes were identified as conveying an increased risk of AS, ARTS1/ERAP1 (aminopeptidase regulator of TNFR1 shedding/endoplasmic reticulum aminopeptidase 1) and IL23R (interleukin-23 receptor) (Burton et al., 2007). ARTS1/ERAP1 has diverse functions, it is involved in the trimming of peptides in the ER for presentation by MHC class I molecules (Saveanu et al., 2005a), and also in the cleavage of cell surface receptors of the pro-inflammatory cytokines TNF, IL-1 and IL-6 (Cui et al., 2002, Cui et al., 2003b, Cui et al., 2003a). Defects, or alterations, in either of these functions have obvious possible consequences relating to the pathogenesis of SpA.

A polymorphism in the IL23R gene has previously been associated with inflammatory bowel disease and psoriasis (Duerr et al., 2006, Cargill et al., 2007) and a new study indicates that the same polymorphism is involved in AS (Rahman et al., 2008). IL-23 is a key cytokine in the regulation of the newly defined effector T-cell subset, Th17. These cells are potent promoters of tissue inflammation and are important in both protective and pathological roles during inflammation (Kurts, 2008, Ouyang et al., 2008).

Th17 cells produce the cytokine IL-17, which has been strongly implicated in the pathogenesis of Rheumatoid arthritis (Lubberts, 2008). A recent study has found increased numbers of Th17 cells in AS and psoriatic arthritis patients, suggesting Th17 cells may also be involved in SpA (Jandus et al., 2008). To this regard it is interesting to

note that Th17 cells are believed to be important in the immune response to extracellular pathogens, of particular interest, considering its association with AS, is the fact that IL-17 is important in the response to *Klebsiella pnuemoniae* infection (Happel et al., 2003). The exact role played by Th17 cells in AS and other SpA has not yet been elucidated but continuing research into this area is likely to be important in the coming years.

There is no doubt that the spondyloarthropathies are multifactoral diseases with both genetic and environmental influences. The association of HLA-B27 and AS remains one of the strongest links between the MHC and disease. A complete understanding of why HLA-B27 is so strongly associated with spondyloarthropathies, in particular AS, will probably only occur by also understanding the detailed molecular properties of this allele and how these relate to both its canonical and non-canonical functions, as well as continuing to study other non-HLA-B27 susceptibility factors.

HLA-B27 and Exosomes

As part of this study we have investigated whether HLA-B27 homodimers, implicated in the pathogenesis of AS and other SpA, are present in immunomodulatory vesicles known as exosomes.

Exosomes – An Overview

Exosomes were first discovered through reticulocyte maturation studies and were believed to be a method of modulating membrane function by removing unnecessary material from the cell (Johnstone et al., 1987). Since their initial discovery, exosomes remained in relative obscurity until 1996 when Raposo et al. showed that exosomes released from B-lymphocytes carry functional MHC molecules and have the ability to stimulate specific T cell responses *in vitro* (Raposo et al., 1996). Since this report was published the interest in exosomes has increased dramatically, and exosomes have now been isolated from many different cell types including; reticulocytes (Johnstone et al., 1987), B- and T-lymphocytes (Raposo et al., 1996, Blanchard et al., 2002), dendritic cells (Zitvogel et al., 1998), mast cells (Raposo et al., 1997), intestinal epithelial cells (van Niel et al., 2001), the placenta (Taylor et al., 2006) and tumour cells (Wolfers et al., 2001). Exosomes have also been purified from human plasma (Caby et al., 2005), breast milk (Admyre et al., 2007b), malignant effusions (Andre et al., 2002), bronchoalveolar lavage (Admyre et al., 2003) and urine (Pisitkun et al., 2004). However, the *in vivo*

function of exosomes still remains poorly defined and work attempting to fully determine how and why these vesicles are formed is ongoing. Nevertheless, they are the subject of great interest for their potential role as 'cell-free' vaccine agents.

Exosomes are small membrane bound vesicles 40-150 nm in diameter, formed by inward budding of endosomes, resulting in the generation of multivesicular bodies (MVB) (Pan et al., 1985). They are involved in intercellular communication through direct signalling (Admyre et al., 2006, Hwang et al., 2003), or through the exchange of proteins (Andre et al., 2004), and mRNA (Valadi et al., 2007) between cells. Exosomes have also been implicated in several disease processes (Admyre et al., 2007a, Gould et al., 2003, Fevrier et al., 2004), and can be immunostimulatory as well as immunosuppressive, and are therefore an extremely important cell-free system that can be manipulated to alter the course of the immune response (Kim et al., 2005, Prado et al., 2008).

The isolation of exosomes from biological fluids demonstrates that exosomes are produced *in vivo* (Admyre et al., 2007b, Caby et al., 2005, Admyre et al., 2003, Andre et al., 2002, Pisitkun et al., 2004), and the isolation of exosomes from human plasma suggests that the blood could act as a physiological fluid for exosome transport, allowing exosomes to reach distant targets (Caby et al., 2005). Indeed, exosomes express CD55 and CD59, proteins that protect them from complement-mediated lysis, and the physical properties of exosomal membranes make them resistant to enzymatic degradation in the circulation (Clayton et al., 2003, Laulagnier et al., 2004).

Morphology and Lipid Composition

As previously mentioned, exosomes are 40–150 nm in diameter. They have been shown to have a 'cup' shaped morphology when imaged using electron microscopy. It has been suggested that this morphology is due to the imaging technique, (Fevrier and Raposo, 2004), however this structure under electron microscopy has become one of the defining characteristics of exosomes.

The lipid composition of exosomal membranes has been reported to be different depending on the cell of origin (Vidal et al., 1989, Laulagnier et al., 2004, Wubbolts et al., 2003). Exosomes from B-lymphocytes are enriched in cholesterol, sphingomyelin and the glycolipid GM3 and are relatively poor in phosphatidylcholine and phosphatidylserine (Wubbolts et al., 2003). The lipids that are enriched in exosomes are characteristic of DRM (detergent-resistant membrames) or raft domains (Ikonen, 2001) and consistent with these raft-like properties, the raft-proteins flotillin, stomatin, and lyn have been identified in exosomes prepared from reticulocytes, erytholeukeamic cells and lymphoblastoid cells (Savina et al., 2002, de Gassart et al., 2003). DRM, or raft domains, may also be important in the segregation of proteins that are destined for exosomes at the limiting membrane of the MVB (Wubbolts et al., 2003) and the lipid composition of exosomes may also help to prevent exosome degradation when released from the originating cell (Laulagnier et al., 2004).

Formation and Sorting

Exosomes form via inward budding of the limiting membrane of the endosome, forming multivesicular bodies (MVB) (Pan et al., 1985), but they are not simply smaller versions of the cell, with the lipid and protein composition of exosomes being different from the plasma membrane (Wubbolts et al., 2003, Laulagnier et al., 2004). The protein composition of exosomes has been extensively studied and several tables of proteins found in exosomes have been published (Mignot et al., 2006, Wubbolts et al., 2003, Thery et al., 2002b, Chaput et al., 2006). The most abundant type of proteins found on exosomes are the tetraspanin family proteins, whilst ER or nuclear resident proteins are typically not found within exosomes.

As well as some common proteins, exosomes also bear a selected subset of proteins that is dependent on the cell they originate from. How this selective incorporation is achieved is not yet fully understood.

Several mechanisms for the targeting of proteins to MVBs have been proposed. For example, there is evidence that molecular clustering of either proteins or lipids within the endosome membrane acts as a sorting signal for targeting to exosomes (Vidal et al., 1997, Geminard et al., 2001). As previously mentioned, DRM or raft domains, may also be important in the segregation of proteins that are destined for exosomes at the limiting membrane of the MVB, particularly domains containing high amounts of sphingolipids which can form ceramide (Wubbolts et al., 2003, Trajkovic et al., 2008).

Monoubiquitination has also been indicated in the targeting of proteins to exosomes, by re-directing proteins from a default pathway that recycles them back to the plasma membrane to the MVB sorting pathway (Katzmann et al., 2001, Reggiori and Pelham, 2001, Urbanowski and Piper, 2001). Monoubiquitinated proteins interact with the endosomal sorting complex required for transport (ESCRT) complexes and are targeted to MVB.

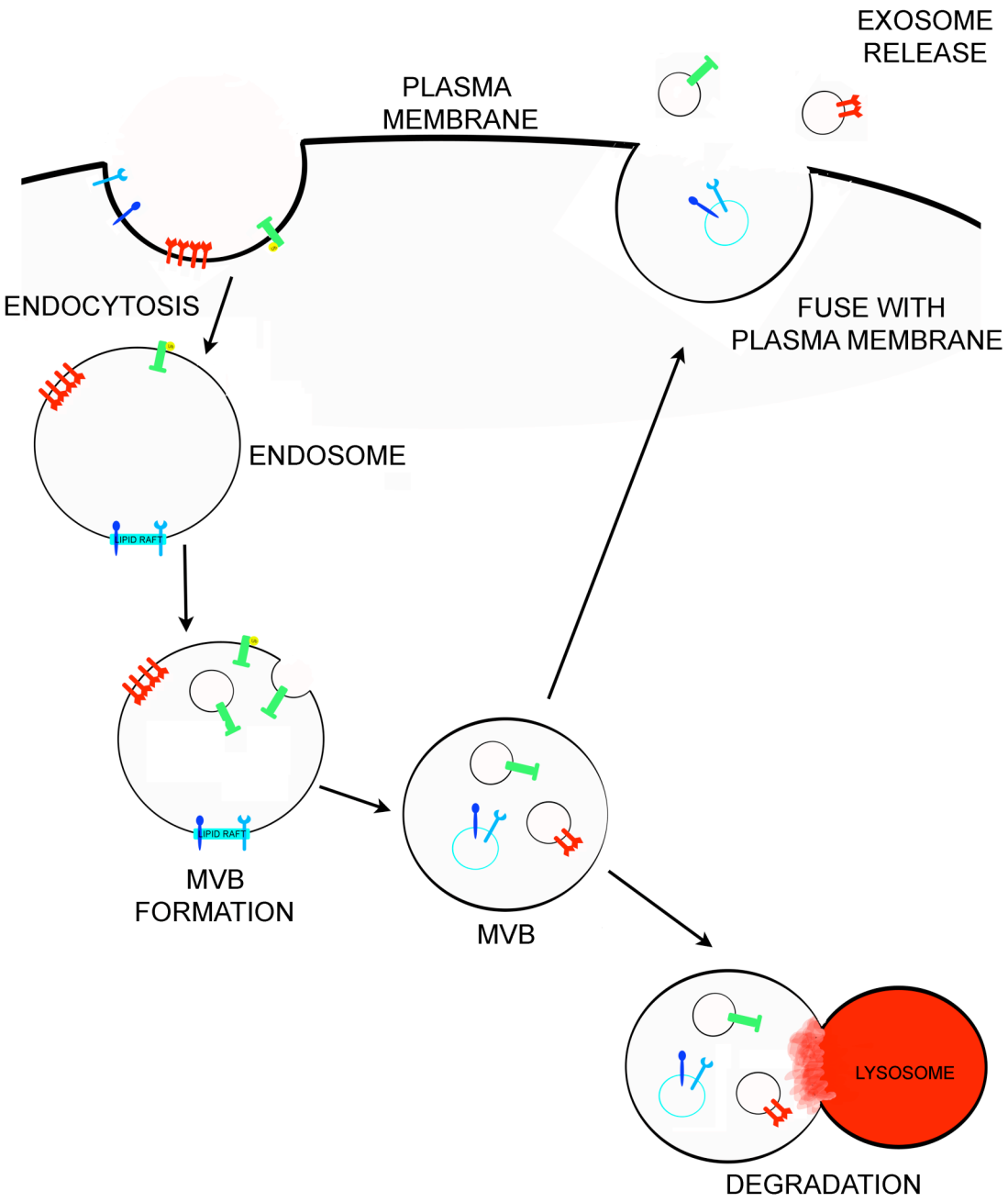


Figure 1. 11: Schematic diagram of exosomes formation and release. Exosomes are formed via inward budding of the limiting membrane of the endosome, forming multivesicular bodies (MVB). MVB can either, fuse with lysosomes and undergo degradation, or they can fuse with the plasma membrane releasing exosomes into the extracellular environment.

ESCRT Pathway

The ESCRT pathway is conserved from yeast to humans and shows relatively few species-specific changes, therefore yeast nomenclature is used here throughout with the human homologue shown in brackets. Monoubiquitinated cargo first interacts with the Vps27-Hse1-complex (HRS-STAM1/STAM2) through ubiquitin interacting motifs (UIM) (Bilodeau et al., 2002). A FYVE domain in the Vps27 component targets the Vps27-Hse1-complex to the endosomal membrane, this domain consists of a compact 60 amino acid Zn^{2+} finger that selectively binds to phosphatidylinositol-3-phosphate (PI(3)P), a lipid enriched in the membrane of the endosome, in preference to all other phosphoinositides (Misra and Hurley, 1999, Burd and Emr, 1998, Stahelin et al., 2002). Vps27 also contains a binding motif that recruits the first of the ESCRT complexes – ESCRT I (Bache et al., 2003, Katzmann et al., 2003).

ESCRT I is a soluble heterotetrameric complex consisting of Vps23 (Tsg101), Vps28 (VPS28), Vps37 (VPS37 A-D) and Mvb12 (MVB12) (Babst et al., 2000, Katzmann et al., 2001, Curtiss et al., 2007). In yeast the C-terminal domain of the ESCRT I subunit, Vps28, recruits the second ESCRT complex by binding to the NZF (Np14 zinc finger) domain in the ESCRT II subunit, Vps36 (EAP45) (Teo et al., 2006). However the human homologue, EAP45, does not contain an NZF region leaving how these complexes interact not fully defined (Slagsvold et al., 2005). The ESCRT II complex consists of Vps22 (EAP45), Vps36 (EAP45), and Vps25 (EAP25) (Babst et al., 2002b).

ESCRT II transiently associates with the endosomal membrane and initiates the formation of ESCRT III (Babst et al., 2002b). ESCRT III consists of soluble monomers that, when recruited to the endosomal membrane, form an insoluble membrane-bound complex, consisting of two sub-complexes made up of Vps2 (CHMP2A, B) bound to Vps24 (CHNP3), and Vps20 (CHMP6) bound to Vps32/Snf7 (CHMP4A, B, C) (Babst et al., 2002a). Vps20 also directly binds to the Vps25 subunit of ESCRT II (Teo et al., 2004), and in humans the ESCRT-associated protein, ALIX, connects Tsg101 of ESCRT I with CHMP4 of ESCRT III, linking the complexes together (Strack et al., 2003, Katoh et al., 2003, von Schwedler et al., 2003).

Vps4 (VPS4A, B), an ESCRT associated protein, is required to catalyse dissociation of the ESCRT complexes in an ATP dependent manner (Babst et al., 1997, Babst et al., 1998). Vta1 (LIP5) is a positive regulator of Vps4 and also interacts with the accessory ESCRT III subunits Vps60/Mos10 (CHMP5) and Did2 (CHMP1) (Azmi et al., 2006, Shiflett et al., 2004, Nickerson et al., 2006).

Ubiquitination is used to target proteins to the MVB, however before cargo is loaded into the MVB it is first deubiquitinated. Doa4 is a deubiquitinating enzyme (DUB) that is targeted to the endosome membrane, the timing of which is helped by an interaction with the ESCRT accessory molecule Bro1 (ALIX) (Amerik et al., 2006, Luhtala and Odorizzi, 2004).

Although the ESCRT complexes have important roles in MVB biogenesis it is still unknown how the complexes promote the invagination of the endosomal membrane. It has been suggested that the lipid composition of the MVB may play a role in vesicle invagination, with mammalian MVBs reported to be rich in 2,2' lysobisphosphatidic acid (Matsuo et al., 2004). However the presence of this lipid has not been reported in yeast, and considering the high conservation of these complexes, it seems unlikely that an entirely different method for budding exists in yeast and mammals. It cannot be excluded however, and lipid composition could be the basis of a second mechanism for MVB formation used by mammals.

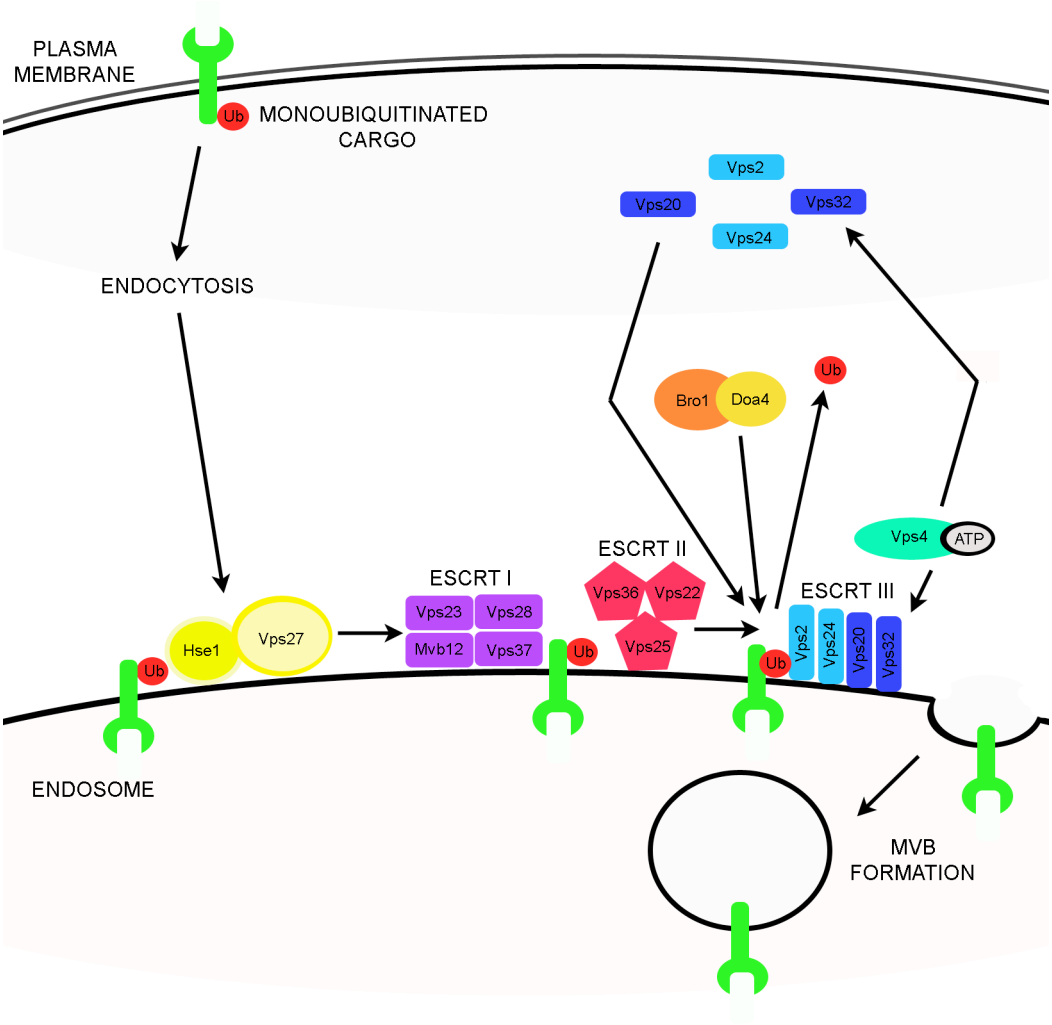


Figure 1. 12: Schematic diagram of the ESCRT machinery. Monoubiquitinated proteins interact with the ESCRT complexes targeting them for inclusion into the MVB pathway.

Exosome Target Cell Stimulation

How exosomes mediate their affects *in vivo* is relatively poorly understood. *In vitro* studies have suggested that exosomes can stimulate T cells directly (Admyre et al., 2006, Hwang et al., 2003) whilst others suggest that they work indirectly by binding to, or

fusing with professional antigen presenting cells (Thery et al., 2002a, Denzer et al., 2000). *In vitro*, in the absence of APCs, the expression of ICAM-1 on the surface of DC-derived exosomes has been shown to be necessary for the binding of exosomes to the surface of naïve T cells (Segura et al., 2005). Segura et al have also shown, both *in vitro* and *in vivo*, that the interaction between ICAM-1 on the surface of the exosomes and LFA-1 (CD11a/CD18) on the recipient DC is essential for exosome capture and presentation (Segura et al., 2007). They propose that LFA-1 on the surface of DCs is a specific receptor allowing the efficient antigen transfer *in vivo* and demonstrate that $CD8^+$ DCs, expressing high levels of LFA-1, capture exosomes more efficiently than $CD8^-$ DCs.

Follicular dendritic cells (FDCs) are important cells in the germinal centre reaction and present antigen to B- and T-lymphocytes. FDCs do not synthesize their own MHC class II molecules but instead passively acquire them (Gray et al., 1991). Immunoelectron microscopy images have shown that MHC class II molecules, on the surface of FDCs, are expressed in discrete vesicles attached to the cell surface, and that these vesicles resemble exosomes (Denzer et al., 2000). Indeed in vitro binding studies have shown that B cell derived exosomes have an increased avidity for FDCs suggesting that these cells could be physiological targets for exosomes *in vivo* (Denzer et al., 2000).

Immunomodulation Through Exosomes

Exosomes that can exert immunotolerogenic effects are sometimes referred to as 'tolerosomes'. It has been suggested that exosomes, or tolerosomes, released from the intestinal epithelial cells of mice are involved in inducing peripheral tolerance to non-self antigens (Karlsson et al., 2001). However the opposite results were obtained in a second study, leaving the exact role played by intestinal epithelial exosomes undetermined (Van Niel et al., 2003).

During pregnancy the fetus demonstrates local immune privilege, thought to be maintained by the placenta. A number of reports have shown that Fas ligand (FasL) is expressed by trophoblast cells during pregnancy (Bamberger et al., 1997, Uckan et al., 1997, Pongcharoen et al., 2004), however two further reports have demonstrated that FasL is not expressed on the cell surface but is instead packaged into small microvesicles that are then secreted into the extracellular space, similar to exosomes (Frangsmyr et al., 2005, Abrahams et al., 2004). It has since been demonstrated that placenta-derived exosomes are found in the circulation of pregnant women and that these exosomes do in fact express FasL, implicating exosomes in maintaining tolerance during pregnancy (Taylor et al., 2006).

Furthermore, in rodent studies it has also been demonstrated that the injection of donor-derived exosomes before, or after, transplantation can significantly prolong cardiac

allograft survival in fully MHC-mismatched hosts, once again suggesting exosomes can have a tolerogenic function (Peche et al., 2003, Peche et al., 2006).

In marked contrast to the above, however, Zitvogel et al. demonstrated that tumours in mice could be eradicated by a single injection of exosomes, implicating exosomes in an immunoactivatory role (Zitvogel et al., 1998). Following the publication of the work by Zitvogel et al. clinical trials in humans began. The first phase I clinical trial was started in melanoma patients and shortly afterwards the second phase I clinical trail began in non-hodgkins lymphoma patients. The results were promising with tumour regression or long-term stabilisation achieved in some patients (Morse et al., 2005, Escudier et al., 2005).

If the factors that determine whether an exosome exerts immunosuppressant or immunoactivatory affects can be elucidated, or if exosomes could be subject to modification, then the possibility of cell free vaccines becomes feasible. Autoimmune diseases and cancers could potentially be treated essentially with the same tool. To this effect, rodent studies have shown that periarticular injection of exosomes derived from BMDCs expressing viral IL-10 or recombinant murine IL-10 are able to suppress delayed-type hypersensitivity responses in both the injected and the contralateral joint, and systemic injection of IL-10 treated DC-derived exosomes in mice was able to prevent the onset of collagen induced arthritis (CIA) and to reduce severity in already established disease (Kim et al., 2005). More recently, similar results were obtained using exosomes derived from dendritic cells genetically modified to express IL-4 (Kim et al., 2007).

Some data suggests that the effects of dendritic cell derived exosomes are dependent upon the maturation state of the originating DC, with exosomes derived from mature DCs being better stimulators of a T cell response than immature DC derived exosomes, possibly due to differing expression of co-stimulatory molecules (Segura et al., 2005, Admyre et al., 2006). However, exosomes isolated from intestinal epithelial cells have been shown to induce a humoral immune response despite their distinct lack of co-stimulatory molecules (Van Niel et al., 2003).

Exosomes in Disease

In order for cancer to develop, tumour cells modulate their environment to promote their expansion and survival. Since the demonstration by Zitvogel et al. that dendritic cell derived exosomes bearing tumour antigens can eradicate developed tumours in mice, the role of exosomes in cancer has been studied in great detail (Zitvogel et al., 1998). However, it now appears that tumour cells can modulate their exosome release to actually help promote growth and survival. To this regard exosomes released from tumours have been shown to express FasL and TRAIL (tumour necrosis factor-related apoptosis-inducing ligand), molecules that stimulate T cell apoptosis, as well as DEL-1 (developmental endothelial locus-1), which can act as a strong angiogenic factor, and HLA-G, a non-classical MHC class I molecule that promotes immune tolerance (Riteau et al., 2003, Huber et al., 2005, Hegmans et al., 2004). Tumour derived exosomes can also impair monocyte differentiation into dendritic cells, promoting instead the development of a myeloid immunosuppressive cell subset (Valenti et al., 2006), and

studies in drug-resistant human ovarian carcinoma cells suggest that the release of exosomes from tumour cells may potentially be a mechanism for drug-expulsion and chemoresistance (Shedden et al., 2003).

Exosomes have also been implicated in prion diseases, infectious neurodegenerative diseases caused by the accumulation of the abnormally folded prion protein scrapie (PrPscr). The mechanism by which PrPscr disseminates from the point of infection is unclear. Exosomes have been isolated that contain regular cellular prion protein (PrPc) and infectious PrPscr before and after infection with sheep prions respectively, and following intracerebral inoculation of mice with PrPscr exosomes, disease development ensued (Fevrier et al., 2004). A further study has demonstrated that PrPc is released in exosomes from activated platelets, suggesting that exosomes released into the blood stream could be a novel mechanism for prion disease transmission from the site of peripheral exposure (Robertson et al., 2006).

A recent in vitro study showing that B cell derived exosomes can present allergen-derived peptides and stimulate allergen-specific T cells to proliferate and release cytokines has also implicated exosomes in allergy (Admyre et al., 2007a). These authors suggest that exosomes may help to potentiate an allergic response. In the future, modulation of exosomes to alter their affects may potentially lead to a new treatment in the management of allergic disease, and a very recent publication demonstrates that, in mice, intranasal treatment with tolerogenic exosomes prior to sensitisation/challenge resulted in sustained tolerance to the antigenic allergen (Prado et al., 2008).

In 2003, The Trojan Exosome hypothesis was postulated, suggesting that retroviruses have evolved to exploit the exosome pathway in order to generate retroviral particles (Gould et al., 2003), and shortly afterwards the first evidence demonstrating HIV budding through the exosome pathway was published (Nguyen et al., 2003). However, although this theory can potentially explain several aspects of retroviral biology, two recent reports claiming that exosomes and retroviral particles are distinct entities has shed some doubt on the theory (Coren et al., 2008, Cantin et al., 2008), and mean that the Trojan Exosome hypothesis remains as yet unproven.

A further novel role for exosomes in immunosurveillance has also recently been suggested. Exosomes released from cells infected with an intracellular pathogen can stimulate macrophage activation both *in vitro* and *in vivo*, suggesting that exosomes may serve an important part in activating an immune response against intracellular pathogens (Bhatnagar et al., 2007).

The above examples demonstrate the diverse effects that have so far been attributed to exosomes. Exosomes can be tolerogenic, or activatory, as well as be involved in immunosurveillance and disease. They are currently being investigated as an attractive cell-free therapy against tumors, and if exosomes can be used to induce immune suppression, they may provide attractive agents in the fight against autoimmunity, inflammatory diseases and even allergy. However, as exosomes have been shown to have roles in both eliciting and suppressing the immune response any use of exosomes *in*

vivo would have to be carefully considered and further research to determine what factors influence the function of different exosomes is necessary.

Chapter II: Materials and Methods

General

Unless otherwise stated all chemicals were obtained from either Sigma or BDH. Materials obtained from elsewhere are indicated in the text. A list of supplier addresses can be found at the end of the chapter.

In vitro Cell Culture

Cell Lines and Media

Table 2.1 describes the various cell lines used in this study. Aspects of the antigen presentation pathways that are mutated or absent, and HLA expression (when determined) are also detailed.

All cell lines were routinely grown in either DMEM (Dulbecco's Modified Eagle's Medium), RPMI (RPMI-1640) or IMDM (Iscoves Modified Dulbecco's Medium), supplemented with FBS (Foetal Bovine Serum) and kanamyicin sulfate (all Invitrogen).

Cell Line	Origin	HLA Expression and Mutations to the Antigen Presentation pathway	Culture Media
HeLa	Derived from human cervical carcinoma. ECACC 93021013.		DMEM + 10 % FBS+ 0.1 mg/ml kanamycin
C58	Rat thymic lymphoma cell line. (Silva et al., 1983)		RPMI + 5 % FBS + 0.1 mg/ml kanamycin
721.220	Human lymphoblastoid cell line.	.220 cells lack HLA-A, HLA-B and functional tapasin. HLA-C remains intact on one chromosome resulting in low expression of HLA-Cw1 (Greenwood et al., 1994, Copeman et al., 1998)	
HOM-2	Human EBV-transformed B cell line. ECACC 98092902.	HLA-A302, HLA-B2705, HLA-Cw1	
Jesthom	Human EBV-transformed B cell line. ECACC 88052004.	HLA-A202, HLA-B2705, and HLA-Cw1.	
KG1	Human dendritic-like cell line. ATCC CCl-246.	HLA-A30, HLA-A31, HLA-B35 and HLA-Cw4.	IMDM + 20 % FBS + 0.1 mg/ml kanamycin
HEK293	Human embryonic kidney cell line. ATCC CRL-1573		DMEM + 10 % FBS+ 0.1 mg/ml kanamycin

Table 2.1: Details of cell lines used in this study. HLA-expression is indicated, this information was obtained from ECACC or ATCC unless otherwise referenced. Details of components of the antigen presentation pathway that are absent or mutated are also listed.

Cell Propagation

Cells were routinely maintained at 37 °C in a humidified atmosphere of 5 % CO_2 in air, in medium supplemented with FBS and kanamycin sulfate. All tissue culture procedures were performed in a class 2 containment cabinet.

Suspension cultures were routinely passaged by 1:1 dilution with fresh medium. Confluent adherent cultures were washed once with 0.5 % trypsin EDTA (ethylene diamine tetraacetic acid) in Hank's balanced salt solution (Invitrogen) before being incubated in 300 μl of the trypsin EDTA solution for 5 minutes to disrupt the cellular monolayer, trypsin was then neutralised with 1 ml of the appropriate medium before passaging.

All plastic plates and flasks used for cell culture were obtained from VWR.

Cell Counting

Cell viability and proliferation were assessed by trypan blue assay (0.4 %) (Sigma) and counted either using a haemocytometer or a Z1 Coulter® Particle Counter (Beckman Coulter).

Transfection of non-adherent cells using Electroporation

In all instances the plasmid vector containing the gene of interest was pCR3 (Invitrogen). Some of the recombinant proteins were C-terminally tagged with SV5 (a 14 amino acid tag with sequence GKPIPNPLLGLDST) allowing isolation and detection with the antibody pk (Hanke et al., 1992).

Transient and stable transfectants of the 721.220 cell line were created using the Amaxa Nucleofactor system (Amaxa). 1 x 10^6 – 5 x 10^6 cells were resuspended in 100 µl RPMI and mixed with 1 µg of DNA before being transferred to an Amaxa cuvette. Cells were electroporated using Amaxa program A-24 before being transferred to 1 ml of pre-warmed medium. For transient transfections the cells were harvested 24 hours after transfection. For stable transfectants the cells were put into selective medium containing 1 mg/ml G418 (Geneticin) (Melford) 24 hours after transfection. Where required, dilution cloning was used to isolate 100 % positive cultures.

Lipid Transfection of adherent cells

Stable and transient transfectants were created using FuGENE 6 (Roche). In all instances the plasmid vector containing the gene of interest was pCR3 or pCDNA3 (Invitrogen). Some of the genes were C-terminally tagged, allowing isolation and detection with the antibody pk.

For all transfections, 1 μg of plasmid DNA was added to 100 μl of DMEM (without serum), and gently mixed before 3 μl of FuGENE 6 was added. The DMEM, DNA and FuGENE 6 was again tapped gently to mix and then left at room temperature for 10 minutes. Following this, the mixture was added dropwise to a semi- confluent well of cells.

For transient transfections the cells were harvested 24 hours after transfection. For stable transfectants the cells were put into selective medium, containing 1 mg/ml G418, 24 hours after transfection. If necessary, positive cultures were enriched using magnetic beads and/or dilution cloning.

Enrichment of Cultures using Magnetic Beads

Magnetic beads expressing sheep anti-mouse IgG (Dynabeads®, Dynal Biotech) were used to separate positively transfected cells. Cell cultures were incubated with 0.01 % of antibody in medium for 10 minutes on ice. Cells were then washed in filter sterile PBS (phosphate buffered saline) (136 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_42H_2O$, 1.5 mM KH_2PO_4) + 0.1 % BSA (bovine serum albumin) (Sigma) and subject to 300 x g centrifugation for 8 minutes. The supernatant was discarded and cells were resuspended in PBS + 0.1 % BSA. Following the addition of magnetic beads samples were incubated on ice for 20 minutes with gentle agitation to ensure the beads did not settle. Positive cells bound to the beads were recovered using a magnet and washed 4 times using PBS +

0.1 % BSA. The bound cells were resuspended in medium and transferred to a fresh culture well. Cells were washed daily with medium until all beads were removed.

Dilution Cloning

100 µl of selective medium (1 mg/ml G418 in DMEM/RPMI) was added to each well of a 96 well plate. Approximately 0.5 – 1 x 10^5 cells were resuspended in 100 µl of medium and added to the top left hand well. A 1:1 dilution was carried out down the first column of wells and then a further 1:1 dilution across each row. Wells that produced a single colony were selected and grown. Positive colonies were assessed by flow cytometry and SDS-PAGE (polyacrylamide gel electrophoresis).

Cell Freezing and Thawing

Cell lines were suspended in chilled freeze medium (90 % FBS + 10 % DMSO (dimethyl sulfoxide)) (Sigma) and frozen to -80 °C using an isopropanol-filled cryo-freezing container (Nalgene) to control the freezing rate. After 24 hours cells were transferred into liquid nitrogen storage. When thawing, cells were warmed in a waterbath at 37 °C and immediately resuspended in the appropriate medium. Cells were plated and returned to the incubator for approximately 6 hours after which the medium was changed.

KG1 Stimulation

KG1 cells were differentiated/matured with 10 ng/ml phorbyl-12-myristate-13-acetate (PMA) (Sigma) and 100 ng/ml ionomycin (Sigma) for 24-48 hours.

Treatment with Chloroquine

Cells were incubated in medium supplemented with 100 μM chloroquine (Sigma) for 24 hours, at 37 °C in a humidified atmosphere of 5 % CO_2 in air, before harvesting. Cell lysates or immunoprecipitations were analysed by SDS-PAGE and western blotting.

Treatment with Diamide

Cells were incubated in medium supplemented with varying concentrations of diamide (10 μM, 100 μM, 250 μM, 500 μM or 1 mM) and incubated on a heating block at 37 °C for 10 minutes before harvesting. Cell lysates were analysed by SDS-PAGE and western blotting.

Biochemistry Techniques

Cell Lysis

0.5×10^6 - 5×10^6 cells were usually lysed using 50-500 µl of lysis buffer (10 mM Tris pH 7.6, 130 mM NaCl, 1 % NP40 (Nonidet P-40) (Sigma), 10 mM NEM (N-ethylmaleimide) and 1 mM PMSF (phenylmethylsulfonyl fluoride)) on ice for 30 minutes. Nuclear and cellular debris were removed by centrifugation at 14,000 rpm (20,000 x g) at 4 °C for 10 minutes.

Whole cell lysates containg the nuclear material were prepared by washing cells twice with PBS before resuspending in approximately 100-300 µl of sample buffer. Samples were passed through a fine gauge syringe five times to shear DNA before heating at 90 °C for 2 minutes.

Protein Quantification

Protein concentrations were determined using the Bradford method. Briefly, a standard curve was set up using known concentrations of BSA (0-8 µg/ml). Sample measurements were taken at a wavelength of 595 nm on a spectrophotometer.

SDS-PAGE

Protein samples were resuspended in SDS sample buffer (2 % SDS, 20 % glycerol, 0.05 % bromophenol blue, 0.5 M Tris pH 6.8) with or without reducing agent, dithiothreitol (DTT) (100 mM) and denatured by heating at 90°C for 2 minutes. Proteins were resolved in 8 % SDS acrylamide gels unless specified. Electrophoresis was carried out using Biorad mini-Protean II electrophoresis equipment (Biorad) in Tris/glycine (25 mM Tris, 192 mM glycine, 0.1 % SDS) running buffer for 15 minutes at 100 V followed by approximately 45 minutes at 150 V or until the dye front reached the bottom of the gel.

8 % Resolving Gel		Stacking Gel	
H_20 (ml)	4.6	H_20 (ml)	2.1
30 % 37.5:1 acrylamide: bisacrylamide (ml) (Thistle Scientific)	2.7	30 % 37.5:1 acrylamide: bisacrylamide (ml)	0.5
1.5 M Tris pH 8.8 (ml)	2.5	0.5 M Tris pH 6.8 (ml)	0.38
10 % SDS (µl)	100	10 % SDS (µl)	30
10 % APS (µl)	100	10 % APS (µl)	30
TEMED (µl)	6	TEMED (µl)	3

Table 2.2: 8 % SDS-PAGE resolving gel and stacking gel components.

Two-Dimensional Gel Electrophoresis

2D gel electophoresis allows the separation of proteins according to charge in the first dimension and molecular weight in the second dimension. The first dimension, isoelectric focusing (IEF), was carried out in single gel tubes using the Bio-Rad mini-Protean II IEF apparatus (Bio-Rad). A pH gradient was set up and maintained by an alkaline buffer (20 mM NaOH) in the upper chamber and an acidic buffer (10 mM H_3PO_4) in the lower chamber. Sample lysates were prepared in 2D-non-reducing sample buffer (9.5 M urea, 2 % Triton X-100, 1.6 % Bio-Lyte 5/7 ampholyte, 0.4 % 3/10 ampolyte) and ran at a constant voltage of 500 V for 4 hours or until the dye front reached the bottom of the gel tube. When the first dimension was complete, the gel was gently removed from the glass tube, loaded on to an 8 % SDS-PAGE gel and covered in 200 µl of SDS sample buffer without reducing agent (2 % SDS, 20 % glycerol, 0.05 %

bromophenol blue, 0.5 M Tris pH 6.8). The second dimension was run at a constant voltage of 50 V in a Tris/glycine buffer (25 mM Tris, 192 mM glycine, 0.1 % SDS) for 25 minutes, or until the dye front had passed from the stacking gel into the resolving gel, and then at a constant voltage of 150 V for 45 minutes, or until the dye front reached the bottom of the gel. 2D gels were then subject to western blotting.

First dimension IEF gel		
Urea	92 M	5 g
30 % 37.5:1 acrylamide: bisacrylamide	4 %	1.33 ml
NP40	2 %	2 ml 10 % NP40
Bio-Lyte 3/10 ampholyte	2 %	0.5 ml
APS	0.02 %	20 μl
TEMED	0.1 %	10 μl
H_2O		1.97 ml

Table 2.3: First dimension IEF gel components.

Western Blot

Protein samples resolved by SDS-PAGE were transferred onto nitrocellulose membranes (Whatman, Protran (VWR)) for 30 – 60 minutes at 100 V in a Tris/glycine buffer (25 mM Tris, 192 mM glycine). Protein transfer was quickly assessed using the non-permanent protein stain, Ponceau S (0.1% Ponceau S in 5% acetic acid). Membranes were briefly washed in PBS containing 0.1% Tween 20 (PBST) before being blocked in

blocking buffer (PBST + 5% (w/v) skimmed milk powder) for 30 minutes. Primary antibody was diluted into PBST and incubated with the membrane for 1 hour at room temperature unless specified. Membranes were given three 10 minute washes with PBST and then if necessary membranes were incubated with the appropriate peroxidase-conjugated secondary antibody diluted in PBST. Membranes were then given three further 10 minute washes with PBST. Antibody binding was visualised using ECL (enhanced chemiluminescence) reagent (Supersignal Femto substrate (Pierce) or Immobilion western HRP substrate (Millipore)) and either exposure to film (Sigma) or using the Fujifilm Intelligent Dark Box LAS-3000 (FUJIFILM). Band intensity measurements were carried out using Aida Image Analyzer software.

Antibodies

Primary antibodies used for this work are listed in Table 2.4.

Antibody	Specificity	Species	Source
HC10	Detects an epitope in the α1 domain of unfolded HLA-B and -C heavy chains not associated with β_2m (Stam et al., 1986).	Mouse	Hybridoma culture supernatants.
ME1	A conformation dependent antibody that detects an epitope in the α1 domain of multiple HLA-B alleles including HLA-B27, -B7, -B42, -B36, -and –Bw22 (Ellis et al., 1982).	Mouse	
W6/32	A conformation-dependent antibody that detects an epitope in the α2 domain of most B2m-associated HLA-A, HLA-B and HLA-C molecules (Barnstable et al., 1978).	Mouse	
BB7.2	A confirmation dependent antibody that detects folded human HLA-A2 (Parham and Brodsky, 1981).	Mouse	
pk	Detects the 14 amino acid tag, SV5 (GKPIPNPLLGLDST), derived from a small epitope on the P and V proteins of the paramyxovirus of simian virus 5 (Hanke et al., 1992).	Mouse	Gift from Professor Rick Randell (St Andrews).
HCA2	Preferentially detects free HLA-A class I heavy chains (Stam et al., 1990).	Mouse	Gift from Professor Jacques Neefjes (Amsterdam).
FITC-B27	A conformation-dependent antibody that labels HLA-B27.	Mouse	One Lamba Inc.
ERp57	Recognises human ERp57.	Rabbit	Gift from N. Bulleid (Manchester, UK)

Antibody	Specificity	Isotype	Source
148.3	Recognises human TAP (transporter associated with antigen processing).	Mouse	Gift from Professor Robert Tampe, Germany.
Anti-human tapasin (Giles)	Recognises human Tapasin.	Rabbit	Gift from T. Elliot (Southampton, UK)
GILT	Recognises precursor and mature chain GILT of mouse, rat and human origin.	Goat	Santa Cruz
CD48	Recognises mouse, rat and human CD48.	Mouse	Santa Cruz
BiP	Recognises the C-terminus of mouse, rat and human BiP.	Rabbit	Santa Cruz
Anti-Flag	Recognises the Flag epitope.	Mouse	Sigma
β-actin	N-terminal peptide of β-actin.	Mouse	Sigma
Alix	Detects full-length Alix	Mouse	Santa Cruz
PDI	Recognises human PDI (protein disulphide isomerase.)	Rabbit	Stressgen
Syntaxin	Recognises human syntaxin 4 aa 1-280	Mouse	Gift from Professor Simon Guild (St Andrews, UK)
Vimentin	Recognises human vimentin	Rabbit	Gift from Professor Simon Guild (St Andrews, UK)

Table 2.4: Primary antibodies used throughout this study. The specificity, species of origin and source of antibodies used in this study are detailed above.

Simply Blue Safe Stain

SDS-PAGE gels were washed three times for 5 minutes in distilled water to remove any excess SDS. Gels were covered with Simply Blue Safe Stain (Invitrogen) and incubated with agitation for 30 minutes. Gels were then destained in repeated changes of distilled water. If necessary, gels were stained once more and destained again.

Immunoprecipitation

Cell lysates that had been subject to centrifugation at 14,000 rpm (20,000 x g) were then pre-cleared with 20 µl protein-G-sepharose beads with rotation at 4 °C for 35 minutes. Samples were then immunoprecipitated using 20 µl protein-G beads and antibody at 4 °C for 1 hour. Beads were washed three times with lysis buffer before being resuspended in non-reducing sample buffer. Samples were heated at 90°C to elute antibody-antigen complexes from the beads. Samples were analysed by SDS-PAGE and western blotting.

Pulse-Chase Analysis

Cells were starved in methionine-free RPMI for 15 minutes at 37 °C. After this time 3.7 MBq [^{35}S] methionine (MP Biomedicals) was added, samples were vortexed to ensure proper mixing before being incubated at 37 °C for 15 minutes. Labelling was stopped by

pelleting the cells and resuspending in medium containing cold methionine. An aliquot was removed and kept on ice (time point zero). The rest of the sample was returned to 37 °C. After 30 and 90 minutes equal aliquots of cells were removed. Cell pellets were resuspended in lysis buffer and immunoprecipitations were performed.

Endoglycosidase H Digestion

Following immunoprecipitation, samples, in 20 µl of lysis buffer, were treated with 1 mU Endoglycosidase H (Roche) at 37 °C for 30 minutes before being resuspended in 20 µl reducing sample buffer. Samples were resolved by SDS-PAGE.

Fixing and Developing Radioactive Gels

Gels were fixed in 30 % methanol and 10 % glacial acetic acid (GAA) for 10 minutes before being dried (Bio-Rad vacuum gel dryer). Dried gels were exposed to x-ray film (Sigma) for 24 hours up to 7 days at -80 °C in a cassette with intensifying screens. For some exposures Kodak BioMax MS film was used (Sigma).

Biotinylation with Streptavidin Pull-Down

Approximately 5-10 x 10^6 cells were washed 3 times in PBS before being resuspended in 10 mM NEM on ice for 10 minutes. Cells were pelleted and washed once in PBS before being incubated with 0.1 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce) in PBS on ice for 10 minutes. Cell pellets were resuspended in TBS + 5 % FCS and kept on ice for a further 10 minutes in order to quench any free biotin. Cell pellets were washed twice in TBS before cell lysis.

Cell lysates were subject to 14,000 rpm (20,000 x g) centrifugation at 4°C to remove cellular and nuclear debris. The remaining lysate was pre-cleared with 20µl of protein G beads for 25 minutes with rotation at 4 °C. Samples were then spun at 14000 rpm (20,000 x g) at 4 °C for 4 minutes, the pre-cleared lysate was removed and tumbled with 20 µl Streptavidin immobilised on agarose (Fluka) for 25 minutes at 4 °C. Beads were washed 3 times with PBS before being resuspended in 20 µl NRSB and heated at 90 °C for 2 minutes. Samples were resolved by 8 % SDS-PAGE.

Flow Cytometry

On ice, cells were washed three times in PFN (PBS + 2 % FBS + 0.1 % sodium azide) before being stained (0.5-1 x 10^6 cells/condition) with appropriate antibody for 35 minutes at 4 °C. Cells were again washed three times to remove any unbound antibody,

followed, if necessary, by incubation with FITC-conjugated secondary antibody. A further three washes were performed with PFN on ice before flow cytometric analysis on a Beckton Dickinson FACScan. Data acquisition and analysis were performed using CellQuest software.

Techniques Used For Exosome Analysis

Isolation of Exosomes

Exosomes were isolated using established procedures. Cells were centrifuged at 1500 rpm (1000 x g) to pellet before washing once in sterile PBS. Cells were recultured in 5 ml of fresh serum-free medium and returned to the incubator for 24 hours. Cells were then pelleted at 1500 rpm (1000 x g) and the supernatent was subject to filtration through a 0.2 μm filter (Nalgene) and ultracentrifugation at 34,000 rpm (100,000 x g) for 2 hours using an SW55Ti rotor in a Beckman L7-65 centrifuge. The supernatent was then discarded and exosome pellets were resuspended in 30-100 μl of PBS or lysis buffer. Protein concentration was determined using the Bradford method. This filtration technique produced the same results as test experiments where supernatants were treated with sequential centrifugation: 1000 x g, to remove cells, 10,000 x g to remove debris, and 100,000 x g to isolate exosomes.

Electron Microscopy

Exosomes were prepared as previously described and resuspended in filter sterile PBS. Electron Microscopy was performed at the University of Dundee by Dr Alan Prescott. Briefly exosomes were loaded onto grids and air dried, then negatively stained.

Mass Spectrometry

Mass spectrometry analysis was performed by Dr Catherine Botting in the Biomedical Sciences Department (BMS), University of St Andrews. Briefly, exosomes run on SDS-PAGE gels were stained with Simply Blue Safe Stain (Invitrogen), washed with distilled water, before the bands of interest were cut out using clean scalpel blades. These samples then underwent in-gel trytpic digestion and mass spec analysis using the in house service at BMS.

Binding of Exosomes to Aldehyde/Sulfate Latex Beads

Approximately 30 μg of exosomes were incubated with 10 μl of 4 μm diameter aldehyde/sulfate latex beads (Interfacial Dynamics) for 15 minutes at room temperature, followed by 1 hour at 4 °C with gentle agitation in 1 ml of PBS. The reaction was stopped by a 30 minute incubation in 100 mM glycine. Exosome-coated beads were washed three times in PFN and resuspended in 500 μl of PFN. Beads were incubated for

35 minutes with each primary antibody, followed when necessary by incubation in FITC-conjugated secondary antibody. Further washes were performed with PFN before cytometric analysis on a Beckton Dickinson FACScan. Data acquisition and analysis were performed using CellQuest software.

Immobilising Exosomes in Agar

Approximately 2.5 x 10^5 cells were washed once before being resuspended in 200 μl of sterile PBS. 3 μl of bodipy ceramide (Molecular Probes, Invitrogen) stain was added to the cells and they were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in air for 20 minutes. Cells were then washed three times in sterile PBS before being resuspended in 50 μl. 3 % agar was melted before being mixed with warmed sterile PBS to create a 0.6 % agar solution. 10 μl of stained cells were mixed with 10 μl of 0.6 % agar solution (final concentration 0.3 %) and mounted on to microscope slides (VWR). Slides were maintained in a humidified chamber at 37 °C in a humidified atmosphere of 5 % CO_2 in air for 24 hrs. Slides were sealed with nail varnish and viewed under 100 x magnification using a fluorescence microscope.

Isolation of Bodipy Stained Exosomes

Cells were washed once in serum free medium before being stained with bodipy ceramide. Cells were incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in air

for 20 minutes. Cells were then washed three times in serum free medium before being resuspended in 5 ml of serum free medium, transferred to a six well plate and incubated at 37 °C in a humidified atmosphere of 5 % CO_2 for 24 hours. Exosomes were isolated as previously described and incubated with KG1 cells grown on coverslips (VWR).

Incubation of Jesthom Exosomes with KG1 Cells

KG1 dendritic-like cells, either grown in 6 well plates or on sterile coverslips, were stimulated with PMA (10 ng/ml) and Ionomycin (100 ng/ml) for 3 hours to start their maturation into dendritic cells. The cells were then incubated with exosomes for 24 hours. Exosomes were isolated from a 70 ml culture of Jesthom cells (usually corresponding to approximately 5-10 μg of exosomes), and half of this preparation was used for immunofluorescence and half for FACS and lysate preparation. Samples were analysed by FACS, or lysed and then analysed by SDS-PAGE and western blotting or stained with appropriate antibody for immunofluorescence microscopy.

Immunofluorescent Microscopy

Cells grown on coverslips were gently washed in PFN on ice before staining with the appropriate antibody for 35 minutes. Cells were washed three times in PFN to remove any unbound antibody and, if necessary, were incubated with the appropriate FITC-conjugated secondary antibody for 35 minutes on ice. Cells were washed a further three

times with PFN before being mounted, using Vectashield with DAPI (Vector Laboratories) on microscope slides. Slides were sealed with nail varnish and viewed under 100 x magnification using a fluorescence microscope.

Incubation with Glutathione (GSH)

Cells were incubated in serum-free medium supplemented with varying concentrations of GSH (10 mM, 1 mM, 0.1 mM) for 6 hours before exosomes were isolated as previously described. Samples were lysed and then analysed by SDS-PAGE and western blotting.

Cholesterol Depletion

Cells were incubated in medium supplemented with 10 mM methyl-β-cyclodextrin (MBC) for 24 hours at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Exosomes were then isolated from the culture supernatent as previously described. Samples were lysed and analysed by SDS-PAGE and western blotting.

Separating Peripheral Blood Mononuclear Cells and Isolating Exosomes

Local ethical approval for this study was obtained from UTREC (University of St Andrews Teaching and Research Ethics Committee).

Peripheral blood was collected from healthy donors, after giving informed consent, in tubes containing 50 units of preservative-free heparin (Vacutainer, BD Bioscience) and PBMCs were isolated by Histopaque (Sigma) density gradient centrifugation. Blood samples were overlaid onto an equal volume of Histopaque and centrifuged for 30 minutes at 250 x g with no brake. The buffy-coat layer was isolated using a sterile Pasteur pipette and washed three times with a large volume of PBS + 0.1 % BSA. Cells were then resuspended in DMEM containing 10 % heat-inactivated serum and 0.1 mg/ml kanamycin sulphate and incubated at 37 °C in a humidified atmosphere of 5 % CO_2 in air for at least 2 hours. This allowed time for the monocytes to adhere to the tissue culture plates leaving the lymphocytes in suspension. Non-adherent cells were then removed by gentle rinsing. Isolated lymphocytes were either left untreated or treated with PHA (phytohaemagglutinin) (1 μg/ml) for 48 hours. Exosomes were isolated from the cell supernatants and the cells were used to make cell lysates.

The adherent monocyte population was stimulated with IL-4 (50 ng/ml) and GM-CSF (50 ng/ml) for 5 days, cells were then either left untreated or treated with LPS

(lipopolysaccharide) (50 ng/ml) for 48 hours. Exosomes were isolated from the cell supernatants and the cells were used to make cell lysates.

Isolating Exosomes from Plasma

Peripheral blood was collected from healthy donors in tubes as above. The blood was separated using Histopaque (Sigma) density gradient centrifugation. Blood samples were overlaid onto an equal volume of Histopaque and centrifuged for 30 minutes at 250 x g with no brake. The plasma was removed from the top of the preparation using a sterile Pasteur pipette. Plasma samples were centrifuged at 500 x g for 30 minutes at 4 °C. The supernatent was then removed and spun again for 45 minutes at 12,000 x g at 4 °C. The top 90 % of the supernatent was removed and spun at 100,000 x g for 2 hours using an SW55Ti rotor in a Beckman L7-65 centrifuge. The resulting pellet was resuspended in approximately 9 ml of 0.22 μm filter-sterile PBS. Samples were filtered through a 0.22 μm filter and subject to ultracentrifugation again at 100,000 x g for 2 hours. The supernatant was then discarded, and exosome pellets were resuspended in lysis buffer before analysis by SDS-PAGE and western blot.

GSH Assay

GSH concentrations were estimated using a Glutathione Assay Kit as per manufacturer's instructions (Sigma). Briefly, exosomes were isolated from Jesthom cell supernatent as

previously described and resuspended in filter-sterile PBS. Jesthom cell lysates were prepared as previously described. Protein concentrations of each sample were estimated using the Bradford method. Samples were deproteinated according to the manufacturer's instructions and the GSH concentration of equal protein concentrations of exosomes and lysates were measured as per manufacturer's instructions at a wavelength of 380 nm using a FLUOstar Optima plate reader (BMG Labtech). This assay measures total GSH content using an assay in which GSH is involved in the reduction of DTNB to TNB, with the resulting oxidised GSSG being recycled by glutathione reductase and NADPH. The yellow colour of the resulting TNB is measured.

Molecular Biology Techniques

Total RNA Isolation

RNA was isolated from cells using the RNeasy® mini kit (Qiagen) as per manufacturer's instructions.

RNA Quantification

RNA was quantified using the Quant-iT™ RNA assay kits and the Qubit™ fluoremeter (Invitrogen) as per manufacturer's instructions.

RT-PCR

RT-PCR (reverse transcription-polymerase chain reaction) was carried out using the Qiagen OneStep RT-PCR kit as per manufacturer's instructions. Briefly, buffer, dH_20, dNTPs, enzyme mix (Reverse Transcriptase and Taq Polymerase) and 0.5 µg of RNA were mixed before the addition of primers. Reverse transcription was carried out at 50°C for 30 minutes before denaturation of the Reverse Transcriptase enzyme at 95°C for 15 minutes.

PCR was performed for 35 amplification cycles with the following protocol: denaturation at 94°C for 30 seconds, primer annealing at 50°C for 30 seconds, primer extension at 72°C for 2 minutes.

Reaction Mix for RT-PCR	
5 x Buffer (µl)	2.5
dH20 (µl)	8 – RNA volume
dNTP (µl)	0.5
Enzyme mix (µl)	0.5
Primers (µl)	0.5 + 0.5
RNA (µg)	0.5

Table 2.5: Components of the reaction mix used for RT-PCR.

PstI Digest

RT-PCR products were treated with a final concentration of 1 U/μl of PstI (NEB) at 37 °C for 90 minutes before being resolved by DNA-PAGE.

DNA-PAGE

DNA samples were diluted 1:6 with loading dye (0.5 ml 0.1M EDTA, 0.4 ml (30 % w/v) ficol 400, 0.05 % bromophenol blue) before being resolved on 5 % poly-acrylamide gels. Gels were run at a constant voltage of 100 V for 45 minutes or until the bromophenol blue dye front reached the bottom of the gel.

5 % Polyacrylamide Gels for DNA-PAGE	
H_20 (ml)	3.67
30 % 29:1 acrylamide: bisacrylamide (ml)	0.83
10 x TBE (ml)	0.5
APS (μl)	50
TEMED (μl)	5

Table 2.6: Components of 5 % polyacrylamide gels for DNA-PAGE.

Silver Staining of DNA-PAGE

The DNA was fixed into the gels by immersing in 7.5 % glacial acetic acid (GAA) for 5 minutes, followed by three 20 minute washes with distilled water. Gels were incubated with silver nitrate solution (0.1 % (w/v) silver nitrate, 0.15 % (v/v) formaldehyde) for 30 minutes, before being rinsed briefly with distilled water to remove any surface silver nitrate. Gels were then immersed in ice-cold developer solution (3 % (w/v) sodium carbonate, 0.15 % (v/v) formaldehyde, 0.00004 % (w/v) sodium thiosulphate). The reaction was stopped with ice-cold 7.5 % GAA when the DNA was sufficiently visible.

Design of Mutagenic Primers

Mutagenic primers were designed using the bioinformatics program, Primer X (http://bioinformatics.org/primerx).

Generation and Expression of Mutant HLA-B27

Mutagenesis experiments were carried out on full-length HLA-B27 cDNA contained within the pCR3 plasmid. HLA B27 cDNA was incubated with the mutagenic primers, dNTPs, Tgo buffer (Roche) and Tgo DNA polymerase (Roche) and temperature cycled for 15 cycles at 95 °C for 30 seconds, 58 °C for 1 minute and 68 °C for 12 minutes. The parental strand was digested using 0.5 U/μl (final concentration) of restriction enzyme

DpnI (NEB) at 37 °C for 1 hour and 30 minutes. The mutated dsDNA was transformed into JM109 competent cells (Promega).

Transforming Competent JM109 Bacterial Cells

Approximately 100 ng of DNA was added to 10-25 µl of JM109 cells and left for 30 minutes on ice. The cells and DNA were then heat shocked at 42 °C for 1 minute and then returned to ice for a further 2 minutes. The transformed bacteria were incubated with 150 µl of Luria Broth (LB) with shaking at 37 °C for 60 minutes, allowing expression of the resistance gene. Samples were plated on LB agar plates containing 100 µg/ml ampicillin. Plates were incubated overnight at 37 °C. Single colonies were selected and grown overnight at 37 °C in 5 ml LB supplemented with 100 µg/ml ampicillin. The bacterial cultures were then used for plasmid purification using Qiagen plasmid mini/midi prep kits as per manufacturer's instructions, or frozen with glycerol to create stocks.

Sca1 Digestion and Agarose Gel Electrophoresis

Purified plasmid DNA was checked by restriction digestion with Sca1 (Roche) which linearises the plasmid. Samples were analysed on 1 % agarose gels, containing 0.05 % ethidium bromide, using 1 x TAE buffer (5 mM sodium acetate, 1 mM EDTA, 40 mM

Tris, pH 7.5) at a constant voltage of 100 V for 45 minutes. DNA was visualised on an ultraviolet (UV) transilluminator.

DNA Sequencing

Plasmids were checked and confirmed by DNA sequencing by the Sequencing Service at the University of Dundee.

Supplier Addresses

Amaxa: Amaxa Biosystems, Cologne, Germany.

BD Bioscience: BD Bioscience, Oxfordshire, UK.

BDH: Supplied by VWR.

Beckman Coulter: Beckman Coulter Ltd, Buckinhamshire, UK.

Becton Dickinson: BD Bioscience, Oxfordshire, UK.

Bio-Rad: Bio-Rad Laboratories Ltd, Hertfordshire, UK.

BMG Labtech: BMG Labtech Ltd, Aylesbury, UK.

Dynal Biotech: Supplied by Invitrogen.

FUJIFILM: Fujifilm UK Ltd, Bedford, UK.

Interfacial Dynamics: Supplied by Invitrogen.

Invitrogen: Invitrogen, Paisley, UK.

Melford: Melford Laboratories Ltd, Suffolk, UK.

Millipore: Millipore (UK) Ltd, Hertfordshire, UK.

MP Biomedicals: MP Biomedicals, Cambridge, UK

Nalgene: Supplied by VWR.

NEB: New England BioLabs, Hertfordshire, UK

Pierce: Perbio Science UK Ltd, Northumberland, UK.

Promega: Promega Corporation, Southampton, UK

Qiagen: Qiagen Ltd, West Sussex, UK.

Roche: Roche Applied Science, Roche Diagnostics Ltd, West Sussex, UK.

Santa Cruz: Santa Cruz Biotechnology Inc, Heidleberg, Germany

Sigma: Sigma Aldrich, Dorset, UK.

Thistle Scientific: Thistle Scientific Ltd, Glasgow, UK.

Vector Laboratories: Vector Laboratories Ltd, Peterborough, UK.

VWR: VWR International, Leicestershire, UK.

Chapter III: HLA-B27 and the UPR

Introduction

The folding of most proteins within the endoplasmic reticulum is tightly regulated by molecular chaperones. If misfolded proteins accumulate within the ER a stress response, known as the Unfolded Protein Response (UPR), is activated. The aim of the UPR is primarily to alleviate stress and re-establish normal physiological conditions. Activation of the UPR results in transcriptional induction of UPR genes, translational attenuation of protein synthesis, and ER-associated degradation (ERAD). The UPR, if not resolved, will eventually result in apoptosis.

HLA-B27 has slower folding kinetics than other class I alleles and is prone to misfolding, resulting in the formation of dimers and multimers (Mear et al., 1999). It was postulated that the accumulation of these misfolded proteins could lead to the activation of a pro-inflammatory ER stress response (Colbert, 2000). The inherent ability of HLA-B27 to misfold could put cells into a state of continuous stress and therefore continuous activation of the UPR.

The Unfolded Protein Response

Within the ER, protein folding is regulated by molecular chaperones. One such key chaperone, which is also of key relevance to the UPR, is the immunoglobulin binding protein (BiP). BiP is involved in the regulation of the three main transducers of the UPR; PKR-like ER kinase (PERK), inositol-requiring enzyme-1 (IRE1) and activating transcription factor 6 (ATF6). Under normal conditions, BiP acts as a negative regulator of IRE1 (Okamura et al., 2000), PERK (Bertolotti et al., 2000) and ATF6 (Shen et al., 2005) activation, but when misfolded proteins accumulate BiP dissociates from the UPR effectors and associates instead with the misfolding proteins.

PERK

PERK is an ER transmembrane protein with protein kinase activity that is involved in the attenuation of protein synthesis in response to ER stress (Harding et al., 1999). PERK is retained in an inactive form through stable binding to BiP, and is released in response to ER stress (Bertolotti et al., 2000). Once released PERK subunits can dimerise, activating their kinase ability. Activated PERK is then able to phosphorylate the α subunit of eukaryotic translation initiation factor-2α (eIF2α) (Harding et al., 2000b), which results in the transient suppression of protein translation, reducing the load of newly synthesised proteins on the ER. Phosphorylated eIF2α also targets cyclin D1 for degradation, preventing stressed cells from progressing through the cell cycle (Raven et al., 2008).

Transcription of some specific UPR target genes also occurs in response to phosphorylated eIF2α, such as the transcription factor ATF4 (Harding et al., 2000a). Translational recovery is mediated by GADD34 (growth arrest and DNA damage-inducible gene 34), which is upregulated by ATF4 and acts by dephosphorylating eIF2α (Novoa et al., 2001). Phosphorylated eIF2α can also activate NF-κB potentially leading to the expression of cell protective genes (Jiang et al., 2003).

If the stress response is not perturbed, the cell will undergo apoptosis, a process that is potentiated by the pro-apoptotic transcription factor CHOP, which is expressed in response to ATF4 (Harding et al., 2000a).

ATF6

There are two isoforms of ATF6 in mammals, ATF6α and ATF6β, both thought to have a role in the UPR (Haze et al., 2001). However evidence suggests ATF6β is a poor transcriptional activator that actually inhibits activation by ATF6α (Thuerauf et al., 2004).

ATF6 is a type I transmembrane protein with its C-terminus located in the ER lumen and its N-terminal domain facing the cytosol (Haze et al., 1999, Haze et al., 2001). It is constitutively expressed and is regulated through stable binding to BiP (Shen et al., 2005). Dissociation from BiP exposes a golgi-localisation signal causing ATF6 to relocate to the golgi apparatus (Shen et al., 2002).

Within the golgi ATF6 undergoes regulated intramembrane proteolysis and is sequentially cleaved by two serine proteases, site-1 protease (S1P) and site-2 protease (S2P). S1P cleaves ATF6 in the luminal domain and S2P cleaves the N-terminal membrane-anchored half (Ye et al., 2000). The resulting cytosolic fragment translocates to the nucleus, where it can bind to the promoter region and initiate translation of ER stress response target genes (Adachi et al., 2008, Kokame et al., 2001, Wang et al., 2000, Yoshida et al., 1998).

ATF6 targets include the molecular chaperone BiP (Haze et al., 1999), XBP1, CHOP (Yoshida et al., 2000), and $P58^{IPK}$ (van Huizen et al., 2003). XBP1 mRNA is a target for activated IRE1 during an UPR, and once translated acts as a transcription factor for UPR target genes (Lee et al., 2003). Overexpression of CHOP leads to growth arrest and promotes apoptosis (reviewed in Oyadomari and Mori, 2004), and it has been suggested that upregulation of $p58^{IPK}$ may serve to regulate the stress response in a negative feedback manner by interacting with PERK and inhibiting its kinase activity (Lee et al., 2003, van Huizen et al., 2003, Yan et al., 2002).

IRE1

IRE1 is an ER transmembrane glycoprotein that contains both kinase and RNase activities in its cytoplasmic domain (Cox et al., 1993, Sidrauski and Walter, 1997). Mammals have two copies of IRE1, IRE1α (Tirasophon et al., 1998) and IRE1β (Wang et al., 1998). IRE1, like PERK, is retained in an inactive state by stable binding to the

molecular chaperone BiP, and is released from BiP when a stress response is activated (Okamura et al., 2000, Bertolotti et al., 2000). This results in auto-phosphorylation and the subsequent activation of IRE1's RNase activity (Shamu and Walter, 1996). The substrate for IRE1 is the mRNA of the transcription factor XBP1 (Yoshida et al., 2001, Calfon et al., 2002). XBP1 transcription is increased in response to ATF6 signaling (Yoshida et al., 2001).

XBP1 mRNA is constitutively produced in a full-length, or unspliced, form (XBP1u). Activated IRE1 cuts XBP1 twice, removing a 26 nucleotide intron from the XBP1 transcript, the two mRNA fragments are ligated together causing a frameshift in the spliced XBP1 transcript (XBP1s), and following translation, XBP1s functions as a potent activator of UPR target genes (Calfon et al., 2002). Both XBP1s and XBP1u mRNA are translated into proteins, with XBP1u acting as an inhibitor of the UPR (Yoshida et al., 2006).

Many XBP1s-specific target genes have been identified (Lee et al., 2003), including; EDEM, a protein involved in the ER-associated degradation (ERAD) pathway (Hosokawa et al., 2001, Oda et al., 2003). ERAD involves the retrotranslocation of misfolded or aggregated proteins from the ER to the cytosol where they undergo proteasomal degradation (reviewed in Meusser et al., 2005); RAMP4, a protein implicated in glycosylation and stabilization of membrane proteins in response to stress (Yamaguchi et al., 1999) and $p58^{IPK}$ which has been implicated in a negative feedback role for controlling the UPR (Lee et al., 2003, van Huizen et al., 2003, Yan et al., 2002).

In addition to the splicing of XBP1 mRNA, IRE1 has also been shown to be involved in the degradation of ER-targeted mRNA transcripts (Hollien and Weissman, 2006) and cleavage of the 28S ribosomal subunit (Iwawaki et al., 2001) suggesting that IRE1 also has a role in translational attenuation in response to ER stress.

If the stress response is not curtailed, IRE1 is also implicated in apoptosis through the recruitment of TRAF2 (TNF receptor-associated factor 2) (Urano et al., 2000). The IRE1/TRAF2 complex recruits ASK1 (apoptosis signal-regulating kinase 1) activating the downstream JNK (c-jun N-terminal kinase) pathway that eventually leads to cell death (Nishitoh et al., 1998, Nishitoh et al., 2002).

As part of an activated UPR an inflammatory response is initiated to help stimulate apoptosis and to repair tissue damage caused by UPR-associated apoptosis. NF-κB is induced in response to both PERK and IRE1 downstream signaling pathways (Jiang et al., 2003, Hu et al., 2006). NF-κB leads to the expression of TNFα, which has an important role in inflammation but also, in the context of ER stress, has an important pro-apoptotic function (Hu et al., 2006).

Homeostasis in the ER is maintained through the coordinated actions of the three branches of the UPR. The main aim is to alleviate stress through enhancing molecular chaperones and reducing further stress by preventing unnecessary transcription. If these mechanisms fail however, the cell will undergo apoptosis removing unhealthy cells from the system.

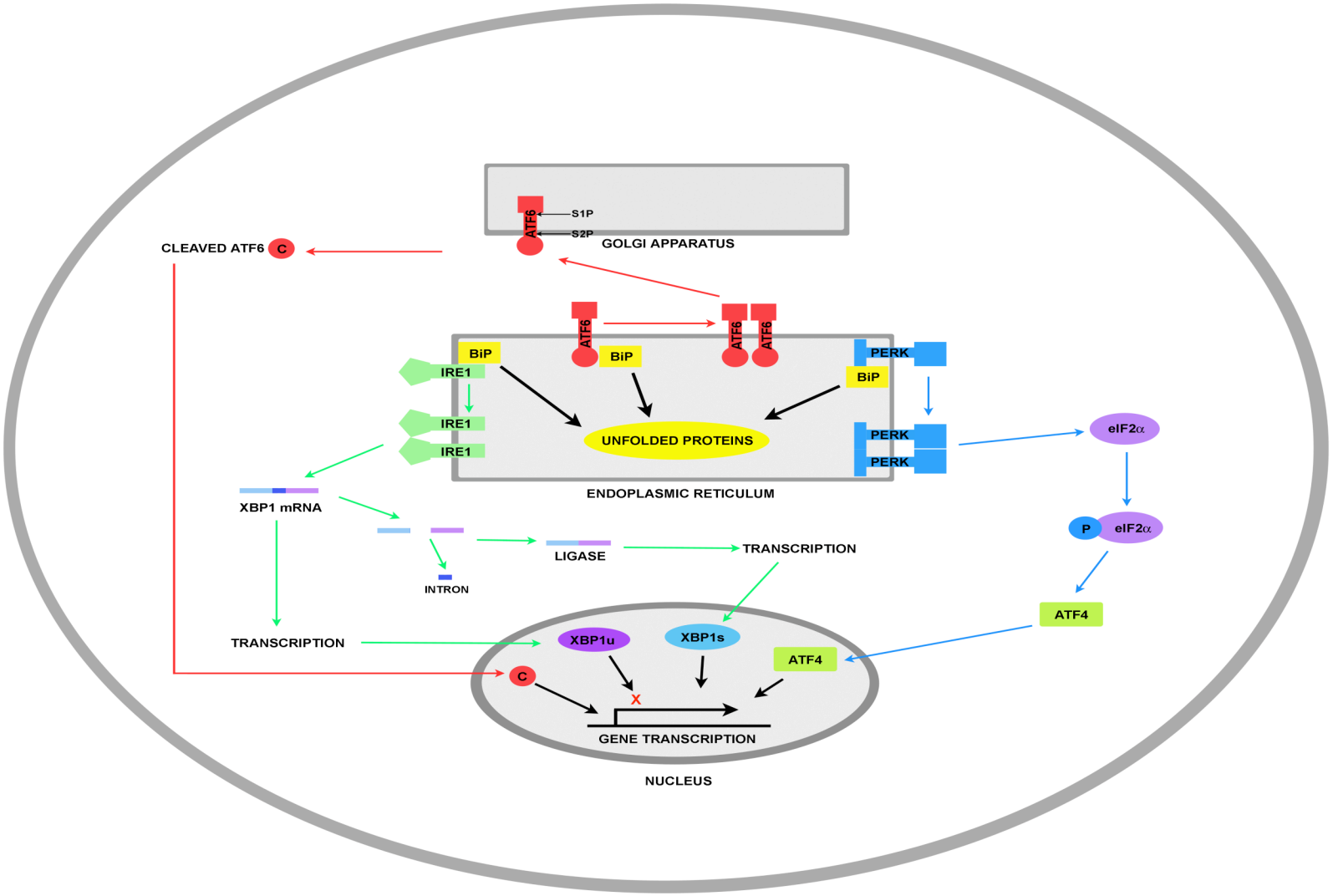


Figure 3.1: The Unfolded Protein Response. When misfolded proteins accumulate in the ER, the UPR is activated. The aim of the UPR is to remove the cause of the stress response and re-establish normal physiological conditions.

HLA-B27 and the UPR

HLA-B27 has slower folding kinetics than other class I alleles and a tendancy to misfold, a characteristic attributed to residues in the B pocket (Mear et al., 1999, Antoniou et al., 2004). This observation formed the basis of the hypothesis proposed by Colbert in 2000, in which he suggested that the misfolding of HLA-B27 was associated with the activation of a pro-inflammatory ER stress response (Colbert, 2000).

The slower folding of B*2705 has been directly linked with the formation of homodimers in the ER, with dimer structures able to be induced in the HLA-A2 molecule when its folding efficiency is reduced at lower temperatures (Antoniou et al., 2004). ER homodimers are distinct from cell surface dimers and do not egress to the cell surface (Dangoria et al., 2002, Antoniou et al., 2004).

The misfolding hypothesis can potentially explain some observations made in transgenic rodent studies. In transgenic rats disease only occurs in those animals that have a high gene copy number with HLA-B27 expression above a certain threshold (Taurog et al., 1993), and in HLA-B27 transgenic mice, spontaneous arthritis occurs in the absence of β_2m (Khare et al., 1995). In both of these situations, HCs could be increasingly more likely to misfold resulting in the activation of a stress response.

In 2004 it was demonstrated in a rat cell line model that HLA-B27 heavy chains are found in association with BiP (Antoniou et al., 2004). Shortly afterwards it was

demonstrated, in splenocytes from HLA-B27 transgenic rats, that HLA-B27 HCs are also extensively bound to BiP (Tran et al., 2004).

Turner et al have since shown that bone marrow-derived macrophages from HLA-B27 transgenic rats show evidence of UPR activation that correlates with the accumulation of misfolded heavy chains (Turner et al., 2005, Turner et al., 2007). They also found evidence of an IFN response in cells from rats suffering with disease, a response that seems to exacerbate the UPR, probably through the up-regulation of HLA-B27 heavy chains (Turner et al., 2005). More recently a study on a human cell line model indicates that expression of HLA-B27 alone is enough to activate a stress response (Lemin et al., 2007).

To date, therefore, UPR induction associated with HLA-B27 has been described in a number of different *in vitro* and *in vivo* systems. However, we had no information on the relative contribution of the UPR, if any, to the systems that have been worked on in this laboratory for a number of years. It was therefore decided to investigate this aspect of HLA-B27 biology.

Results

Transient Expression Model for HLA-B27 and the UPR

Cells expressing HLA-B27 do not express enhanced levels of BiP compared to other alleles.

To investigate whether the expression of HLA-B27 resulted in an increase in the level of BiP, HeLa cells were transiently transfected with HLA-B27 and HLA-A2. In addition, a series of mutants of these alleles that contain a cysteine to serine mutation at the position of a structural cysteine residue, and thus fail to fold efficiently in the ER, were also utilised. All alleles contain the 14 amino acid tag SV5 (also termed V5 when commercially used from Invitrogen) on their C-terminus. This tag is recognized by the antibody pk, allowing specific detection of the transfected alleles.

HLA-A2 is a non-disease associated allele that folds efficiently within the ER and would therefore not be expected to be associated with an UPR. The structural mutants are unable to fold correctly and therefore do not exit from the ER. Accumulation of these molecules may be expected to lead to the activation of an UPR. HLA-B27 has slower folding kinetics than other class I alleles and is prone to misfolding with the ER, it has been suggested that these factors may lead to ER stress and activation of the UPR.

BiP is a major player in the UPR, and binds to unfolded proteins in an attempt to aid their folding, and an activated UPR results in a characteristic increase in the level of expression of this chaperone. If HLA-B27 is constantly misfolding this may put the cells in a state of continuous stress. To determine whether expression of this allele increases the level of the stress responsive chaperone BiP, cell lysates were subject to SDS-PAGE and western blotting with antibodies against β-actin (loading control), pk (transfection control) and BiP (Figure 3.2 A). The BiP levels were normalized against the β-actin and relative BiP levels were plotted (Figure 3.2 B).

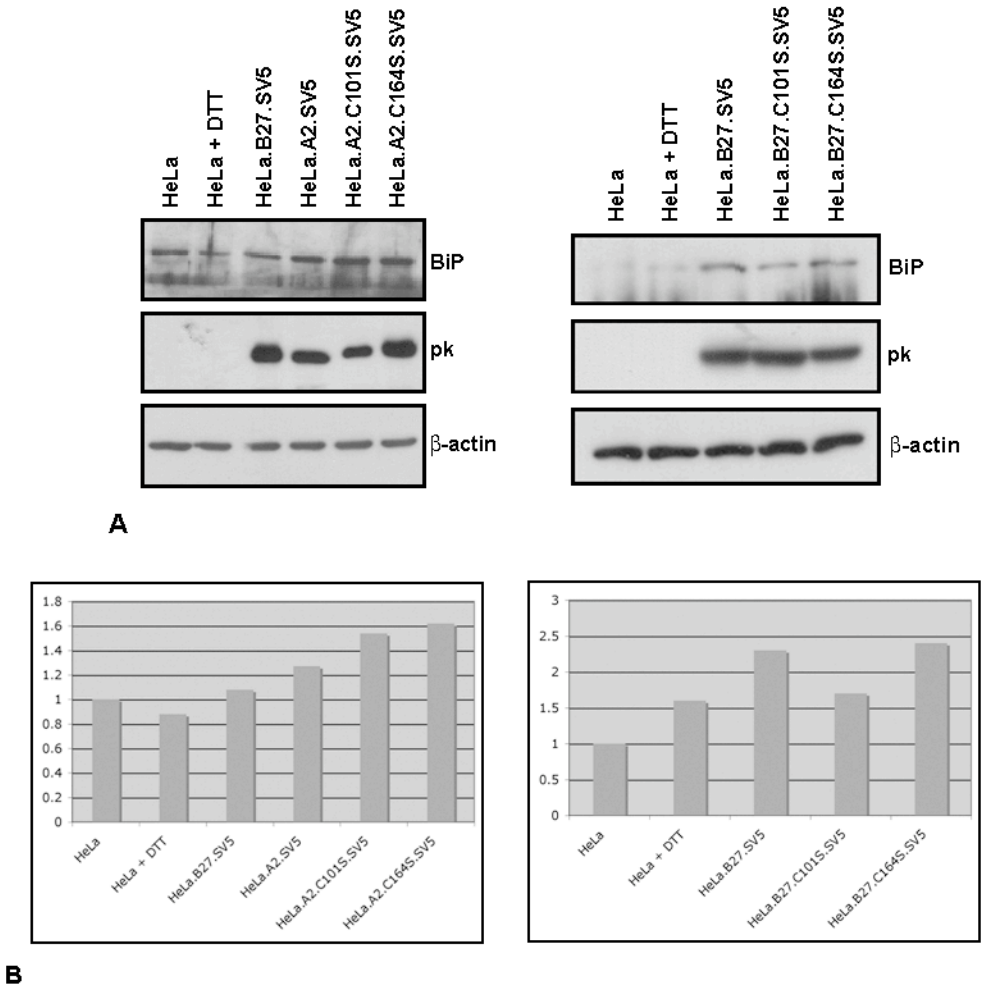


Figure 3.2: BiP levels are increased in cell lines transiently expressing disease associated and non-disease associated alleles. Cells were transfected using Fugene 6 with 1 μg of pCR3 plasmid DNA containing the appropriate class I allele cDNA. Transient transfectants were harvested 24 hours after transfection. (A) Cells were lysed and then protein quantified using the Bradford method. Samples were resolved by 8 % SDS-PAGE before western blotting. Membranes were probed with the indicated antibodies. (B) The relative BiP levels for (A) are shown after being normalised for β-actin. Results are representative of at least 3 experiments.

BiP levels were consistently higher in the transfected cell lines compared with the HeLa wild-type (wt) samples, however levels between different experiments were not consistent (Figure 3.2 A & B). Somewhat unexpectedly, HLA-A2 showed higher levels of BiP than HLA-B27. Furthermore, whilst the structural cysteine mutants usually expressed increased BiP levels, this was not always true, despite good levels of mutant expression, for example with mutant HLA-B27.C101S.SV5 (Figure 3.2 B).

Treatment of HeLa cells with DTT, a chemical that causes global stress, did not always increase the level of BiP. Possibly, the length of time of treatment with DTT (50 minutes) was not long enough to cause an increase in protein synthesis of BiP in these conditions.

XBP1 splicing occurs in cell lines expressing disease associated, non-disease associated and structural cysteine mutant alleles.

XBP1 mRNA is constitutively expressed in the cell (XBP1u). When a stress response is activated XBP1 mRNA undergoes splicing by IRE1, removing 26 bp, leaving the spliced XBP1 product (XBP1s). In order to investigate whether XBP1 was spliced or unspliced, and therefore whether a UPR was active or not, we extracted RNA from cell lines transiently expressing different HLA class I alleles and RT-PCR was performed with XBP1 specific primers (Figure 3.3), kindly donated by Dr Antony Antoniou (UCL, London).

The XBP1 primers amplify a region of XBP1 that contains the 26 bp that are spliced by IRE1 (indicated in red, Figure 3.3). Primer binding sites are indicated in blue on Figure 3.3. If XBP1 is unspliced, the amplified region of XBP1 would be 473 bp in length, and if splicing has occurred the amplified product would be 447 bp in length. The amplified region of unspliced XBP1 also contains a PstI restriction site (underlined in black, Figure 3.3). If XBP1 is cut with PstI the two resulting fragments would be 282 bp and 191 bp in length. This restriction site is lost if splicing has occurred resulting in spliced XBP1 being resistant to PstI digestion.

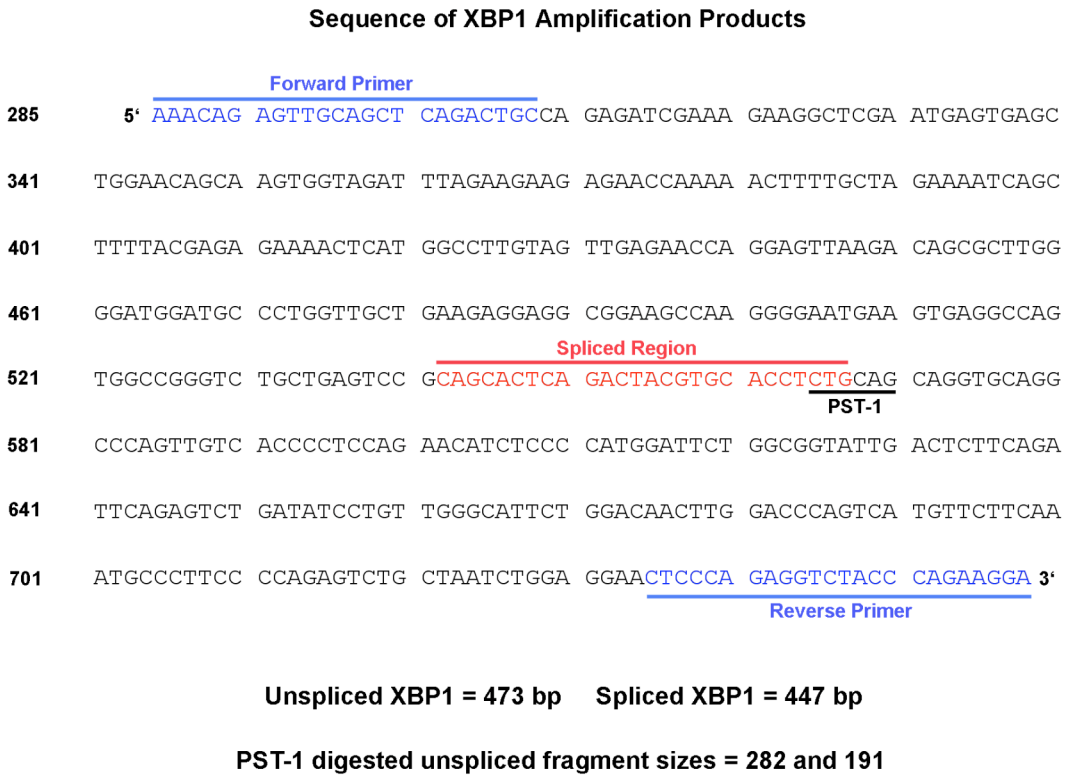


Figure 3.3: Sequence of XBP1 amplification products. Forward and reverse primers are indicated in blue. If the UPR is not active the entire region will be amplified resulting in a 473 bp product. When XBP1 is spliced during a stress response, 26 bp (indicated in red) are removed from the sequence resulting in a smaller 447 bp product. The 6 bp recognition site of PstI is indicated with a black line. If the UPR is active, and XBP1 undergoes splicing, part of this recognition site is lost resulting in the spliced product being resistant to PstI digestion. If XBP1 is unspliced then PstI cuts in the middle of the recognition site resulting in two fragments of 282 bp and 191 bp.

Amplified RT-PCR product was resolved by DNA-PAGE and silver staining. All of the transfected alleles showed splicing of XBP1 (Figure 3.4 A & B). Untransfected HeLa cells did not seem to have undergone XBP1 splicing, indicating that prior to transfection

the cells were not under going an active UPR. Overall, however, both disease and non-disease associated alleles, and structural mutants all activate XBP1 splicing events.

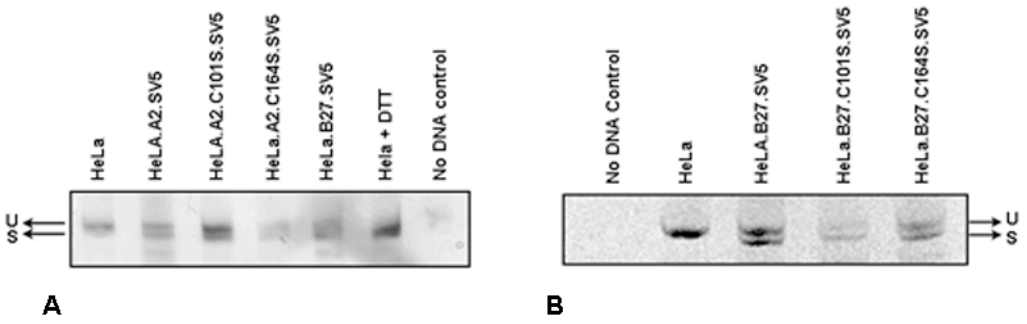


Figure 3.4: XBP1 splicing occurs in cell lines transiently expressing disease associated and non-disease associated alleles. Cells were transfected using Fugene 6 with 1 μg of pCR3 plasmid DNA containing the appropriate class I allele cDNA. Transient transfectants were harvested 24 hours after transfection. RNA was isolated from the cells and quantified. RT-PCR was carried out using the Qiagen OneStep RT-PCR kit with 0.5 μg of RNA and XBP1 specific primers. The RT-PCR product was resolved using DNA-PAGE. Gels were fixed and RT-PCR product was visualized by silver staining. Results are representative of at least 3 experiments.

To further confirm this observation, that XBP1 is present in the spliced form in the transfectants, RT-PCR products were subject to PstI digestion (Figure 3.5 A). Digestion of RT-PCR product from all of the alleles resulted in the formation of some digest products, as expected, because not all of the XBP1 was in the spliced form (Figure 3.4 A & B). However, densitometry analysis of both the unspliced and spliced XBP1 reveals that in transfected alleles at least 60 % of the XBP1 was in the spliced form compared with the HeLa cells alone where just over 20 % of the XBP1 was spliced (Figure 3.5 B). However, both the disease and non-disease associated alleles appear to have an activated stress response compared to the HeLa wt cells.

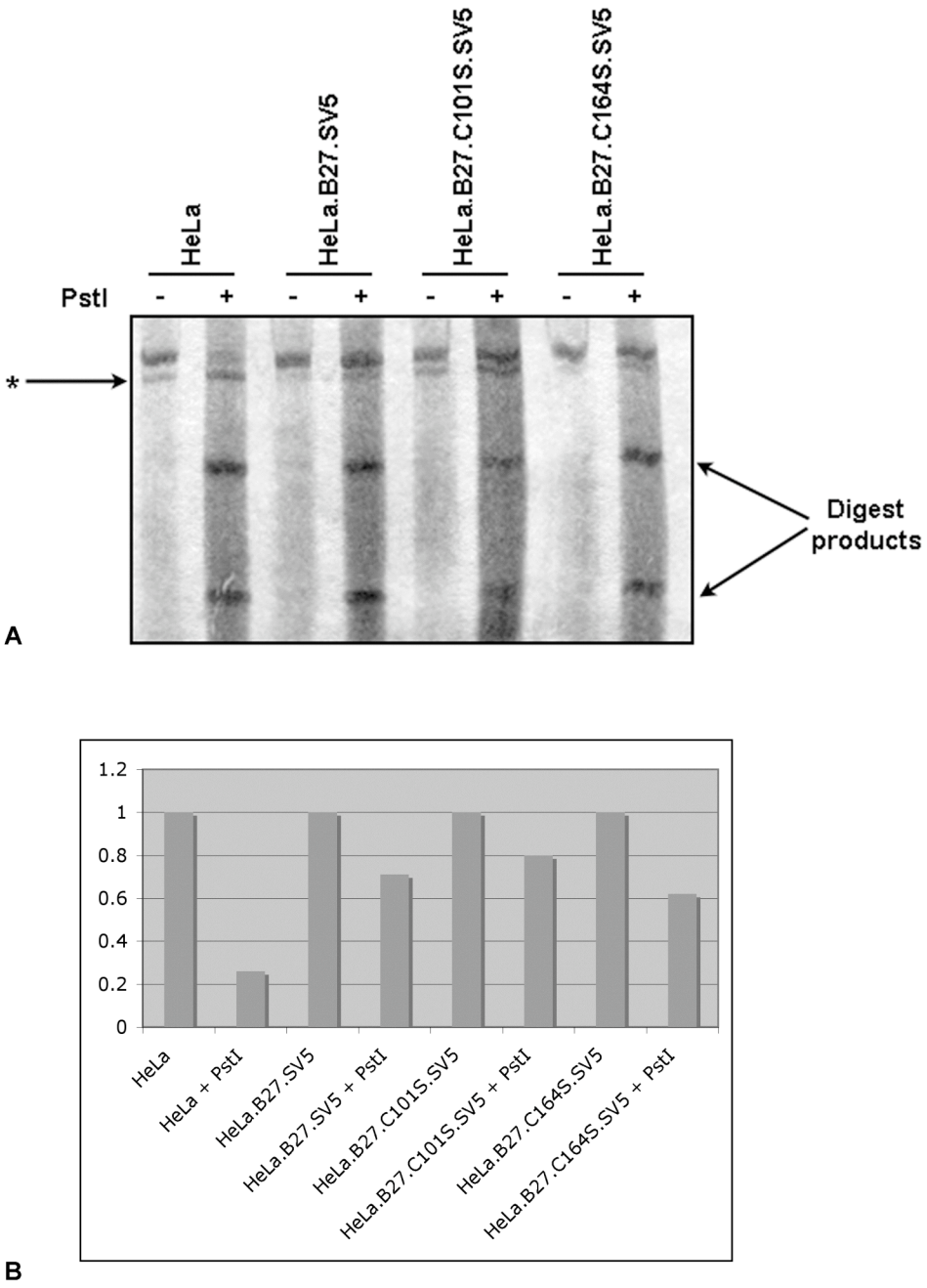


Figure 3.5: PstI digest of RT-PCR products from transiently transfected cells. (A) RT-PCR products were treated with a final concentration of 1 U/μl of PstI at 37 °C for 90 minutes before being resolved by DNA-PAGE. Gels were fixed and RT-PCR products were visualised by silver staining. A contaminant band running just below the region of spliced and unspliced XBP1 is indicated with an asterisk. (B) Relative levels of unspliced and spliced XBP1 are compared before and after PST1 digestion. Results are representative of at least 3 experiments.

These results suggest that the use of a transiently transfected cell-model to assess the effects of HLA-B27 on the activation of the UPR may not be appropriate. It may be that all of the alleles cause, to some extent, the activation of a stress response or the activation may be a response to the transfection process itself. Although HeLa wt cells underwent a mock transfection (Fugene 6 and serum free DMEM but no DNA) it may be that the DNA is responsible for the activation of the stress response. Therefore, in order to further investigate the activation of an UPR in response to the presence of HLA-B27 we repeated the experiments using cells stably transfected with HLA-A2 and HLA-B27.

Stable Expression Model for HLA-B27 and the UPR

BiP levels are enhanced in both HLA-A2 and HLA-B27 expressing cell lines compared to wild-type HeLa cells.

Hela cells were stably transfected with either HLA-A2 or HLA-B27. In order to aid detection, both alleles contain the 14 amino acid SV5 tag on their C-terminus. To confirm the expression of the transfected alleles, cells were assessed by flow cytometry using monoclonal antibodies ME1 (folded B27) or BB7.2 (folded A2) (Figure 3.6 A) and western blotting with the pk antibody (Figure 3.6 B). The HLA-B27 allele is expressed at slightly higher levels than the HLA-A2 allele.

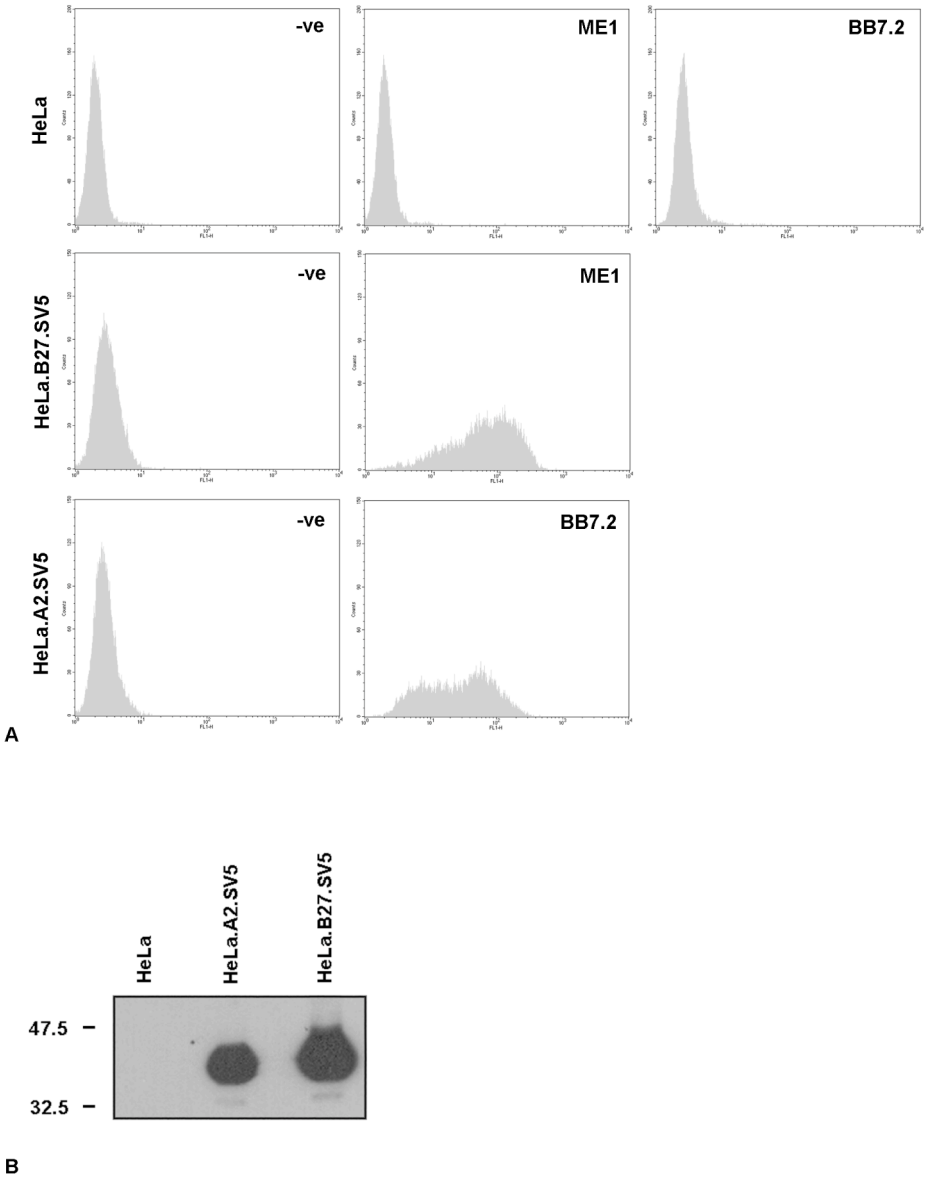


Figure 3.6: Characterisation of HeLa cells stably expressing HLA-A2 or HLA-B27. Cells were transfected using Fugene 6 with 1 μg of pCR3 plasmid DNA containing the appropriate class I allele cDNA with a 14 amino acid C-terminal tag (SV5). Cells were put into selective medium 24 hours after transfection. If necessary, positive cultures were enriched using magnetic beads and/or dilution cloning. (A) The expression of the transfected allele on the cell surface was analysed by flow cytometry using the indicated antibodies. (B) Whole cell lysates were resolved using SDS-PAGE and western blotting. The membrane was probed with the pk antibody that detects the C-terminal SV5 tag. Results are representative of at least 3 experiments.

To determine whether stable expression of these alleles increases the level of the stress responsive chaperone BiP, cell lysates were subject to SDS-PAGE and western blotting with antibodies against β-actin and BiP (Figure 3.7 A). The BiP levels were normalized against the β-actin and relative BiP levels were plotted (Figure 3.7 B).

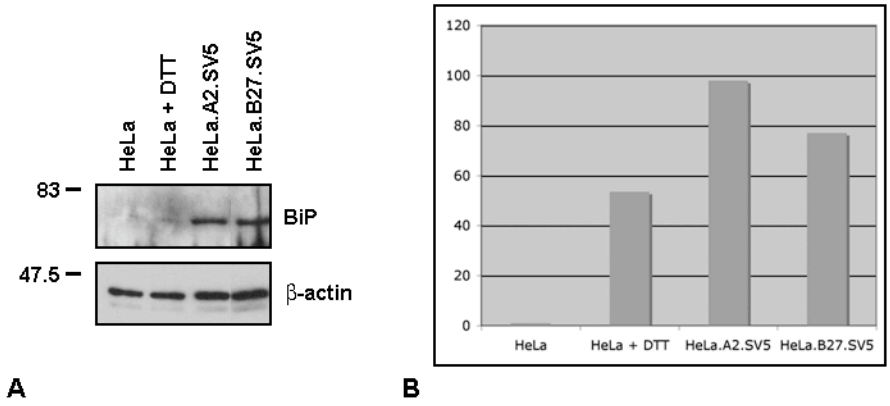


Figure 3.7: BiP levels are increased in cell lines stably expressing disease associated and non-disease associated alleles. (A) Cells were lysed and then protein quantified using the Bradford method. Samples were resolved by 8 % SDS PAGE before western blotting. Membranes were probed with the indicated antibodies. (B) The relative BiP levels for (A) are shown after being normalised for β-actin. Results are representative of at least 3 experiments.

BiP levels were increased in both the HLA-A2 and HLA-B27 expressing cell lines, with the -A2 expressing cells having higher levels of BiP than those cells expressing the disease associated allele -B27. In both cases the level of BiP was enhanced compared to the HeLa wt sample. HeLa cells treated with DTT also contained an increased amount of

the protein BiP compared to the HeLa wt cells, however the level of BiP in the HLA-A2 and HLA-B27 expressing cell lines was further elevated.

XBP1 is predominantly unspliced in stable cell lines expressing HLA-A2 and HLA-B27.

To determine whether these enhanced levels of BiP were associated with an activated stress response, the status of XBP1 (spliced or unspliced) was investigated. In order to do this, RNA was extracted from the cells and RT-PCR was performed with XBP1 specific primers (Figure 3.3). The amplified RT-PCR product was analysed by DNA-PAGE and silver staining (Figure 3.8).

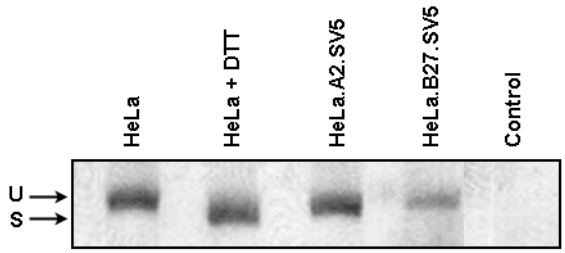


Figure 3.8: XBP1 splicing does not occur in cell lines stably expressing disease associated and non-disease associated alleles. RNA was isolated from the cells and quantified. RT-PCR was carried out using the Qiagen OneStep RT-PCR kit with 0.5 μg of RNA and XBP1 specific primers. The RT-PCR product was resolved using DNA-PAGE. Gels were fixed and RT-PCR product was visualized by silver staining. Results are representative of at least 3 experiments.

The majority of XBP1 occurs in the unspliced form in cell lines expressing either HLA-A2 or HLA-B27, comparable to the HeLa wt cells (Figure 3.8). Treatment of the cells with DTT shifts the majority in the opposite direction, with a higher proportion of XBP1 being in the spliced form (Figure 3.8). To further assess the status of XBP1 in these cell lines RT-PCR product was subject to digestion with PstI (Figure 3.9 A).

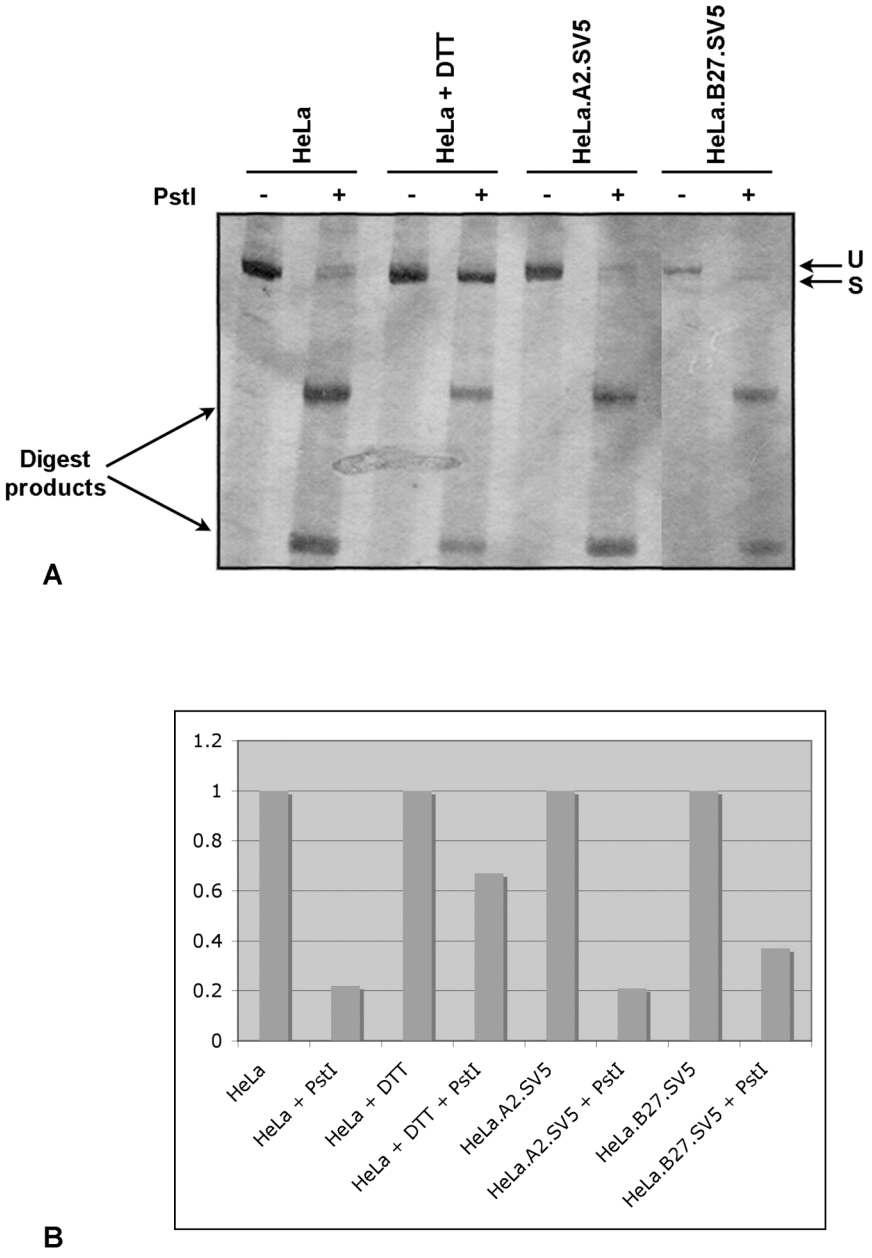


Figure 3.9: PstI digest of RT-PCR products from stably transfected cells. (A) RT-PCR products were treated with a final concentration of 1 U/µl of PstI at 37 °C for 90 minutes before being resolved by DNA-PAGE. Gels were fixed and RT-PCR products were visualised by silver staining. (B) Relative levels of unspliced and spliced XBP1 are compared before and after PstI digestion. Results are representative of at least 3 experiments.

Digestion of RT-PCR product from both alleles resulted in the formation of digest products. Densitometry analysis of both the unspliced and spliced XBP1 bands show that in both Hela wt cells and HeLa cells expressing the HLA-A2 allele, approximately 20 % of the total XBP1 is spliced (Figure 3.9 B). These results are comparable with the level of splicing seen in HeLa wt cells in the transient transfection model (Figure 3.5 A + B). A higher proportion of XBP1 in HeLa cells expressing HLA-B27 was in the spliced form, approximately 38 % compared to 20 % in the wt and HLA-A2 cells, but was lower than the level of spliced XBP1 in the DTT treated cells (Figure 3.9 B).

ATF6 is not cleaved in HeLa cells stably expressing HLA-A2 or HLA-B27.

Attempts were made to immunoblot for endogenous ATF6 as another indicator of UPR. However, several commercially available antisera were tested, but produced no easily identifiable bands. Therefore wild-type HeLa cells and HeLa cells stably expressing either HLA-B27 or HLA-A2 were transiently transfected with ATF6. The ATF6 cDNA (a gift from Dr A. Antoniou, UCL, London) contained a 3 x flag tag sequence to allow detection of the transfected ATF6 with anti-flag tag antibody.

When an UPR is activated, ATF6 translocates from the ER to the golgi and undergoes sequential cleavage. The resulting product then translocates to the nucleus and functions as a transcription factor. In order to investigate whether an UPR was activated in these cells, lysates containing the cell nuclei were prepared and analysed by SDS-PAGE and

western blotting. As a positive control, cells transfected with ATF6 were also treated with DTT before lysis (Figure 3.10).

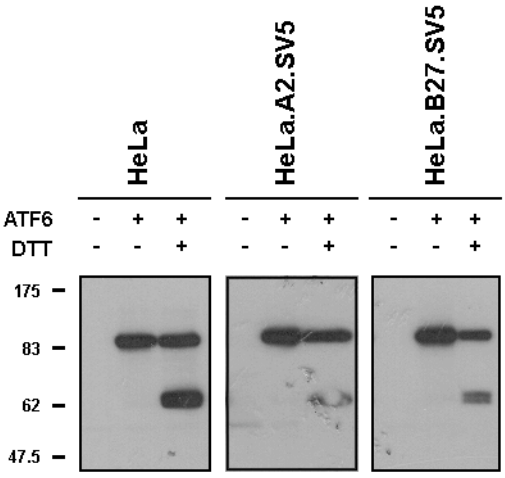


Figure 3.10: Cleavage of ATF6 was not increased in cell lines stably expressing HLA-A2 or HLA-B27. Cells were transfected using Fugene 6 with 1 μg of plasmid DNA containing flag-tagged ATF6. Transient transfectants were harvested 24 hours after transfection. Cells lysates containing the nuclear fraction were prepared and then resolved by 8 % SDS-PAGE before western blotting. Membranes were probed with anti-flag tag antibody. Results are representative of at least 3 experiments.

Transfection of ATF6 into the cell lines was successful and treatment with DTT produced the ATF6 cleavage product (Figure 3.10). In non-DTT treated cells the majority of ATF6 is not cleaved. On longer exposure faint bands representing the cleavage product can be seen in the HLA-B27 expressing cell line but also in the HeLa wt cells suggesting that some cleavage may be occurring due to other stress triggers and not because of the expression of HLA-B27 allele alone (data not shown).

In stable transfectants of the rat cell line, C58, the expression of BiP varies between alleles.

Much of the previous published work on HLA-B27 and the UPR came from work carried out on transgenic rats. To investigate whether rat cells in culture showed an increase in the molecular chaperone BiP in response to the expression of HLA-B27, we used stable C58 cell lines expressing HLA-B27, HLA-A2 or various structural cysteine mutants, all containing the C-terminal SV5 tag. Cell lysates were analysed by SDS-PAGE and western blotting with β-actin, pk and BiP (Figure 3.11 A). The BiP levels were normalized against the β-actin and relative BiP levels were plotted (Figure 3.11 B).

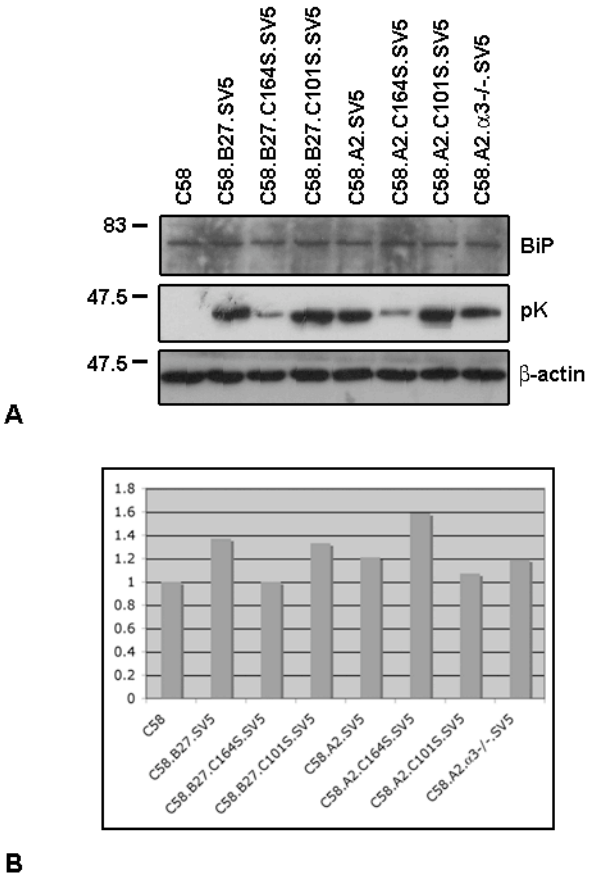


Figure 3.11: BiP levels vary in stably transfected C58 cell lines. (A) Cells were lysed and then protein quantified using the Bradford method. Samples were resolved by 8 % SDS-PAGE before western blotting. Membranes were probed with the indicated antibodies. (B) The relative BiP levels for (A) are shown after being normalised for β-actin. Mutant A2.α3-/-SV5 denotes a double mutant HLA-A2.C203S+C259S, removing both α3 domain conserved cysteines. Results are representative of at least 2 experiments.

BiP levels vary between the different alleles (Figure 3.11 B). The HLA-A2 allele with a cysteine to serine mutation at position 164 has the highest relative level of BiP. However, the corresponding mutation in the HLA-B27 allele has a BiP level comparable to wild-type C58 cells. These results suggest that BiP levels do differ between cell lines

expressing different HLA alleles but that the differing HLA expression may not be the reason for this observed difference.

Discussion

HLA-B27 has a tendency to misfold, forming high molecular weight dimeric and multimeric complexes that have been implicated in the pathogenesis of disease. One current theory suggests that accumulation of misfolded proteins in the ER can result in the activation of a proinflammatory stress response.

BiP Expression

One of the key chaperones involved in the regulation of protein folding in the ER is the immunological binding protein, BiP. The association of HLA-B27 and BiP has been previously reported in rat cell line model and in transgenic rats (Antoniou et al., 2004, Tran et al., 2004). We have tried to determine whether, in a human cell line model, the expression of HLA-B27 is associated with an increased expression of the chaperone BiP.

We transiently transfected HeLa cells with the non-disease associated allele HLA-A2, the disease-associated HLA-B27 or an HLA-A2 or -B27 mutant containing a cysteine to serine substitution at the position of a structural cysteine. These structural mutants are unable to fold correctly and are therefore unable to exit from the ER. Cells were transfected 24 hours prior to harvesting and samples were analysed by western blotting.

Bip levels were consistently higher in the all of the transiently transfected HeLa cells compared with the wild-type HeLa cells (Figure 3.2 A & B). BiP expression is regulated at a post-transcriptional level, in a stressed cell protein level will increase through enhanced translation efficiency (Gulow et al., 2002). However the amount of BiP expressed by HeLa cells transfected with the same allele, or in the HeLa wild type sample, was not consistent between experiments (Figure 3.2 A & B).

HeLa wild-type cells were mock transfected using the same transfection agent, suggesting the difference seen between wild-type cells and transient transfectants cannot be to do with the transfection agent, but it could however, be caused by the DNA, as mock transfectants did not contain DNA. It is possible that the differing effects seen between experiments for the transfected alleles may be associated with the transfection efficiency however this would not explain why differences were also observerd for the wild-type HeLa cells.

As a positive control we treated wild-type cells with DTT, a chemical that will cause global protein misfolding. In some cases however we were unable to detect an increase in BiP following DTT treatment. This is possibly because the short duration of DTT treatment was not long enough to allow increased protein synthesis of BiP. Significantly we did see major effects with DTT treatment subsequently on XBP1 splicing. Thus the immediate DTT impact upon mRNA splicing takes a longer timescale to impact upon overall protein expression levels.

We had hypothesised that BiP expression would be influenced by the folding capacity of the HLA alleles we had transfected, with HLA-A2 expressing the lowest level of BiP and HLA-B27 and the cysteine mutants expressing an increased amount of BiP. However, we were unable to detect this with both the wild-type and transfected alleles differing in their BiP expression between experiments.

We have also examined BiP expression in cells stably transfected with either the disease-associated allele HLA-B27, or the non-disease associated allele HLA-A2. In these cell lines the expression of HLA-B27 was slightly higher than of HLA-A2 as determined by flow cytometry and western blotting (Figure 3.6 A & B). In both cell lines BiP levels were increased above basal levels (Figure 3.7 A & B). However, the cell line expressing the more efficient folding allele, HLA-A2, had a higher level of BiP than the HLA-B27 expressing cell line.

The previous work studying the relationship between BiP and HLA-B27 has been carried out on transgenic rats or on rat cell lines. We therefore tried to determine whether the expression of HLA-B27 in rat cells resulted in an enhanced expression of BiP. We used stably transfected C58 cells expressing HLA-B27, HLA-A2 or a mutant cell line that does not fold properly. Although in most cases BiP levels were increased above that of wt levels there did not seem to be a correlation between HLA-B27 and BiP levels, nor did the mutant cell lines show higher levels of BiP expression compared with HLA-A2 (Figure 3.11 A & B).

These results indicate that the overall expression of BiP in cell-lines may not be appropriate to determine whether an allele is misfolding. For the purpose of investigations with HLA-B27, BiP binding may be a more useful indicator, and it has previously been reported that HLA-B27 is more likely to be bound to BiP than the non-disease associated allele HLA-B7 (Tran et al., 2004).

XBP1 Splicing

XBP1 splicing in HeLa cells transiently expressing HLA-B27 has been reported previously (Lemin et al., 2007). To further investigate this we determined the state of XBP1 in HeLa cells transiently expressing HLA-B27, HLA-A2 as well as mutant cell lines expressing a structural cysteine mutation.

All of the transiently transfected alleles showed splicing of XBP1, however this does not appear to be the case for the untransfected HeLa cells (Figure 3.4). These results indicate that before transfection the cells were not undergoing an active UPR. We were unable to detect a difference in XBP1 splicing between the different alleles, perhaps suggesting that the transfection process itself, rather than the transfected alleles, is responsible for the activated stress response.

As with the BiP experiments, we also studied the state of XBP1 in stably transfected cell lines expressing HLA-A2 or HLA-B27. In these cell lines, XBP-1 is predominantly

unspliced (Figure 3.8). However densitometry analysis determined that in cells expressing HLA-B27 more XBP1 is spliced than in wt or HLA-A2 expressing cells.

ATF6 Cleavage

The previous study carried out on transiently transfected HeLa cells also looked at the status of ATF6 (Lemin et al., 2007). ATF6 is cleaved during an UPR to produce a cyosolic fragment that translocates to the nucleus causing downstream effects. The data presented here perhaps suggest that transiently transfected cells do not make a good model for studying the activation of a stress response. We therefore investigated the status of ATF6 in stably transfected HeLa cells expressing either HLA-A2 or HLA-B27.

We were unable to detect any obvious ATF6 cleavage products in either cell line. On longer exposure a faint cleavage band was detectable in the HLA-B27 cell line, however it was also detectable in the HeLa wt cell line suggesting another factor other than the expression of HLA-B27 was responsible for this.

Summary

The results presented here demonstrate that the use of cell line models to demonstrate an activated UPR should be interpreted with great care. We obtained different results using transiently expressing and stably expressing cell lines. The report by Lemin et al was

intriguing as it suggested that the expression of HLA-B27 alone was enough to activate a stress response in the ER (Lemin et al., 2007). However we have demonstrated that the transient expression of other alleles in HeLa cells also show similar signs of an activated UPR. Such UPR data, when obtained from 'real' cells, ie from patient cells, or animal models, may have relevance, but *in vitro* expression models based on these observations, must be treated with additional analysis.

Chapter IV: Non-HLA-B27 Homodimers

Introduction

As previously discussed, classical MHC class I molecules are expressed at the cell surface of every nucleated cell, with the main function of presenting peptide to $CD8^+$ T cells for immunosurveilance. Conventional fully folded class I molecules are heterotrimeric complexes consisting of a class I heavy chain, β_2m, and an 8-10 amino acid peptide. The vast majority of current data supports a role for this fully folded structure to exist and function essentially as a monomer, one single MHC class I molecules being recognised by a single receptor, eg TCR or NK receptor. However, the expression of non-conventional MHC class I structures at the cell surface has been widely documented (reviewed in Arosa et al., 2007).

Free Heavy Chains

In 1990, Schnabl et al. showed that MHC class I heavy chains not associated with β_2m (free HCs) were present on the surface of activated T-lymphocytes (Schnabl et al., 1990). This was confirmed by Madrigal and colleagues, who also showed the presence of similar molecules on EBV-transformed B-lymphocytes (Madrigal et al., 1991). More recently, it has again been demonstrated that free HCs are not readily detectable on the surface of

normal human T cells but are enhanced in response to T cell activation and proliferation (Santos et al., 2004). There is evidence to suggest that small stable populations of MHC class I heavy chains not associated with β_2m and/or peptide exist ubiquitously on the surface of all MHC class I-expressing cells (Carreno ref 1994), however abundant expression is predominantly seen at the cell surface of activated lymphocytes (Bix and Raulet, 1992, Matko et al., 1994, Schnabl et al., 1990, Madrigal et al., 1991, Santos et al., 2004).

MHC class I folding in the ER, enabling a stable association with β_2m and peptide to occur, is a tightly regulated process involving several chaperones and accessory molecules. Nascent MHC class I heavy chains not associated with β_2m are usually bound to calnexin and are prevented from exiting the ER (Rajagopalan and Brenner, 1994). This would imply that most free HCs originate from the unfolding of cell surface heterotrimers. In this regard, Pickl *et al*. showed that free HCs on the surface of activated T cells arise following internalisation of fully folded class I molecules (Pickl et al., 1996), and Santos *et al*. demonstrated that free HCs on the surface of normal T-lymphocytes were resistant to Endo H digestion, indicating they had achieved successful transit from the ER (Santos et al., 2004). However, in β_2m knockout mice, some surface expression of class I molecules has been detected, suggesting that, at least in mice, some free heavy chains can exit from the ER without associating with β_2m (Bix and Raulet, 1992).

The exact role of free heavy chains has not yet been determined although some evidence suggests they are important for the stimulation of T-cells. Bodnar *et al*. have

demonstrated that free HCs are important in the clustering of MHC class I molecules at the cell surface, a process that can be decreased in response to the addition of exogenous β_2m, reducing the number of free HCs, and therefore clustering, lowers the effectiveness of cytotoxic T cells (Bodnar et al., 2003). Beretta *et al*. showed that blocking of a cell surface protein on monocytes, that was later shown to be free MHC class I heavy chains, efficiently inhibits the proliferation of responsive lymphocytes (Beretta et al., 1987, Grassi et al., 1991). Taken overall these data suggest that free HCs can be functionally important for the stimulation of some T cells.

MHC Class I Homodimers

Dimeric HLA class I complexes were first identified from human cell lines in the early 1970s (Cresswell and Dawson, 1975). These dimers were thought to consist of two heavy chains disulfide bonded together, non-covalently associated with β_2m. However a second report published by the Strominger lab suggested these dimeric complexes were not naturally found in the membrane of the cell but were instead a product formed during the purification method (Springer et al., 1977).

Several years later dimeric mouse class I heavy chains were isolated from the surface of a number of cell lines, the formation of which is believed to be regulated by the availability of β_2m (Capps et al., 1993). These dimers were disulfide bonded through a free cysteine residue in the cytoplasmic tail (Capps et al., 1993).

The formation of homodimers has functional consequences for immune recognition. The non classical MHC class I molecule, HLA-G, is predominantly expressed during pregnancy on fetal trophoblast cells that invade the decidua during placentation (McMaster et al., 1995). It is unusual amongst class I molecules because of its ability to form β_2m-associated homodimers through an unpaired cysteine residue at position 42, situated on an exposed loop beneath the α1 peptide-binding helical region (Boyson et al., 2002, Gonen-Gross et al., 2003). A cysteine at this position is unique to HLA-G alleles, implying this type of complex is only formed by HLA-G. There is convincing evidence that LILRB1 and LILRB2 recognise the dimeric form of HLA-G more strongly than the monomeric form (Shiroishi et al., 2006, Gonen-Gross et al., 2003, Apps et al., 2007) and a chimeric LILRB1 reporter cell assay showed that dimeric HLA-G was required at 100-fold lower concentrations than monomeric HLA-G to induce signalling (Shiroishi et al., 2006).

HLA-B27 Cell Surface Homodimers

HLA-B27 homodimers were first isolated in 1999 during *in vitro* folding studies (Allen et al., 1999). Misfolded HLA-B27, particularly homodimers, have been implicated in the pathogenesis of AS and related spondyloarthropathies, either by misfolding in the ER which may result in the activation of a proinflammatory stress response (Colbert, 2000), or through their presence at the cell surface. Cell surface homodimers may be involved in peptide presentation or be recognised themselves as neoantigens by some immune receptors (Allen et al., 1999).

Homodimers can be recognised by both HC10 and W6/32 monoclonal antibodies, and it has been demonstrated that these are distinct populations (Allen et al., 1999, Dangoria et al., 2002). HC10 reactive dimers form early after heavy chain synthesis suggesting they form within the ER, whereas W6/32 reactive dimers are formed much later suggesting they form following exit from the ER (Dangoria et al., 2002). However, both populations can be isolated from the cell surface, indicating HC10 reactive dimers can also form at a later time point (Dangoria et al., 2002). Consistent with this finding, Bird et al. demonstrated that HC10 reactive cell surface homodimers could form from fully folded heterotrimers that are internalised and recycled back to the cell surface via an endosomal compartment (Bird et al., 2003).

Comparative analysis of dimer formation in cell lines expressing HLA-B8, -A2 or -B53 showed no evidence of dimer formation although some HLA-B7 dimers were detected, dimers formed by HLA-B7 were fewer in total than those formed by HLA-B27, and more of the HLA-B7 species were W6/32 reactive (Dangoria et al., 2002). These data indicate that dimer formation is not unique to HLA-B27 but may differ quantitatively and qualitatively between MHC class I alleles.

HLA-B27 possess the relatively rare cysteine residue at position 67 in its α1 domain, pointing into the peptide-binding groove. This cysteine has been strongly implicated in the formation of HC10 reactive homodimers (Allen et al., 1999, Dangoria et al., 2002, Bird et al., 2003). Since their initial discovery, HLA-B27 homodimers have subsequently been detected in HLA-B27 transgenic rats, as well as on populations of peripheral blood

and synovial fluid monocytes from patients with spondyloarthritis (Kollnberger et al., 2002, Tran et al., 2004, Kollnberger et al., 2004). These HLA-B27 homodimers have been identified as ligands for several killer immunoglobulin-like receptors (KIR) and leukocyte immunoglobulin-like receptors, notably KIR3DL2 (Kollnberger et al., 2002, Allen and Trowsdale, 2004).

Supporting the theory that an abnormal interaction between immune receptors and homodimers are implicated in disease, Chan et al have detected increased numbers of NK cells and $CD4^+$ T cells expressing KIR3DL2 from patients suffering with spondyloarthritis (Chan et al., 2005). These results demonstrate that B27 homodimers are recognised by a specific, although overlapping, subset of receptors compared with traditional, fully folded B27 molecules, and that at least one of these receptors is enhanced in patients suffering with spondyloarthritis.

In addition to the above, we also recently published data showing that, in the KG1 DC-like cell line and human monocyte-derived DCs from a HLA-$B27^+$ individual, HLA-B27 homodimers are a transiently expressed population that are induced in response to activation (Santos et al., 2008). Overexpression of an HLA allele alone is most likely not enough to confer disease susceptibility, since the HLA-B7 transgenic rats express -B7 to a similar level as the disease-prone HLA-B27 transgenic rats, but remain healthy (Taurog et al., 1999). Overall, HLA-B27 appears to be more susceptible to form dimers than other alleles. Defining the conditions under which non-disease associated MHC class I molecules can form dimers may therefore provide crucial information to our

understanding of the factors that predispose HLA-B27 to form dimers. Indeed, if HLA-B27 dimers are a significant driving force in the development of AS, then we may gain important insights from studying how other MHC class I dimers may contribute to disease, since we must always be aware that the incidence of HLA-B27 in AS patients is around 90-95 %, showing that AS can develop in the context of numerous other MHC class I alleles present in the human population.

Results

HLA-B8 forms homodimers in the .220 cell line when expressed in combination with human tapasin.

The 721.220 cell line (referred to forthwith as .220) lacks HLA-A and HLA-B alleles but retains partial expression of HLA-Cw1 (Greenwood et al., 1994). The cell line is also tapasin deficient (Copeman et al., 1998) resulting in many MHC class I alleles being expressed at the cell surface after transfection at relatively low levels. In fact this system has been used to define the tapasin dependency of several MHC class I alleles, and also to determine residues in the peptide-binding groove which are important for this activity (Park et al., 2003). HLA-B27 is a relatively tapasin-independent allele whereas HLA-B8 is more dependent on the function of this accessory molecule (Peh et al., 1998). Normal cell-surface expression of tapasin dependent alleles can be restored by transfection with tapasin cDNA (Peh et al., 1998) (Park et al., 2003).

Stable .220 cell lines expressing either HLA-B27, HLA-B8 or HLA-B8 and human tapasin (htpn) were generated by electroporation (B27), or were already available for use in the laboratory from previous studies (B8 and B8.htpn). The cell lines were characterised by flow cytometry (Figure 4.1 A) and western blotting (Figure 4.1 B).

Wild-type .220 cells display low levels of background staining with the W6/32 antibody, however the transfected alleles are clearly expressed on the cell surface above this

background level (Figure 4.1 A). The .220.B8 cell line expresses the least class I on the cell surface, in line with its reported dependence on tapasin (Peh et al., 1998). Restoring tapasin in .220.B8.htpn cells significantly increases this cell surface expression. The .220.B27 cell line expresses at the cell surface at a level intermediate between .220.B8 and .220.B8.htpn cells. Immunoblotting with HC10 of quantified detergent cell lysates indicate that the .220.B8.htpn cells have much higher levels of heavy chain in comparison to .220.B8 cells (Figure 4.1 B). In part this may be due to the known ability of tapasin to stabilise MHC class I molecules, which otherwise would be unstable and undergo degradation. In contrast .220.B27 cells express the least amount of detectable heavy chain, but nevertheless express more of this at the cell surface in a folded conformation than .220.B8 cells (Figure 4.1 A), confirming the ability of HLA-B27 to assemble in a relatively tapasin independent manner.

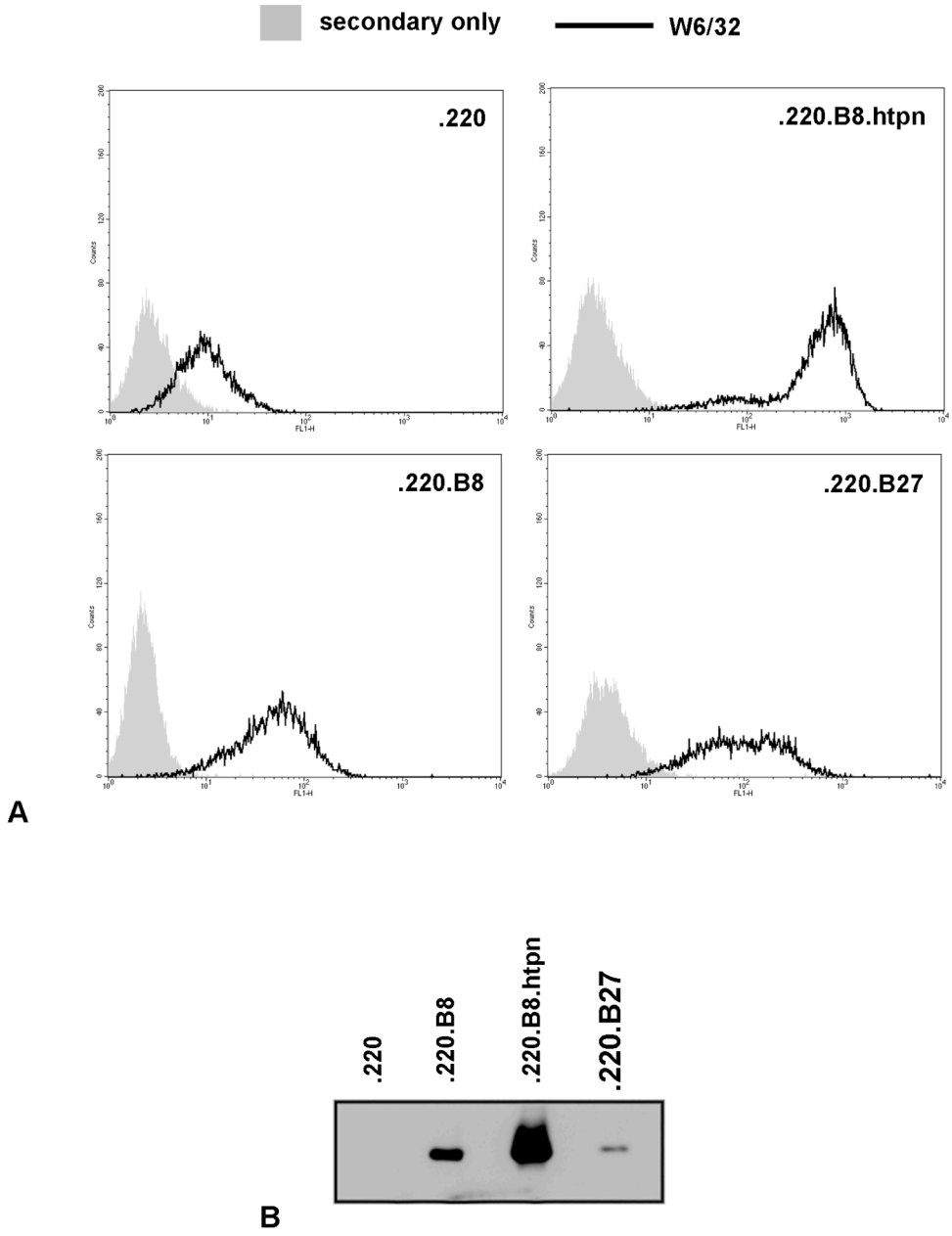


Figure 4.1: Characterisation of stably transfected .220 cell lines. (A) The expression of the indicated transfected MHC class I allele on the cell surface was analysed by flow cytometry. Cells were stained with W6/32 and FITC anti-mouse IgG. (B) Whole cell lysates were quantified using the Bradford method. 20 ug of protein was loaded in each case. Samples were resolved by 8 % SDS-PAGE, transferred to membrane, and visualized through western blotting with HC10. Results are representative of at least 3 experiments.

The formation of HLA-B27 homodimers has been well characterised when expressed in .220 cells (Bird et al., 2003, Kollnberger et al., 2007). However, in the .220.B27 cells we generated we were unable to detect higher molecular weight complexes when analysed in non-reducing conditions. In marked contrast, .220.B8.htpn cells display a high molecular weight species in the size range expected for a MHC class I dimer of around 80 kDa under non-reducing conditions, which, under reducing conditions, resolves to form a single monomeric band (Figure 4.2).

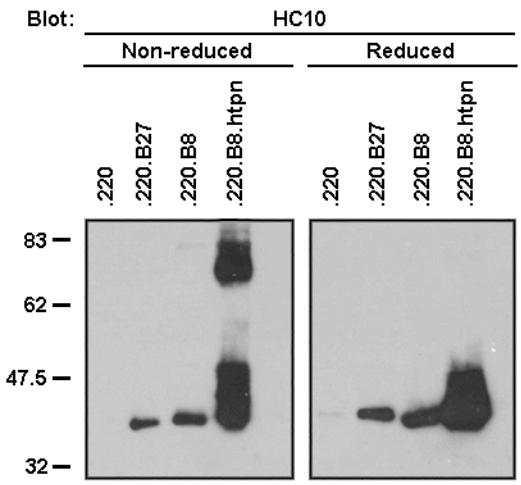


Figure 4.2: .220.B8.htpn cell lysates, but not .220.B27 lysates, contain a higher molecular weight complex that resolves to a monomer upon reduction. Detergent cell lysates were quantified using the Bradford method. 20 μg of protein was loaded in each lane. Samples were resolved by 8 % SDS-PAGE, in non-reducing and reducing conditions, before western blotting. The membrane was probed with HC10. Results are representative of at least 3 experiments.

This high molecular weight complex is also not present in the .220.B8 cell line, suggesting that the formation of this complex is either as a result of the expression of tapasin, or due to the increased amount of class I heavy chain, which as mentioned above, may in itself be a result of the presence of tapasin.

The presence of a high molecular weight species does not prove the formation of a MHC class I dimer, since a similar result could be obtained by an MHC class I molecule being disulfide bonded to another (non-MHC class I) molecule of similar size. Therefore, in order to further characterise this higher molecular weight complex, cell lysates were subject to 2-dimensional electrophoresis. The principle behind this analysis is that two MHC class I heavy chains linked together would have the same isolelectric point as a monomer MHC class I heavy chain, thus a true dimer spot would focus directly above the monomer. However, a heavy chain linked to another species would, more likely than not, result in a different focusing dimer spot. Detergent lysate samples of the .220.B8.htpn cells were therefore separated in the first dimension by isoelectric focusing (IEF) and in the second dimension by non-reducing SDS-PAGE, followed by western blotting with HC10.

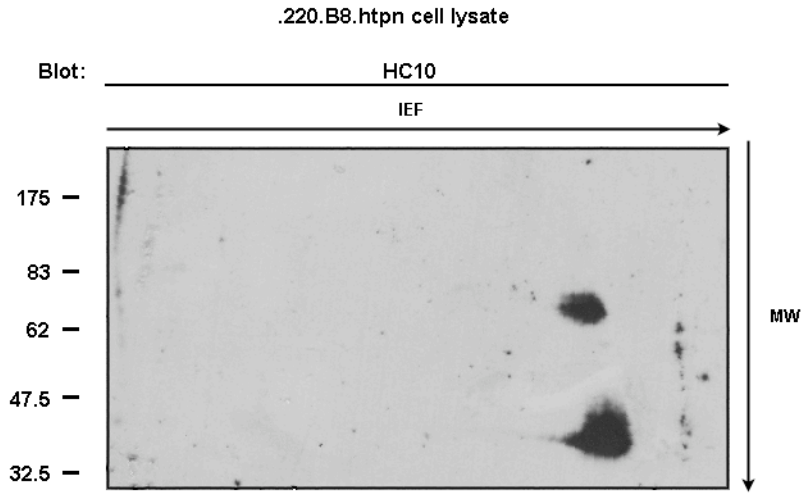


Figure 4.3: The higher molecular weight band represents a pool of B8 homodimers. Non-reducing two-dimensional electrophoresis and immunoblottting was carried out to determine if the higher molecular weight complex was a B8 homodimer. Cell lysates were resolved, in the first dimension by isoelectric point and in the second dimension by molecular weight, and spots revealed with HC10. Results are representative of at least 2 experiments.

Figure 4.3 shows the presence of two HC10 reactive spots with essentially the same isoelectric point, allowing for minor distortions of the gel, one corresponding to the monomeric species seen in the cell lysates and the other running at the same molecular weight as the higher molecular weight complex also seen in the lysates (figure 4.2). This is strong evidence that the high molecular weight species represents a HLA-B8 heavy chain dimer.

HLA-B8 homodimers are expressed at the cell surface.

To determine whether the HLA-B8 dimers are present on the cell surface we utilised a technique involving global biotinylation of cell surface proteins, followed by detergent lysis and specific isolation of the biotin-lablled cell surface species with streptavidin coupled beads. Immunoblotting is then used to detect the presence of the protein of interest, in this case MHC class I heavy chains. As a control, to check that only cell surface MHC class I was being isolated we took advantage of a transfectant of the C58 cell line expressing HLA-B27 with a cysteine to serine mutation at position 101 (one of the conserved structural cysteine residues). Mutation of this cysteine prevents the class I allele from folding correctly and it does not egress from the ER to the cell surface, and should therefore not be labelled with the sulfo-derivative of biotin used in this technique.

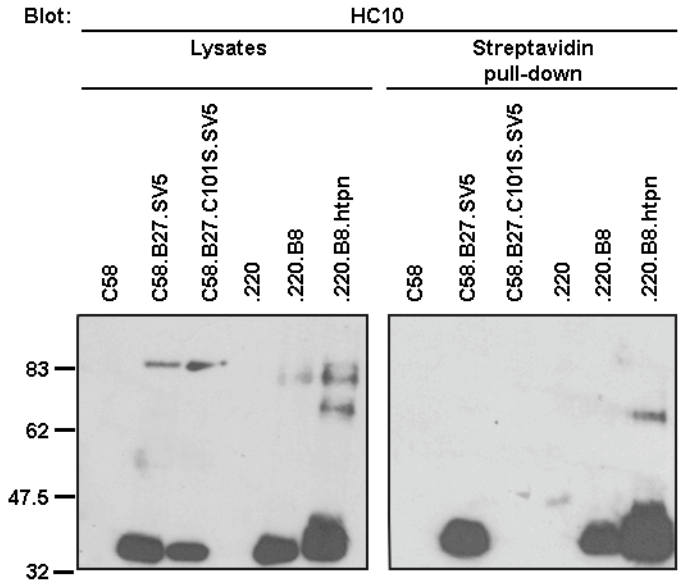


Figure 4.4: .220.B8.htpn homodimers are expressed at the cell surface. Cells were alkylated with NEM for 10 minutes on ice before being incubated with EZ-Link sulfo-NHS-LC-Biotin for a further 10 minutes on ice. Free biotin was quenched with TBS 5 % FBS before cell lysis. Lysates were then subject to streptavidin-agarose pull-down. Samples were resolved by 8 % SDS-PAGE before western blotting with HC10. Results are representative of at least 3 experiments.

Figure 4.4 shows the results of the cell surface biotinylation and streptavidin pull-down. The right-hand panel shows that monomeric class I was isolated from the surface of both the .220.B8 and .220.B8.htpn cell lines, and also from the C58.B27.SV5 control cell line. Significantly, the B8 dimer molecule described in the .220.B8.htpn cell line was also isolated from the cell surface. The control lysate blot (left panel) demonstrates that the C58.B27.C101S.SV5 cell line is expressing the transfected HLA-B27 construct, but it is not detected on the cell surface (streptavidin pull-down), indicating that our technique is

specifically labelling cell-surface proteins. This experiment clearly demonstrates that MHC class I dimers formed by non-HLA-B27 MHC class I molecules can be expressed at the cell surface.

To try to determine what factors influence dimer formation in the .220.B8.htpn cell line we first looked at the assembly kinetics of HLA-B8 in the transfectants by determining how fast HLA-B8 alleles in the .220.B8.htpn cell line acquired Endo H resistance, an indicator of assembly and exit from the ER. HLA-B27 is known to have slower folding kinetics than many other class I alleles, a factor which may influence dimer formation within the ER (Antoniou et al., 2004, Mear et al., 1999).

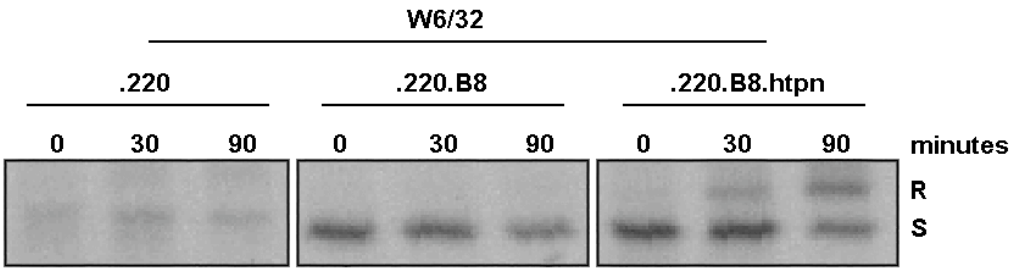


Figure 4.5: Class I molecules in the .220.B8.htpn cell line exit the ER within 30 minutes after synthesis. Cells were incubated in methionine-free RPMI for 15 minutes prior to labelling with [^{35}S] methionine. Labelling was stopped by resuspending the cells in media containing cold methionine. Aliquots were taken at 0, 30 and 90 minutes post labelling. Cells were lysed and immunoprecipitated with antibody W6/32. After immunoprecipitations samples were treated with Endo H. Samples were resolved by SDS-PAGE and gels were fixed and dried. Dried gels were exposed to X-ray film. Endo H sensitive bands are indicated with an S and resistant bands are indicated with an R. Results are representative of at least 2 experiments.

In the .220.B8.htpn cell line, HLA-B8 can be seen to begin to exit the ER after 30 minutes (Figure 4.5). After 90 minutes approximately half of the labelled molecules had gained Endo H resistance. In marked contrast HLA-B8 molecules in the .220.B8 cell line failed to gain Endo H resistance even after 90 minutes of chase. These results indicate that the presence of tapasin results in rapid folding kinetics for the HLA-B8 allele. In the context of this system it does not, however, lend support to the theory that slow folding kinetics predispose to MHC class I dimer formation, as has been proposed for HLA-B27.

It has been reported that the treatment of HLA-B27 expressing cells with chloroquine prevents homodimer expression at the cell surface (Bird et al., 2003). Chloroquine is a lysosomotrophic agent that neutralises the pH in endosomal and lysosomal compartments. We first investigated the affect of chloroquine on the total pool of HLA-B8 dimers in cell lysates before investigating any specific effects on cell surface dimers.

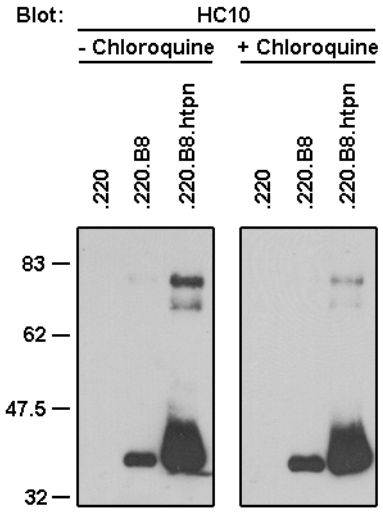


Figure 4.6: Chloroquine treatment influences the level of dimers in whole cell lysates. Cells were grown in RPMI with or without 100 μM chloroquine for 24 hours at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Cells were lysed in the presence of NEM and samples were resolved by 8 % SDS-PAGE before HC10 western blot.

Pre-treatment of cells with chloroquine decreased the detectable pool of B8 dimers (Figure 4.6). However, cell surface biotinylation and streptavidin pull-down studies show that although decreased, dimers at the cell surface were not abolished when cells were pre-treated with chloroquine (Figure 4.7).

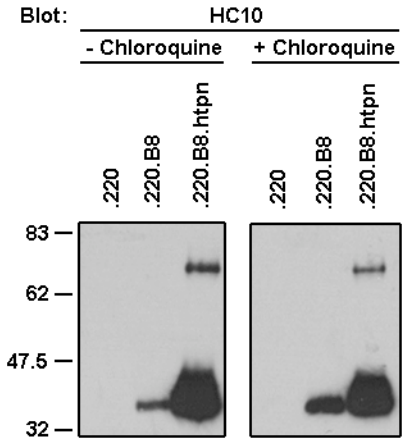


Figure 4.7: Chloroquine treatment decreases but does not abolish dimers at the cell surface. Cells were grown in RPMI with or without 100 μM chloroquine for 24 hours at 37 °C in a humidified atmosphere of 5 % CO_2 in air. Cells were alkylated with NEM for 10 minutes on ice before being incubated with EZ-Link sulfo-NHS-LC-Biotin for a further 10 minutes on ice. Free biotin was quenched with TBS 5 % FBS before cell lysis. Lysates were then subject to streptavidin pull-down. Samples were resolved by 8 % SDS-PAGE and HC10 western blot.

By altering the pH of the endosomal compartment, chloroquine is believed to disrupt the endosomal recycling process that is necessary for the formation of HLA-B27 homodimers at the cell surface. These results indicate that HLA-B8 dimer formation at the cell surface in our system may not be occurring in the same way as for the HLA-B27 homodimers so far described in the literature (Bird et al., 2003).

Further cell treatments were carried out in order to investigate what may influence dimer formation in the HLA-B8 allele. This laboratory has previously used the oxidising agent diamide to probe interactions within the components of the MHC class I peptide loading complex (Santos et al., 2007). The addition of diamide rapidly depletes cellular stocks of

glutathione (GSH), thus generating highly oxidising conditions. Treatment of cells with the oxidising agent diamide revealed interesting results. Cells were treated with increasing concentrations of diamide for a period of 10 minutes at 37 ^{o}C, and detergent cell lysates were then resolved by SDS-PAGE before HC10 western blotting.

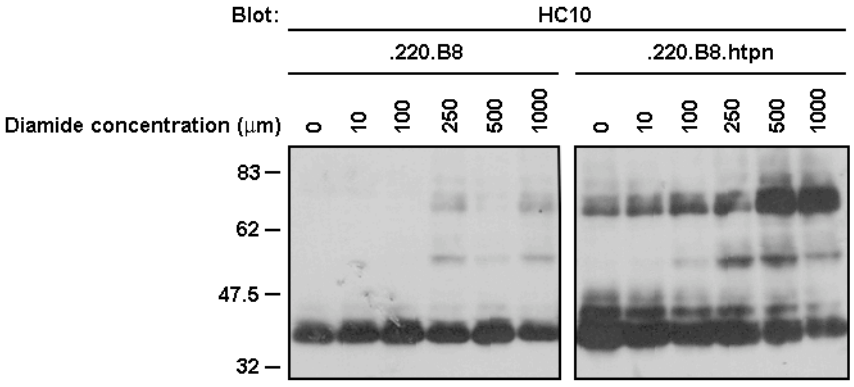


Figure 4.8: Incubation of cells with diamide can enhance the formation of dimers. Cells were treated with diamide in RPMI for 10 minutes at 37 °C. Cells were lysed and then protein quantified using the Bradford method. Samples were resolved by 8 % SDS-PAGE before western blotting. The membrane was probed with HC10. Results are representative of at least 3 experiments.

Changing the cellular environment using the oxidising agent diamide enhances the formation of HLA-B8 dimers in a concentration dependent manner (Figure 4.8). In the .220.B8 cell line, where dimers are not usually seen, higher concentrations of diamide induces such dimers. Dimers are also enhanced in the .220.B8.htpn cell line by the addition of diamide. These data indicate that the redox environment is important in the formation of the HLA-B8 dimers characterised in the .220 cells.

Introduction of the HLA-G-specific cysteine at position 42 into HLA-B27

HLA-G is a non-classical MHC class I allele that has the capacity to form dimers between two fully folded monomers. These dimers form through a unique cysteine residue at position 42 in the α1 domain. Site-directed mutagenesis of HLA-B27 cDNA was utilised to generate a HLA-B27 mutant (denoted B27.S42C) with this extra cysteine at position 42. Mutagenic primers were designed using the program Primer X to generate the alteration indicated in figure 4.9.

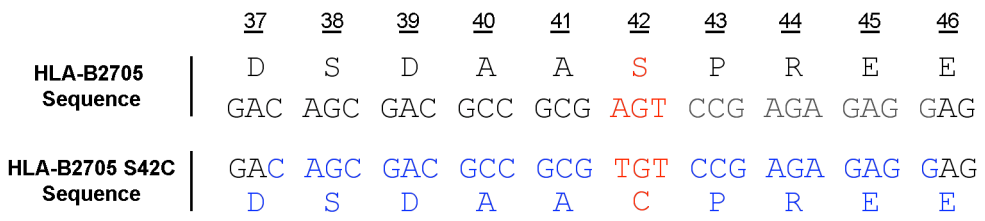


Figure 4.9: Sequence of region affected by cysteine 42 mutation. Mutagenic primers were designed using the bioinformatics program, Primer X. Mutagenesis experiments were carried out on full-length HLA-B27 cDNA contained within the pCR3 plasmid. The mutated dsDNA was transformed into JM109 competent cells. The bacterial cultures were then used for plasmid purification. Purified plasmid DNA was checked by restriction digestion and confirmed by DNA sequencing.

HLA-B27.S42C cDNA and wt HLA-B27 cDNA were transiently transfected into HeLa cells. Cell lysates were resolved by SDS-PAGE and immunoblotted with HC10. Lysate derived form .220.B8.htpn cells, which display good levels of HLA-B8 dimers, were included as a control.

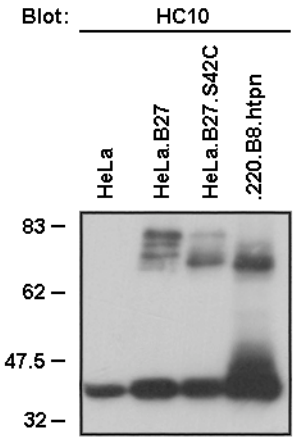


Figure 4.10: Introduction of an HLA-G-specific cysteine at position 42 in HLA-B27 enhances the formation of dimers of a similar molecular weight to B8 homodimers. Cells were transfected using Fugene 6 with 1 μg of pCR3 plasmid DNA containing the mutated HLA-B27 cDNA or the wild type HLA-B27 cDNA. Transient transfectants were harvested 24 hours after transfection. Cells were lysed and samples resolved by 8 % SDS-PAGE before western blotting. The membrane was probed with HC10.

The introduction of the HLA-G-specific cysteine at position 42 to form B27.S42C significantly altered the pattern of dimer bands in comparison to wt HLA-B27 (Figure 4.10). A predominant new dimer band resolved in B27.S42C, most likely produced by the formation of cys42-linked dimers, which also closely matched the size of HLA-B8 dimers seen in .220.B8.htpn cells. Taken together, these data suggest that the dimers formed by both HLA-B8 and B27.S42C are folded dimers.

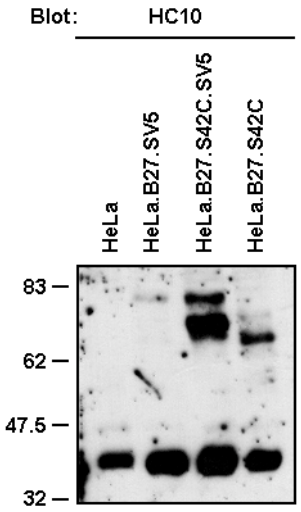


Figure 4.11: Stable expression of mutant HLA-B27.S42C. HeLa cells were transfected using Fugene 6 with 1 μg of pCR3 plasmid DNA containing the mutated HLA-B27 cDNA, mutated HLA-B27 cDNA with a 14 amino acid SV5 tag, or the wild type HLA-B27 cDNA. Cells were put into selective medium 24 hours after transfection. If necessary, positive cultures were enriched using magnetic beads and/or dilution cloning. Cells were lysed and samples resolved by 8 % SDS-PAGE before western blotting. The membrane was probed with HC10. Results are representative of at least 2 experiments.

Stable cell lines were also created and analysed by western blot (Figure 4.11). The B27.S42C dimer band was visible in cell lines created using untagged and SV5 epitope tagged B27.S42C cDNA. The addition of the 14 amino acid SV5 tag created a small increase in size of the new dimer band (lane 3). Higher molecular weight bands were also visible in the B27.S42C mutants, which likely represent the already described 'unfolded' homodimer structure.

To confirm that the B27.S42C dimer band was in fact a true homodimer formed between HLA-B27 heavy chains, we performed two-dimensional electrophoresis, separating in the first dimension by isoelectric focusing and in the second dimension by molecular weight, followed by immunoblotting with either HC10 or the SV5 tag specific monoclonal antibody pk.

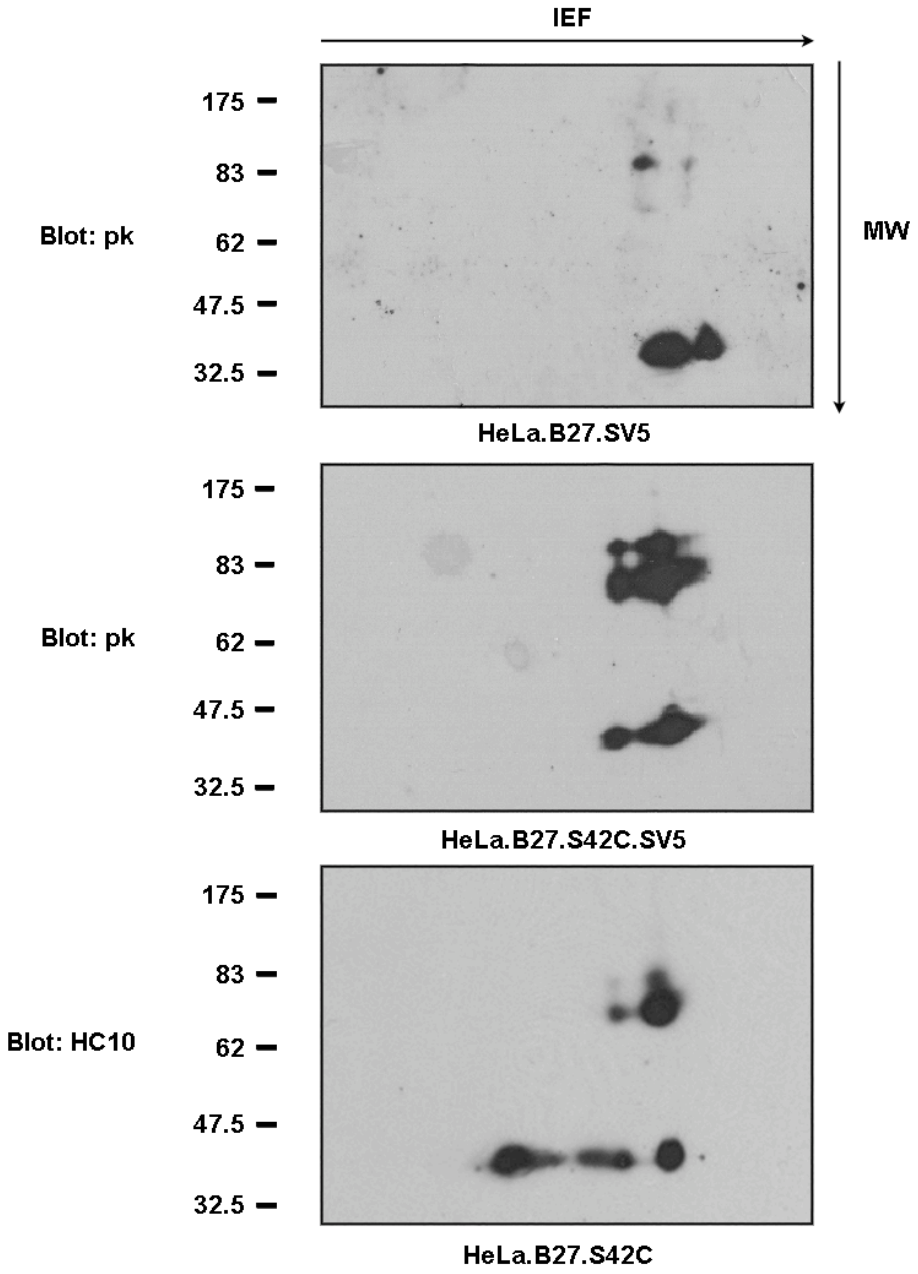


Figure 4.12: HLA-B27.S42C dimers are confirmed by 2-dimensional electrophoresis. Stably expressing cell lines were lysed and resolved in the first dimension by isoelectric point, and in the second dimension by molecular weight, followed by western blot with the either HC10 or, the anti-SV5 tag antibody, pk. Results are representative of at least 2 experiments.

A higher molecular weight spot visible in the HeLa.B27.SV5 cell line migrated with the same isoelectric point as the HLA-B27 monomer (Figure 4.12, top panel) confirming this spot as a HLA-B27 dimer. In both cell lines expressing HLA-B27 with the S42C mutation this same spot remained (Figure 4.12, middle and lower panels) but in addition, a new dominant set of spots apperared. These new S42C-induced spots, also retained the same isolelectric point as mononmeric HLA-B27 heavy chain, and are therefore representative of heavy chian dimers. Furthermore the relative molecular mass of the wtB27 and B27.S42C dimers in these gels corresponds to that seen in normal SDS-PAGE, as shown in Figures 4.10 and 4.11. The HC10 immunoblot of HeLa.B27.S42C lysate also indicates the MHC class I signal derived from the endogenous HeLa MHC class molecules that focus to the left of the HLA-B27 spots, but do not form dimer spots. The multiple spots generated by HLA-B27 with the same size, for example in the middle panel of Figure 4.12, are usually indicative of either phosporylation of some of the proteins, or small charge modifications that occur during the loading and running of the gels.

It was next investigated whether or not the B27.S42C dimer detected in HeLa transfectants was expressed at the cell surface, using the cell surface biotinylation and streptavidin pull-down technique already described.

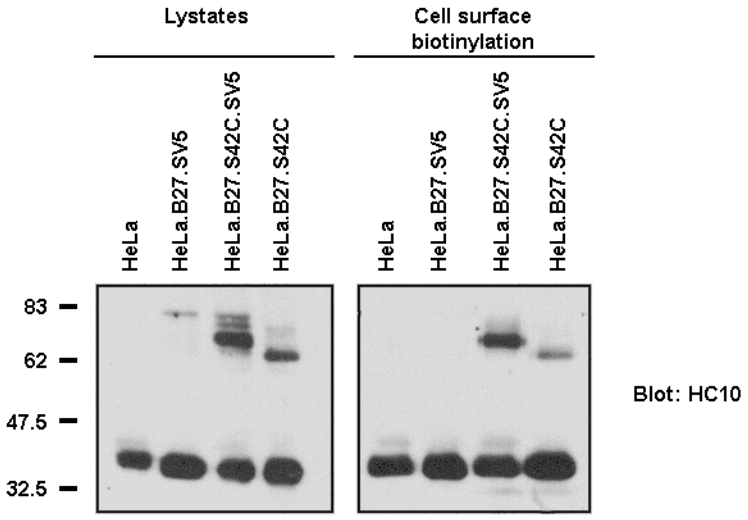


Figure 4.13: B27.S42C homodimers are expressed at the cell surface. Cells were alkylated with NEM for 10 minutes on ice before being incubated with EZ-Link sulfo-NHS-LC-Biotin for a further 10 minutes on ice. Free biotin was quenched with TBS 5 % FBS before cell lysis. Lysates were then subject to streptavidin-agarose bead pull-down. Samples were resolved by 8 % SDS-PAGE and HC10 western blot. Results are representative of at least 2 experiments.

Control lysates from the samples used for the streptavidin pull down (Figure 4.13, left panel) show the same pattern of dimers as previously seen for these cell lines (Figures 4.10 & 4.11). Streptavidin pull-down indicates that the dimers that are formed in the B27.S42C mutant are expressed at the cell surface. The higher molecular weight dimers seen in lysates are not detected at the cell surface, suggesting that these are dimer species that do not exit the ER.

Formation of dimer structures in other non-disease related MHC class I alleles.

The data presented so far in this chapter suggest that HLA-B8 molecules can be detected as homodimer structures when expressed in .220.B8.htpn cells, but not in .220.B8 cells. Furthermore the .220.B27 cells generated in this chapter also do not appear to form significant dimer structures. An observation that arises from these experiments is whether the high level of HLA-B8 heavy chain expression present in the .220.B8.htpn cells is a contributing factor in the formation of these dimers, and that the lower levels of HLA-B8 and -B27 in the other .220 lines prevents them from forming dimers.

To address this question transient transfection of MHC class I molecules into HEK293 cells, a well-established system used for over-expression studies, was investigated. Rat C58 cell lines stably expressing the human MHC class I alleles HLA-B*2705, -B*0702, B*5101, and B*3501 were created, and the same alleles were transiently transfected into HEK293 cells. In C58 cells, only HLA-B27 formed dimer-like bands, whereas in marked contrast, in HEK293 cells all the tested class I alleles produced complex, multiple dimer bands. This suggest that over expression of MHC class I alleles may be a contributory factor in the formation of MHC class I homodimers, and may explain why HLA-B8 dimers are detectd in .220.B8.htpn cells.

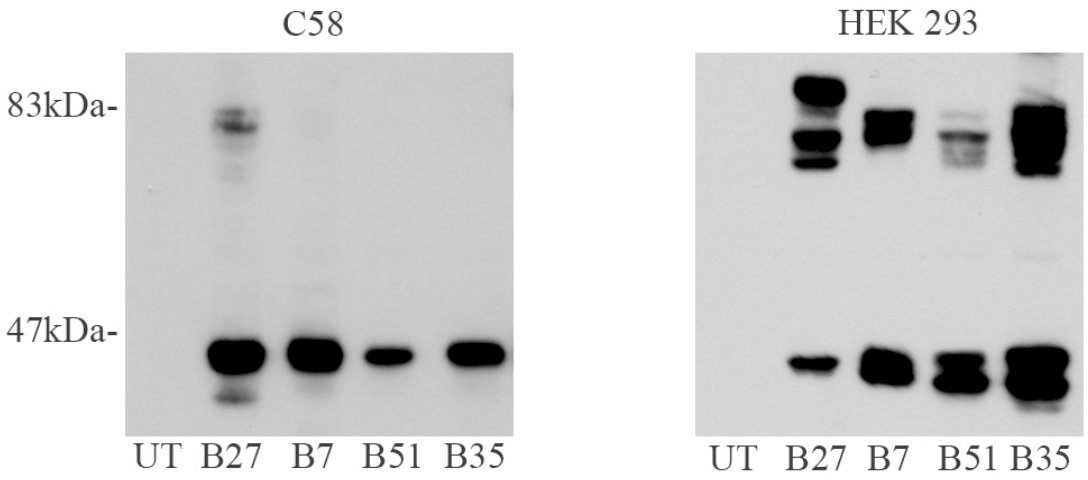


Figure 4.14: Dimer formation by non-AS associated HLA class I alleles in HEK293 cells. All the HLA class I alleles were cloned into the pCR3 or pCDNA3 mammalian expression vectors. C58 cell lines were generated through electroporation and stable lines selected by G418 selection. HEK293 cells were transiently transfected with 1μg DNA and Fugene 6. After 24 hours, cell lysates were prepared and immunoblotted with HC10. UT represents untransfected cells. HEK293 cells express only very low levels of endogenous MHC class I, and the endogenous rat MHC class I in C58 cells is not detected by HC10. This experiment was performed in conjunction with Dr S Powis.

Discussion

The discovery that HLA-B27 misfolds forming non-conventional structures (Mear et al., 1999), has formed the basis of a new avenue of research into AS, focussing on the intrinsic properties of HLA-B27 folding rather than its antigen presenting abilities. In particular the formation of HLA-B27 homodimers has been implicated in the pathogenesis of disease (Allen et al., 1999, Colbert, 2000). Nevertheless, if HLA-B27 dimers are involved in driving forward the inflammatory process that leads to AS, there must also be an explanation as to why 5-l0 % of AS patients do not carry the HLA-B27 gene. If it were to be the case that other MHC class I alleles could also, under specific conditions, form dimeric structures, then this may support the dimer-disease hypothesis.

The formation of homodimers in other class I alleles has been reported previously (Dangoria et al., 2002, Santos et al., 2008). However, the circumstances in which they form and what influences their formation has not been fully investigated. Using primarily the .220 cell line, that has previously been used to study the formation HLA-B27 homodimers (Bird et al., 2003), the formation of HLA-B8 homodimers has been studied in this chapter.

Stable .220 cell lines expressing HLA-B27, -B8 or -B8 and human tapasin (B8.htpn) were characterised by western blotting and flow cytometry (Figure 4.1). In the .220.B27 cell line that we used, low total HLA-B27 expression was detected although HLA-B27 was still expressed at the cell surface consistent with this allele being relatively tapasin

independent (Peh et al., 1998). In a previous report Bird and colleagues showed that HLA-B27 transfected into .220 cells forms dimers (Bird et al., 2003). These results significantly differ from the data shown here, and it is likely, based on the other data presented here that this is due to the low levels of HLA-B27 expression in our .220.B27 cell line. However, it cannot be ruled out that dimers are forming but at a level lower than our current system is able to detect.

HLA-B8 was detected on the surface of .220.B8 cells, and it has previously been demonstrated that cell surface expression of this allele shows an intermediate level of tapasin dependency (compared with HLA-B27 and other alleles) (Peh et al., 1998). We were unable to detect dimers in the .220.B8 cell line (Figure 4.2), similarly Dangoria *et al*. were also unable to detect HLA-B8 dimers in another human lymphoblastoid cell line, C1R (Dangoria et al., 2002). However in our system, co-expression of HLA-B8 with human tapasin enhances the expression of HLA-B8 heavy chain and also results in the formation of a higher molecular weight complex that we have confirmed to be a HLA-B8 homodimer (Figures 4.2 & 4.3). This dimer formation could either be related to the presence of tapasin or to the enhanced expression of HLA-B8. Re-expression of tapasin in a .220 cell line expressing HLA-B27 resulted in an overall reduction in dimer formation (Dangoria et al., 2002), suggesting that the increase in HLA-B8 dimers is more likely to be due to the enhanced expression of the HLA-B8 heavy chain.

To determine whether HLA-B8 dimers are present at the cell surface we performed cell surface biotinylation experiments (Figure 4.4), which revealed that HLA-B8 homodimers are present at the cell surface.

A higher molecular weight band is also visible in .220.B8.htpn and .220.B8 cell lines as well as in the two C58 cell lines expressing HLA-B27 alleles (Figure 4.4). It is interesting to speculate that these bands may represent a more unfolded class I dimer structure, however it is not clear whether this band is also present in the .220.B8.htpn 2D gel which would confirm it as a class I dimer band. Previously two structures for dimers have been observed, a more folded structure that is detected by W6/32 and an unfolded structure detected by HC10 (Dangoria et al., 2002, Allen et al., 1999). In the study by Dangoria *et al.*, the pool of HC10 reactive dimers were more heterogenous than the W6/32 reactive pool, with some bands being of a higher molecular weight than the W6/32 dimers.

The slower folding of HLA-B27 is one of the factors that have been directly implicated in the formation of HLA-B27 homodimers (Mear et al., 1999, Antoniou et al., 2004). To determine whether the folding of HLA-B8 in the .220.B8.htpn cell line could be involved in the formation of HLA-B8 dimers, we compared the folding efficiency of HLA-B8 in the .220.B8 cell line and the .220.B8.htpn cell line (Figure 4.5). Pulse chase analysis showed that HLA-B8 folded more efficiently in the .220.B8.htpn cell line. In the .220.B8 cell line, HLA-B8 molecules had not gained Endo H resistance 90 minutes post chase, consistent with the fact that in the absence of tapasin most class I alleles are unable to

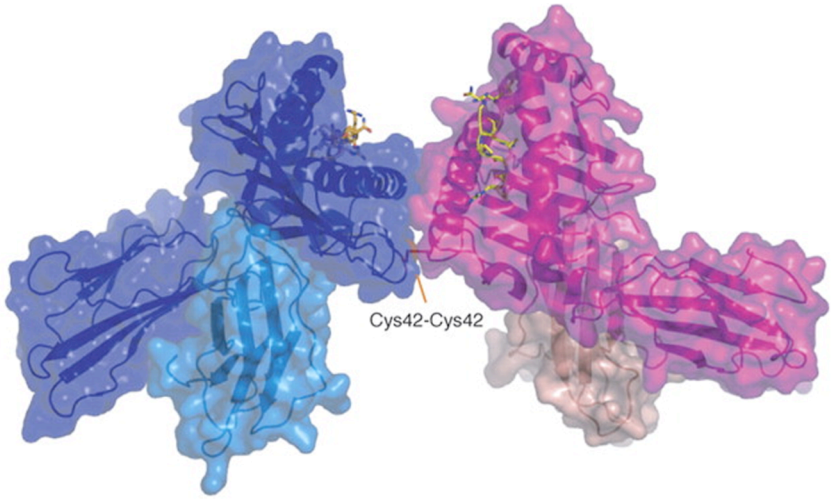
fold properly, preventing them from exiting the ER. This result suggests that slower folding does not influence the formation of HLA-B8 homodimers in the .220 cell line, which is therefore in contrast to the published data on this point.

Cell surface expression of HLA-B27 homodimers in the .220.B27 cell line is dependent on the internalisation and recycling of cell surface heterotrimers via an endocytic compartment, a process that can be blocked using lysomotrophic agents such as chloroquine (Bird et al., 2003). To determine whether chloroquine could influence the formation of HLA-B8 homodimers we incubated cells with chloroquine for 24 hours. In our system chloroquine was able to decrease the total amount of dimers but did not prevent their presence at the cell surface altogether (Figure 4.6 & 4.7).

Incubation of cells with diamide, to dramatically alter the redox balance within the cells, led to an interesting result (Figure 4.8). Homodimers were enhanced in a concentration dependent manner in the .220.B8.htpn cell line, and could be induced at higher diamide concentrations in the .220.B8 cell line. This suggests that the redox status of cells is normally balanced towards the prevention of MHC class I dimer formation.

Dimer formation of the non-classical HLA-G allele occurs *in vivo*, and these homodimers are known to be involved in immune recognition (Shiroishi et al., 2006, Gonen-Gross et al., 2003, Apps et al., 2007). This allele is unique amongst class I alleles because of the expression of a cysteine residue at position 42 (Cys^{42}), and HLA-G homodimers form through Cys^{42}-Cys^{42} interactions. These dimers are fully folded and retain their

association with β_2m (Boyson et al., 2002, Gonen-Gross et al., 2003). They are therefore, by nature, significantly different from the partially unfolded HLA-B27 dimers proposed by Bowness and colleagues (Figure 1.9), which may be involved in AS (Bowness, 2002).



Shiroishi, M. et al. J. Biol. Chem. 2006;281:10439-10447

Figure 4.15: Crystal structure of a β_2m-associated HLA-G dimer. The crystal structure of the disulfide-bonded dimer of the HLA-G-peptide (RIIPRHLQL) complex was solved in 2006. The HLA-G heavy chain and β_2m are represented in ribbon model and encased in a semitransparent surface (dark blue and pink, heavy chain; light blue and light pink, β_2m; yellow stick model, peptide RIIPRHLQL) (Shiroishi et al., 2006).

More recent data has shown that HLA-G can also form a β_2m-free dimer that is similar in structure to β_2m-containing HLA-G dimers, but these structures are differentially recognised by LILRB1 (Gonen-Gross et al., 2005).

To create a version of HLA-B27 that might mimic the dimer structure formed by HLA-G, HLA-B27 cDNA was mutated at position 42, changing a serine residue to a cysteine residue (Figure 4.9). We compared HLA-B27.S42C dimers in HeLa cells with the HLA-B8.htpn dimers by western blotting (Figure 4.10). Both dimer species migrated with virtually identical size, indicating that HLA-B8 dimers in the .220.B8.htpn adopt a more folded structure, similar to that of HLA-G dimers. Higher molecular weight species could also be detected in the HeLa.B27 and HeLa.B27.S42C samples. Two-dimensional gel analysis indicated both the higher molecular weight band and the S42C-induced band were both homodimers (Figure 4.12). However, cell surface biotinylation of these cell lines indicated that only the lower molecular weight dimer was present at the cell surface suggesting the other dimers may represent ER resident homodimers (Figure 4.13).

The hypotheis put forward from this chapter is that the dimers formed by HLA-B8 in the .220.B8.htpn cell line form because of the increased expression of HLA-B8. In support of this observation, over-expression of several other non-disease associated HLA class I alleles in HEK293 cells resulted in dimer-like structures forming. We have recently shown that HLA-B27 dimers form in the KG1 dendritic cell-like cell line and in peripheral monocyte derived dendritic cells following cell activation, a process that results in the increase of total class I, suggesting enhanced class I expression can also influence HLA-B27 dimer formation (Santos et al., 2008).

Increased expression of HLA-B27 has already been shown to be important in transgenic rat models, with only those animals expressing a high gene copy number developing

disease (Taurog et al., 1993) and HLA-B27$^+$ AS patients have been shown to express an increased level of HLA-B27 heavy chains compared with healthy HLA-B27$^+$ controls (Cauli et al., 2002). It is proposed therefore that under some circumstances, increased expression of non-HLA-B27 alleles can result in the formation of MHC class I dimers, which may act as recognition structures at the cell surface for immune receptors, or contribute to ER stress activation within the environment of the ER. It could therefore conceivably contribute to the disease process in a similar way as proposed for HLA-B27 homodimers.

Chapter V: HLA-B27 and Exosomes

Introduction

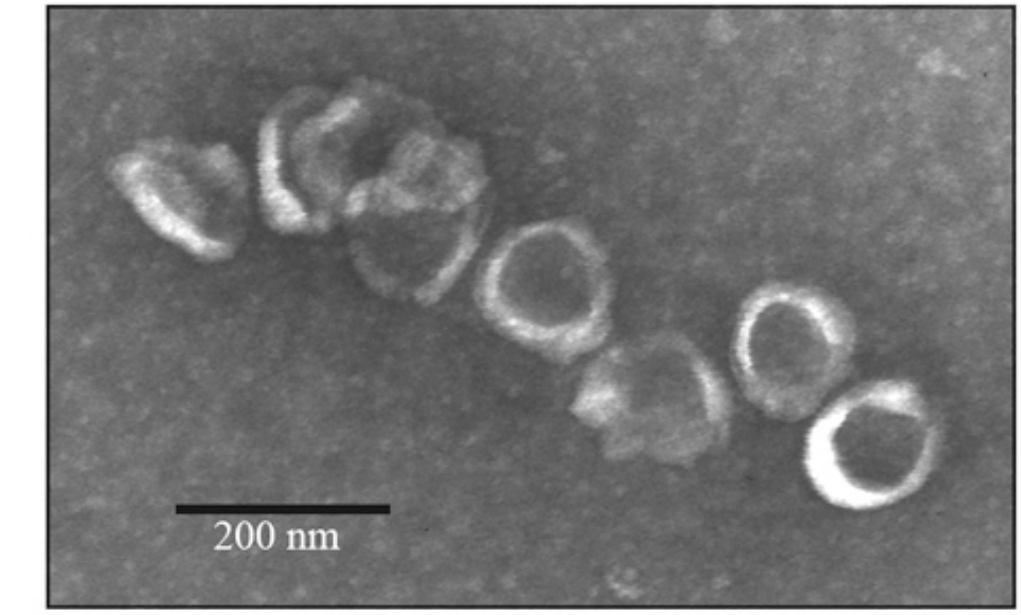
Exosomes are small vesicles released into the extracellular environment that can have both immunostimulatory and immunoinhibitory effects. Although they are secreted by many cell types, those released by cells of the immune system are of particular importance to immunologists because of their potential use as immunomodulatory agents.

This laboratory initiated a study into whether exosomes also bear the HLA-B27 dimer structures that have been implicated in the pathogenesis of AS, and therefore might be agents that would drive forward the disease process. Initial studies were performed on the human EBV-transformed B cell cell-line Jesthom, which has been characterised as undergoing features of the UPR (Lemin et al., 2007), a feature relevant to the previous work undertaken in this thesis.

Results

Exosomes enriched in high molecular weight MHC class I complexes can be isolated from the supernatent of Jesthom cells.

Exosomes were initially isolated, by filtration and ultracentrifugation, from the human B cell line, Jesthom (a gift from Adam Benham, University of Durham), which naturally expresses HLA-B27 and for comparison, were also isolated from the KG1 dendritic cell-like cell-line. Exosomes were imaged via electron microscopy.



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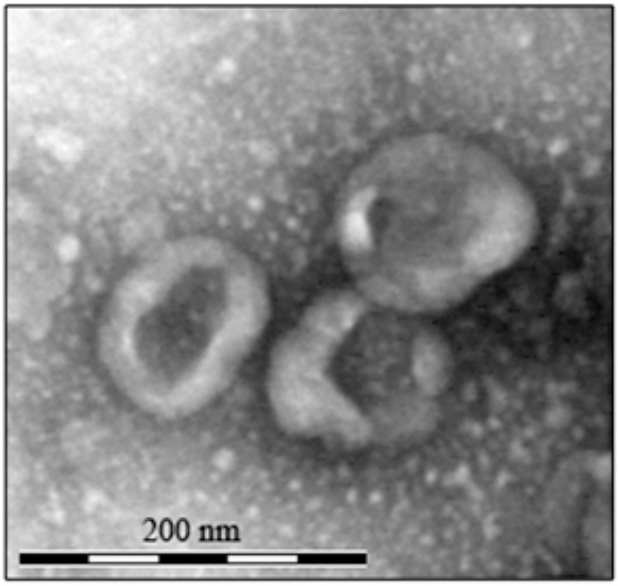


B

Figure 5.1: Electron microscopy image of isolated exosomes. Exosomes were isolated from (A) Jesthom cells or (B) KG1 cells, resuspended in 0.22 μm filter-sterile PBS and visualised using electron microscopy. Electron microscopy was performed by Dr Alan Prescott, University of Dundee. Results are representative of at least 2 experiments.

Electron microscopy revealed vesicles with 'cup' shape morphology, the characteristic shape of exosomes viewed under electron microscopy, with a diameter of approximately 100 nm, also typical of exosomes, although exosomes from Jesthom cells appeared slightly larger overall (Figure 5.1).

Exosomes usually contain a specific subset of proteins, differing in content from whole cell lysates, and are often enriched in MHC molecules and other molecules relevant to immune signalling. We isolated exosomes from Jesthom cells and compared equal protein quantities of whole cell lysates with exosome lysates. Samples were resolved by SDS-PAGE before western blotting with HC10.

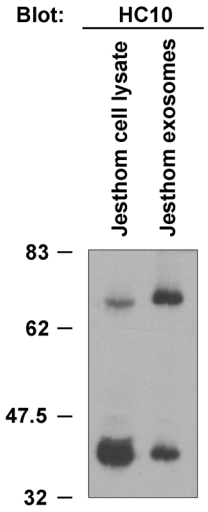
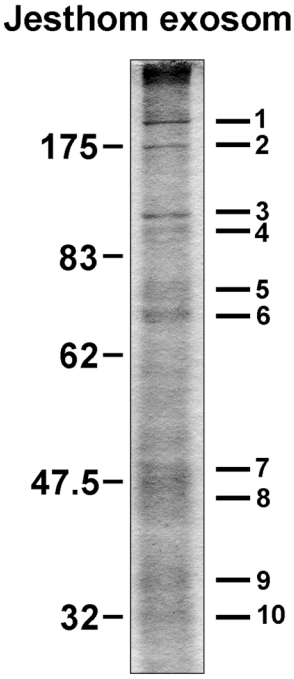


Figure 5.2: Exosomes contain enhanced amounts of HLA-B27 heavy chain dimers. Cell lysates and isolated exosomes were protein quantified using the Bradford method. 3 μg of each sample was loaded and resolved by 8 % SDS-PAGE and western blotting. Results are representative of at least 3 experiments.

We found that, compared with whole cell lysates, Jesthom exosomes are enriched in MHC class I dimers (Figure 5.2). This is an intriguing observation considering the speculative role of HLA-B27 homodimers in spondyloarthropathies and the reported abilities of exosomes to stimulate the immune system.

To further characterise the exosomes from the Jesthom cell line, exosomes were analysed by mass spectrometry (Figure 5.3). Bands were excised from a SDS-PAGE gel of exosomes, and submitted for in-gel tryptic digestion and mass fingerprinting.



1 (>175 kDa)	Myosin 9, transferrin receptor
2 (~ 175 kDa)	Clathrin heavy chain
3 (~ 130 kDa)	MVP, EBNA2
4 (~ 120 kDa)	Na+/K+ ATPase
5 (~ 80 kDa)	HLA class I, ezrin
6 (~ 70 kDa)	Annexin 6, HSP70, Moesin
7 (~ 47 kDa)	Actin, HLA class I, CD48
8 (~ 45 kDa)	HLA-A2, HLA-B27
9 (~ 34 kDa)	HLA-DR, CD20
10 (~ 30 kDa)	HLA-DR, annexin 4

Figure 5.3: Mass spectrometry of exosomes isolated from Jesthom cells. Exosomes isolated from Jesthom cells were resuspened in lysis buffer and quantified using the Bradford method. Approximately 3 μg of exosomes were resolved using 10 % SDS-PAGE. Gels were washed to remove any excess SDS before staining with Simply Blue Safe Stain. Ten bands were cut out and analysed by mass spectrometry. Mass spectrometry was performed by Dr Catherine Botting, University of St Andrews.

Mass spectrometry revealed that exosomes isolated from Jesthom cell supernatent contained proteins characteristically known to be present in exosomes, (Figure 5.3). In addition, we also identified the presence of CD48, a protein not previously reported in association with exosomes. CD48 binds to the 2B4 molecule, which is expressed on NK

cells and some T cell subsets and mediates non-MHC-restricted toxicity (Valiante and Trinchieri, 1993) (Garni-Wagner et al., 1993).

We also confirmed some of the mass spectrometry results, and further characterised the Jesthom exosomes, through western blotting (Figure 5.4).

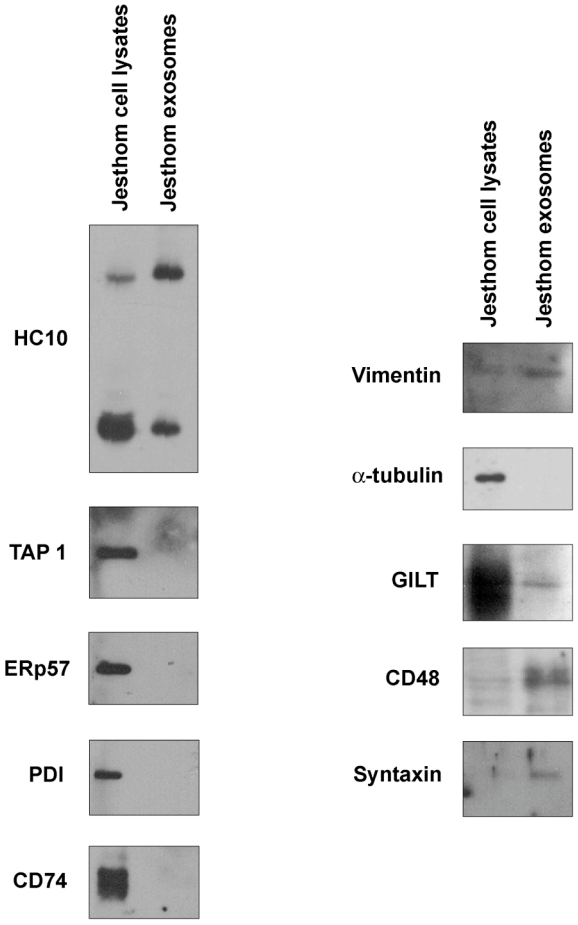


Figure 5.4: Characterising proteins in exosomes and whole cell lysates. Cell lysates and isolated exosomes were protein quantified using the Bradford method. Samples were then resolved by 8 % or 10 % SDS-PAGE and western blotting. Membranes were probed with antibodies to the indicated proteins. Results are representative of at least 2 experiments.

Jesthom exosomes, as would be expected, are devoid of proteins normally resident in the ER (TAP1, ERp57, PDI). They were also essentially devoid of CD74 (invariant chain), which has been previously reported to be absent in B cell-derived exosomes (reviewed in

Thery et al., 2002b), and also α-tubulin. Western blotting reveals the presence of low levels of GILT (γ-interferon-inducible lysosomal thioredoxin) an enzyme involved in disulfide bond reduction in endosomal compartments, and also expected exosomal proteins such as vimentin and syntaxin (Mignot et al., 2006). The mass spectrometry identification of CD48 was also confirmed by western blotting.

To further investigate the MHC class I molecules on the surface of exosomes, we bound the exosomes to latex beads to permit analysis by flow cytometry (Figure 5.5).

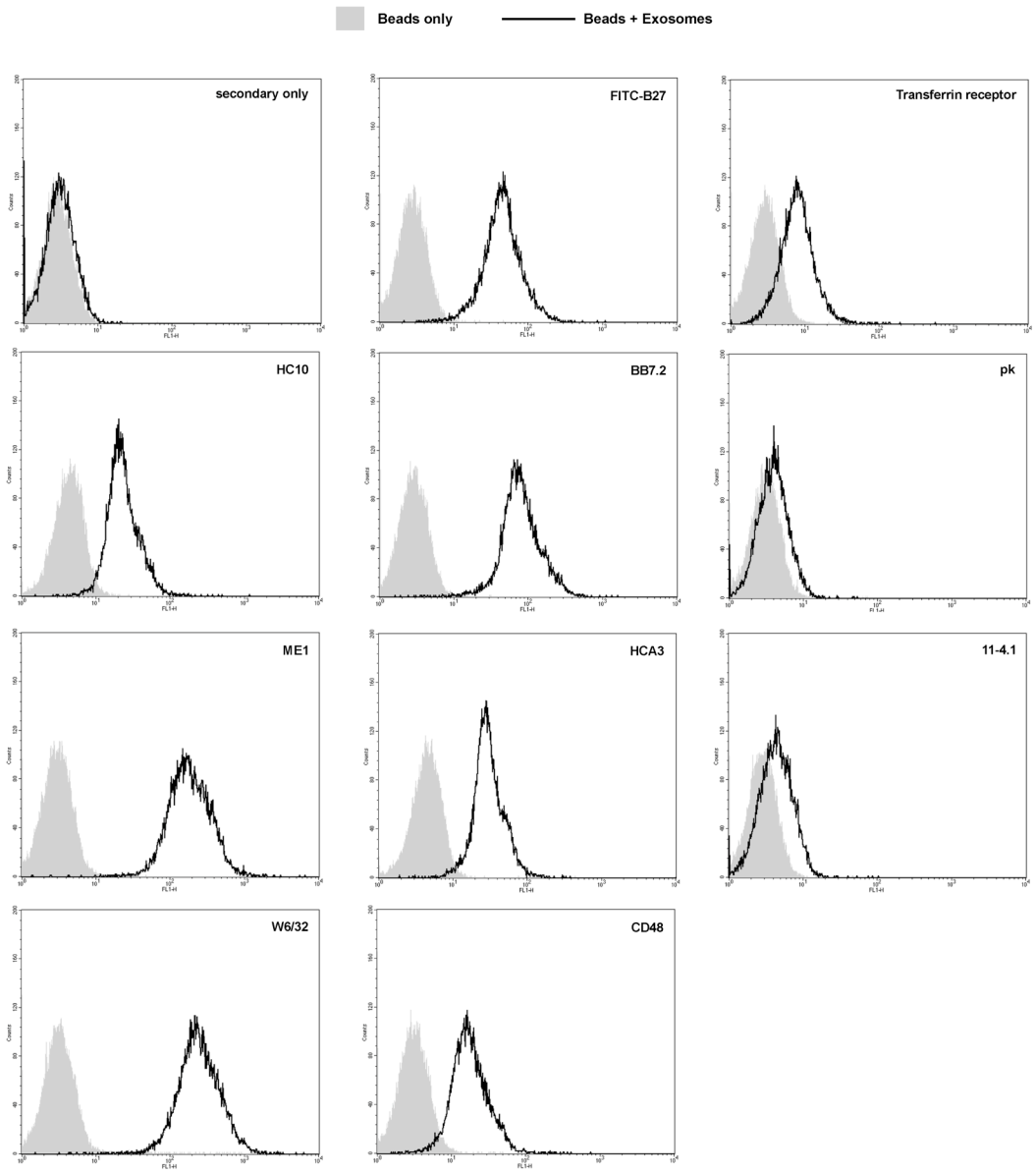


Figure 5.5: Proteins on the surface of exosomes were analysed by flow cytometry. Exosomes isolated from Jesthom cell supernatant were bound to aldehyde-latex beads before analysis with flow cytometry. Beads (solid grey) and beads with exosomes bound (black line) were both stained to show any non-specific binding. Results are representative of at least 3 experiments.

At the surface of exosomes, MHC class I can be detected in both a folded (eg ME1, BB7.2 and W6/32 reactive) and unfolded (HC10 reactive) conformation. Somewhat unexpectedly, in contrast to the western blotting, that had revealed that exosomes contained a higher proportion of MHC class I dimers in comparison to monomers, the observed HC10 signal was relatively low in comparison to that of ME1. This observation led us to further characterise the nature of these proposed MHC class I dimer structures.

The high molecular weight band is an MHC class I homodimer.

To confirm that this high molecular weight complex, believed to be dimers, was made up of two class I heavy chains, we analysed exosomes using two-dimensional electrophoresis. Exosome lysate was separated in the first dimension by isoelectric focusing and in the second dimension by molecular weight (Figure 5.6).

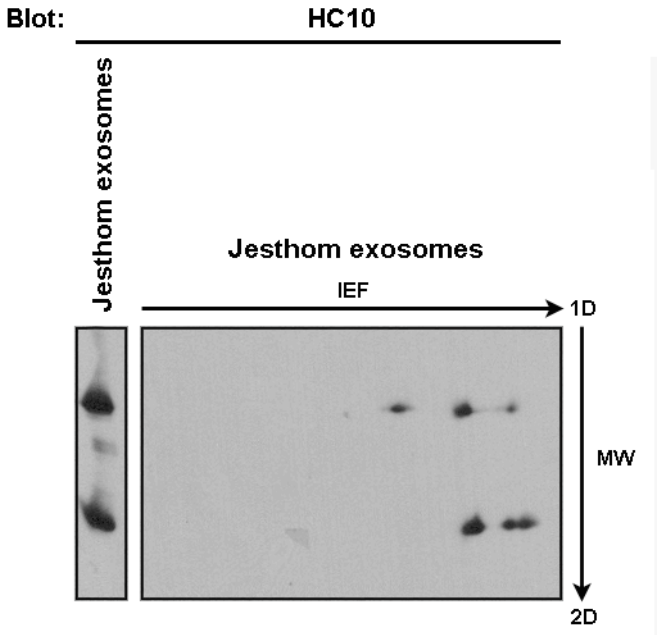


Figure 5.6: Heavy chain dimers were confirmed by 2D electrophoresis. Exosomes were isolated from Jesthom cell supernatants and were subject to lysis. Samples were resolved in the first dimension by isoelectric point and in the second dimension by molecular weight before western blotting. Membranes were probed with HC10. Results are representative of at least 3 experiments.

Figure 5.6 shows the presence of two HC10 reactive spots with the same isoelectric point, one corresponding to the monomeric species seen in the exosome lysates (left panel) and the other running at the same position as the higher molecular weight complex also seen in the lysate. Having the same isoelectric point as the monomeric class I molecules make the chance of this complex being a class I monomer bound to another molecule unlikely, and indicate that this higher molecular weight species is a HLA-B27 homodimer.

Unexpectedly, another spot was also identified, migrating to the left and very slightly higher than the dimer spot. Jesthom cells also express the class I allele, HLA-A2, and from previously published work, we knew that the HLA-A2 allele migrates to the left of HLA-B27 on this type of two-dimensional gel system (Antoniou et al., 2002b). It was therefore hypothesised that this spot may be a mixed dimer consisting of one HLA-B27 heavy chain and one HLA-A2 heavy chain. The HC10 antibody used in the blot is capable of detecting only HLA-B and -C heavy chains. Therefore, to confirm this hypothesis, we obtained monoclonal antibody HCA2, which recognises unfolded HLA-A heavy chains, and performed further two-dimensional electrophoresis, this time blotting with HC10 (unfolded B alleles), HCA2 (unfolded A alleles) or both HC10 and HCA2 (Figure 5.7).

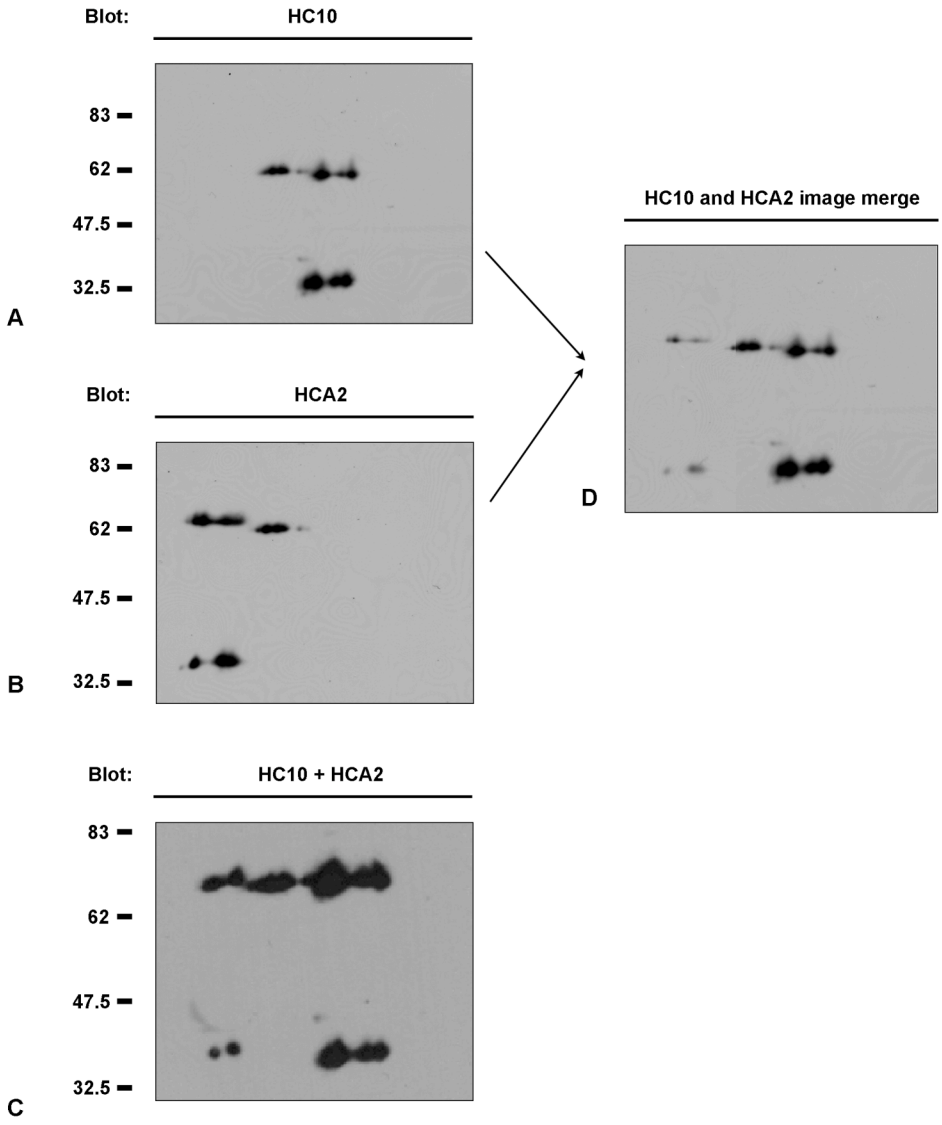


Figure 5.7: 2D electrophoresis reveals HLA-A2 dimers and novel A2-B27 dimers. (A) (B) and (C) Exosomes were isolated from Jesthom cell supernatants and were subject to lysis. Samples were resolved in the first dimension by isoelectric point and in the second dimension by molecular weight before western blotting. Membranes were probed with the indicated antibodies. (D) Images from (A) and (B) were merged overlaying the central band. Results are representative of at least 3 experiments.

Figure 5.7 A once again reveals the presence of dimeric HLA-B27 molecules with a further spot slightly higher and to the left of this dimer band. When probed with HCA2 (Figure 5.7 B) monomeric and dimeric HLA-A2 heavy chains are detected alongside another spot that migrates to the right of these dimers and slightly lower. Membranes probed with HC10 and HCA2 (Figure 5.7 C) show monomeric and dimeric HLA-B27 and HLA-A2 heavy chains, as in figures 5.7 A and B. A single spot located between HLA-A2 dimers and HLA-B27 dimers migrates slightly lower than HLA-A2 dimers and slightly higher than HLA-B27 dimers. Figure 5.7 D is the merged images of figure 5.7 A and B, this reveals the same pattern as the HC10 and HCA2 double blot (Figure 5.7 C). Taken together, the evidence strongly supports that this central spot represents a novel mixed dimer, consisting of an HLA-B27 heavy chain and an HLA-A2 heavy chain. The identification of this mixed dimer immediately raises questions about these structures in comparison to those of HLA-B27 dimers reported to be involved in AS, which rely on a disulfide bond between Cys^{67} residues in HLA-B27. HLA-A2 does not contain Cys^{67}, thus the dimers identified here must be significantly different, warranting further characterisation.

To further confirm the presence of both HC10 and HCA2 reactive dimers in exosomes, we compared Jesthom cell lysate with Jesthom exosomes by SDS-PAGE and western blotting (Figure 5.8).

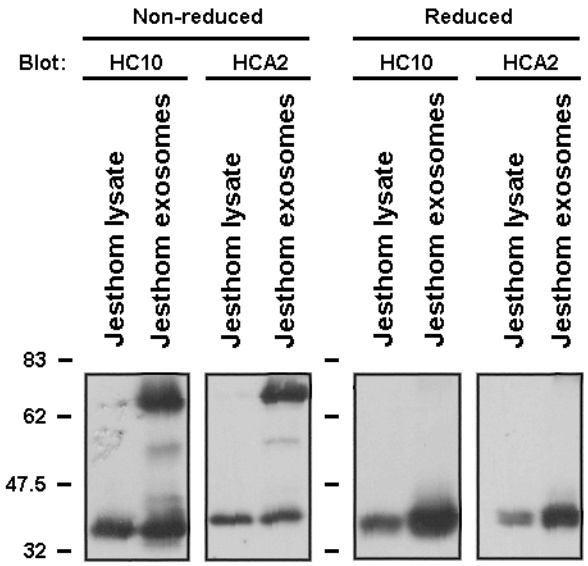


Figure 5.8: HC10 and HCA2 reactive dimers resolve to form a single monomeric species in the presence of DTT. Cell lysates and isolated exosomes were protein quantified using the Bradford method. Samples were resolved, with or without DTT, by 8 % SDS-PAGE and western blotting. Membranes were probed with HC10 or HCA2 as indicated. Results are representative of at least 3 experiments.

Figure 5.8 confirms that both HC10 and HCA2 reactive dimers are enhanced in Jesthom exosomes compared with whole cell lysates. In the presence of DTT both HC10 and HCA2 reactive dimers resolve to a single monomeric band.

We further investigated the formation of HLA-A dimers using the KG1 dendritic cell-like cell line. KG1 cells naturally express HLA-A3 and HLA-B35. We prepared exosomes from KG1 cell supernatants, which were resolved by SDS-PAGE, before western blotting.

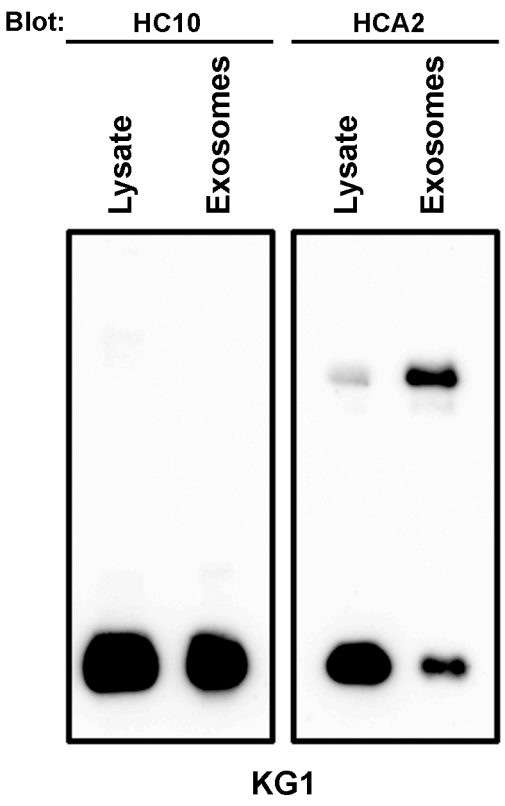


Figure 5.9: KG1 cells also contain HCA2 reactive dimers. Exosomes were isolated from cell supernatants and were subject to lysis, whole cell lysates were prepared and samples were protein quantified using the Bradford method. Samples were resolved by 8 % SDS-PAGE before western blotting. Membranes were probed with HC10 or HCA2 as indicated. Results are representative of at least 2 experiments.

Interestingly, KG1 cells contained HCA2 reactive dimers but not HC10 reactive dimers. This result suggests that some alleles are more prone to forming dimers than others, utilising a yet to be defined mechanism.

The formation of MHC class I dimers, dependent on disulfide bonds, can be prone to the formation of artefactual structures, mostly occurring post-lysis. To prevent this from occurring, it is routine to include the cell permeable alkylating agent NEM in pre-lysis

wash buffers and in the lysis buffers. When isolating exosomes from cell supernatents, the supernatants were normally pre-treated with NEM before centrifugation to isolate exosomes. To determine whether these dimers could be forming as a post lysis event due to inefficient alkylation, we compared exosomes that had only been treated pre-spin with NEM to those treated pre- and post- spin with NEM (Figure 5.10).

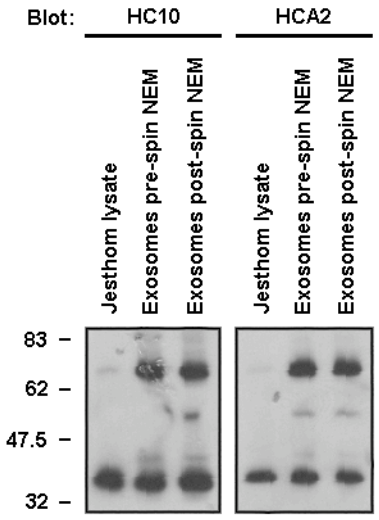


Figure 5.10: Dimers in exosomes are not a post lysis event. Jesthom exosomes were isolated from cell supernatents and treated prior to ultracentrifugation with NEM, an aliquot of exosomes was also treated with NEM post-centrifugation. Samples were then resolved by 8 % SDS-PAGE and western blotting. Membranes were probed with HC10 or HCA2 as indicated. Results are representative of at least 2 experiments.

Treating exosomes post-spin with NEM did not affect dimer formation indicating that these dimeric structures were not forming as a post lysis event. Dimers in exosomes were also not enhanced if exosomes were only treated post-spin (data not shown). Together

these results indicate that dimers in exosomes are not occurring due to a post-lysis event or because of the method used to isolate the exosomes.

Dimeric complexes in exosomes are immunoprecipitated with conformation dependent antibodies.

The identification of a mixed B27-A2 dimeric structure suggested that the exosomal MHC class I dimers were different to unfolded HLA-B27 dimers. To gain further information about the structure of the dimers found in exosomes, we therefore performed immunoprecipitations (IP) using different MHC class I reactive antibodies, both conformation-dependent and -independent, and also an antibody against β_2m.

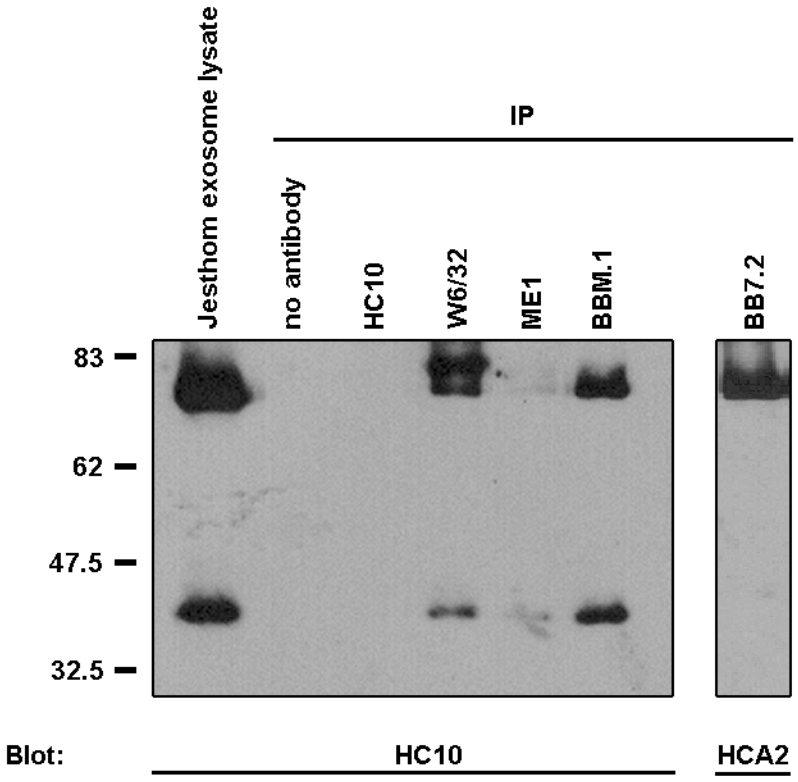


Figure 5.11: Exosome dimers are immunoprecipitated with conformation dependent antibodies. Exosomes were isolated from Jesthom cell supernatents and resuspended in lysis buffer. Lysates were then subject to immunoprecipitation with the indicated antibodies. Samples were resolved by 8 % SDS-PAGE and western blot. Membranes were probed with HC10 or HCA2 as indicated. Results are representative of at least 2 experiments.

HLA-B27 homodimers that have previously been reported are primarily HC10 reactive and consist of two unfolded heavy chains not associated with β_2m. It was therefore intriguing to discover that the dimers isolated from exosomes were recognised by W6/32, ME1 and BB7.2, and also by the β_2m specific-BBM.1 (note that ME1 is of relatively low affinity and is often poor in immunoprecipitation studies). These results indicate the

HLA-A and -B dimers in exosomes retain their confirmation and are associated with β_2m. These results have highly significant consequences for how these dimeric structures might be formed and recognised.

Dimers in exosomes may be forming through interactions between the cysteines present in the cytoplasmic tail.

The cysteine residue at position 67 in the HLA-B27 heavy chain has been strongly implicated in the formation of HLA-B27 dimers, although interactions between the structural cysteines residues could also play a part (Allen et al., 1999, Dangoria et al., 2002, Antoniou et al., 2004, Saleki et al., 2006). Some dimers have been reported to be W6/32 reactive indicating that they retain some of their normal confirmation (Allen et al., 1999, Dangoria et al., 2002), however fully folded HLA-B27 dimers that are still associated with β_2m, have not previously been described.

We took advantage of a stable KG1 cell line available in the lab, expressing HLA-B27 with cysteine to serine mutation at position 67, to investigate the importance of Cys^{67} on the formation of dimers in exosomes.

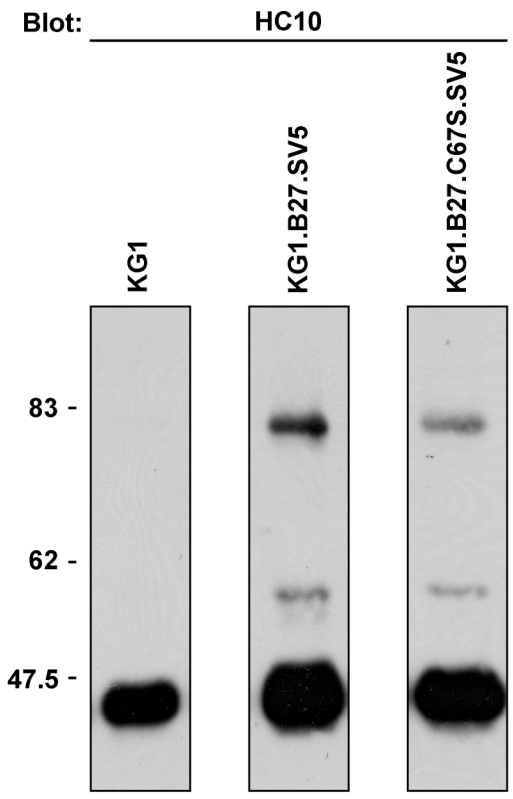


Figure 5.12: Exosome dimers are not dependent on Cysteine 67. Exosomes were isolated from the indicated KG1 cell line supernatant and were subject to lysis. Samples were resolved by 8 % SDS-PAGE, before HC10 western blotting. Results are representative of at least 2 experiments.

Exosomal dimers still formed in the absence of Cys^{67} suggesting that another cysteine residue, or residues, are important for their formation. If dimers were forming through an interaction between one of the structural cysteine residues, however, it is unlikely that they would be recognised by the confirmation dependent antibodies W6/32 and ME1, which has been shown to be the case in figure 5.11. Thus the exosomal MHC class I dimers described here must be forming through available cysteine residues other than the conserved structural residues at positions 101, 164, 203 and 259. For this reason we

hypothesised that the MHC class I dimers in exosomes may be forming through interactions between the cysteine residues in the cytoplasmic tail, which a significant number of MHC class I molecules possess. For example HLA-B27 contains two unpaired cysteines at positions 308 and 325 (although 308 may be on the very edge of the transmembrane region, lying just immediately prior to the RRK stop-transfer signal), whilst HLA-A2 contains a single cysteine in its cytoplasmic tail at position 339 as shown below:

Figure 5.13: Cytoplasmic tail cysteines which are present in HLA-B27 and HLA-A2. HLA-B27 contains two cysteine residues in its cytoplasmic tail, at positions 308 and 325 (indicated in red). HLA-A2 contains only one cysteine residue at position 339 (indicated in red). The last 25 residues that are missing from the C58.A2.tailless cell line are indicated in blue.

To begin the investigation of the role of the cytoplasmic tail in MHC class I dimer formation we used a transfectant of the rat C58 cell line expressing an HLA-A2 allele with a cytoplasmic tail truncation removing the last 25 residues (Figure 5.13). It was hypothesised that removal of this region would prevent dimer formation in exosomes.

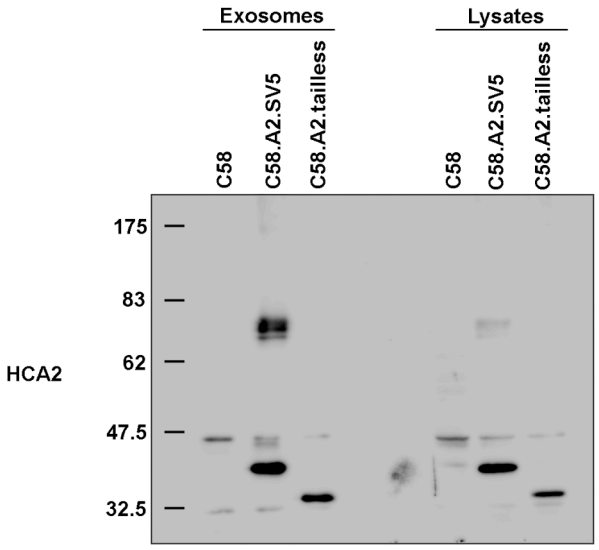


Figure 5.14: Exosomes isolated from C58 cells expressing an A2 allele with a tail truncation do not contain dimers. Exosomes were isolated from the supernatents of C58 cells expressing either a normal HLA-A2 allele or an HLA-A2 allele with a tail truncation. Exosomes were resuspended in lysis buffer and samples were resolved by 8 % SDS-PAGE and HCA2 western blot. Results are representative of at least 2 experiments.

Figure 5.14 shows that HLA-A2 dimers are present in exosomes isolated from the C58.A2.SV5 cell line but that these dimers are undetectable in the C58.A2.tailless cell line. This result suggests that W6/32 reactive dimers in exosomes are forming through interactions between the cysteine residues in the cytoplasmic tails. Further support for this hypothesis is present in figures 5.9, 5.12 and 5.23. In these figures immunoblotting with HC10 of KG1 derived exosomes reveals no dimer structures, whereas blotting with HCA2 does reveal dimers (Figure 5.9). Whilst this result was initially confusing, in light of the data presented above on the role of cytoplasmic cysteines, a check of the published

sequence of the HLA-A3 and HLA-B35 alleles present in KG1 cells reveals that HLA-A3 contains Cys^{339}, whereas HLA-B35 does not have Cys^{325}, thus explaining the observation. This also suggests that Cys^{308} is not significantly involved in exosomal dimer formation.

Depleting Jesthom cells of cholesterol may alter exosome composistion or release.

The use of methyl-β-cyclodextrin (MBC) to deplete cells of cholesterol has been reported to enhance clustering of MHC class I molecules. If exosomal MHC class I dimer formation involves cytoplasmic tail cysteines, we hypothesised that by depleting cholesterol from exosomes we may increase the clustering of MHC class I and therefore increase dimer formation.

In an attempt to deplete cholesterol from the exosomes, cells, and supernatant, were treated with an increasing concentration of MBC for 30 minutes before exosomes were isolated from the supernatent.

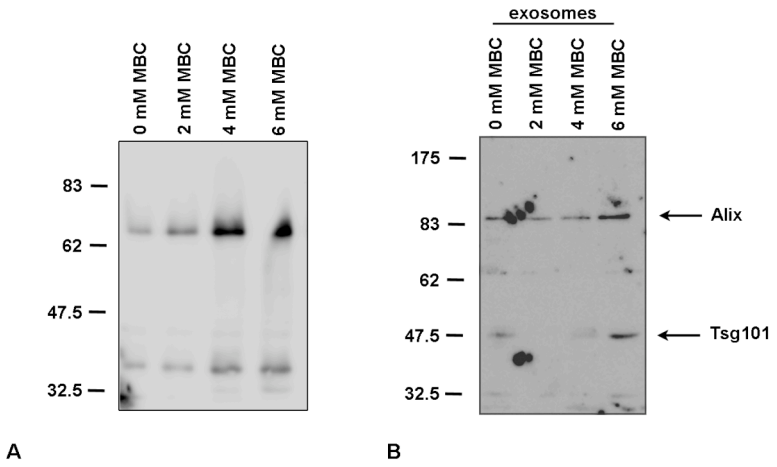


Figure 5.15: Depleting Jesthom cells of Cholesterol alters exosome formation. Jesthom cells were treated with the indicated concentrations of MBC for 30 minutes. Exosomes were then isolated from the cell supernatant. Exosomes were resuspended in lysis buffer and samples were resolved by 8 % SDS-PAGE and western blotting. (A) Samples were run non-reduced and the membrane was probed with HC10. (B) Samples were run reduced and the membrane was probed with Alix and Tsg101. Results are representative of at least 2 experiments.

Unexpectedly, treatment with the higher concentrations of MBC (4 mM and 6 mM) increased the total class I signal but did not seem to alter the monomer to dimer ratio (Figure 5.15 A). To try to determine whether this was a class I specific increase or whether other proteins were also increased, we blotted the same samples again using antibodies to detect Alix and Tsg101. Alix and Tsg101 are involved in the formation of exosomes and we had only weakly been able to detect them in previous blots (data not shown). Figure 5.15 B shows that both Alix and Tsg101 are increased following treatment with MBC. These data indicate that depletion of cholesterol from Jesthom cells may alter either exosome release or exosome composition.

To determine whether the treatment of isolated exosomes with MBC could enhance dimer formation we treated isolated exosomes with the same concentration of MBC as was previously used to treat cells and supernatants.

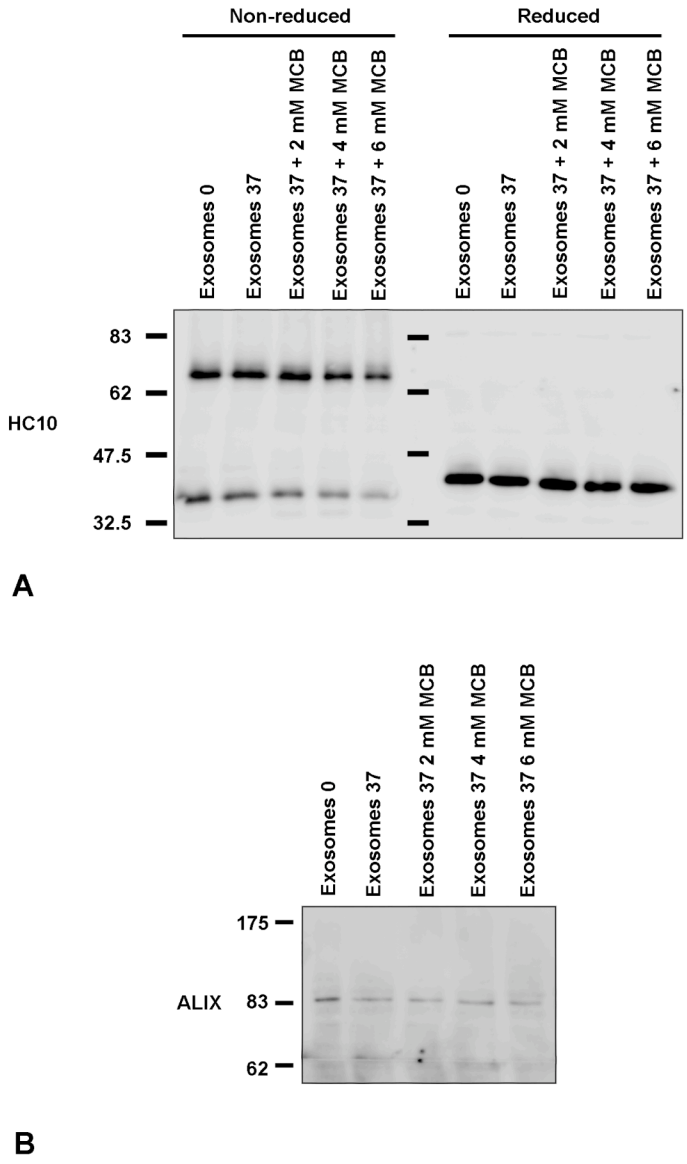


Figure 5.16: Treatment of exosomes with MBC does not affect dimer formation. Exosomes were isolated from the supernatant of Jesthom cells and resuspended in 0.2 µm filter-sterile PBS. Exosomes were protein quantified using the Bradford method. Equal quantities were treated with different concentrations of MBC at 37 °C for 30 minutes. Samples were resolved by 8 % SDS-PAGE before western blotting. (A) Samples were run non-reduced (left) and reduced (right) and membranes were blotted with HC10. (B) Samples were run reduced and the membrane was blotted with Alix. Results are representative of at least 3 experiments.

Treating isolated exosomes with MBC had no effect on the monomer to dimer ratio (Figure 5.16 A). Samples were also blotted using Alix to determine whether there was any change to another exosome protein (Figure 5.16 B). Alix levels were also unaffected by MBC treatment.

These data indicate that cholesterol depletion and therefore clustering of MHC class I does not affect the formation of dimers in exosomes. However, treatment of Jesthom cells with the higher concentration of MBC appears to enhance exosome release or alter exosomes composistion.

Increasing extracellular glutathione concentration prevents dimer formation in exosomes isolated from Jesthom cell supernatants.

To try to answer the question of why these dimers were able to form more readily in exosomes than in cells we carried out a number of experiments designed to alter the exosomal environment.

Reduced glutathione (GSH) is the major free thiol in most living cells and is involved in the maintenance of the oxidation state of protein sulfhydryls. We hypothesised that the cysteines in the cytoplasmic tail of the MHC class I alleles may be kept in a reduced state through the action of GSH. If exosomes were unable to maintain the GSH levels then this may allow the cysteine residues to become oxidised allowing disulfide bonds to form. The concentration of GSH alters significantly in different cellular compartments.

Within the cytoplasm it can be as much as 10 mM, dropping to around 1 mM in the ER, hence the ER is a much more oxidising environment. Outside cells the GSH concentration drops dramatically to around 20 μM. Jesthom cells were therefore allowed to secrete exosomes into an extracellular environment where the GSH level had been raised to levels as high as those present in the cytoplasm. Under these conditions MHC class I dimers were essentially unable to form (Figure 5.17).

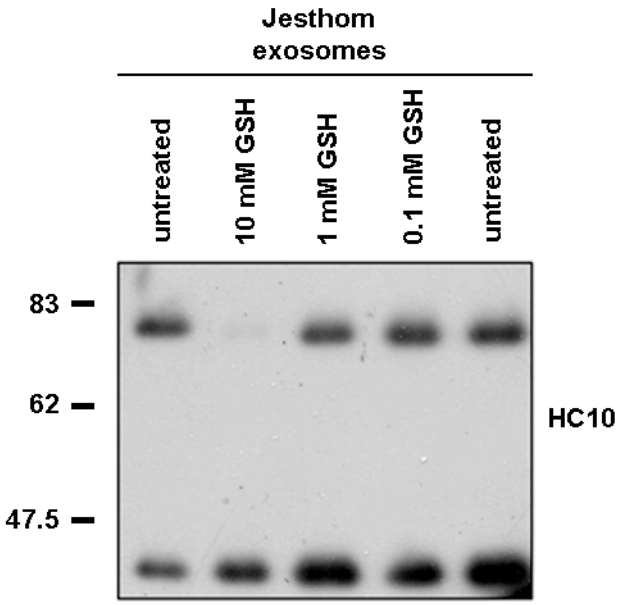


Figure 5.17: Increasing GSH concentration reduces dimer formation in exosomes. Supernatants from Jesthom cells were treated with varying concentrations of GSH for 6 hours. Exosomes were isolated from the supernatant and resuspended in lysis buffer. Samples were resolved by 8 % SDS-PAGE and HC10 western blot. Results are representative of at least 2 experiments.

Diamide induces the formation of W6/32 reactive dimers in cells.

Leading on from the above observation, if the reducing capacity of the cytoplasm, in terms of GSH levels, prevents the formation of MHC class I dimers at the cell surface of normal cells, then it is logical to assume that changing the cellular environment to a more oxidative environment would drive the formation of these complexes. To investigate this hypothesis, we incubated Jesthom cells with increasing concentrations of diamide, which acts by rapidly depleting free GSH levels within cells.

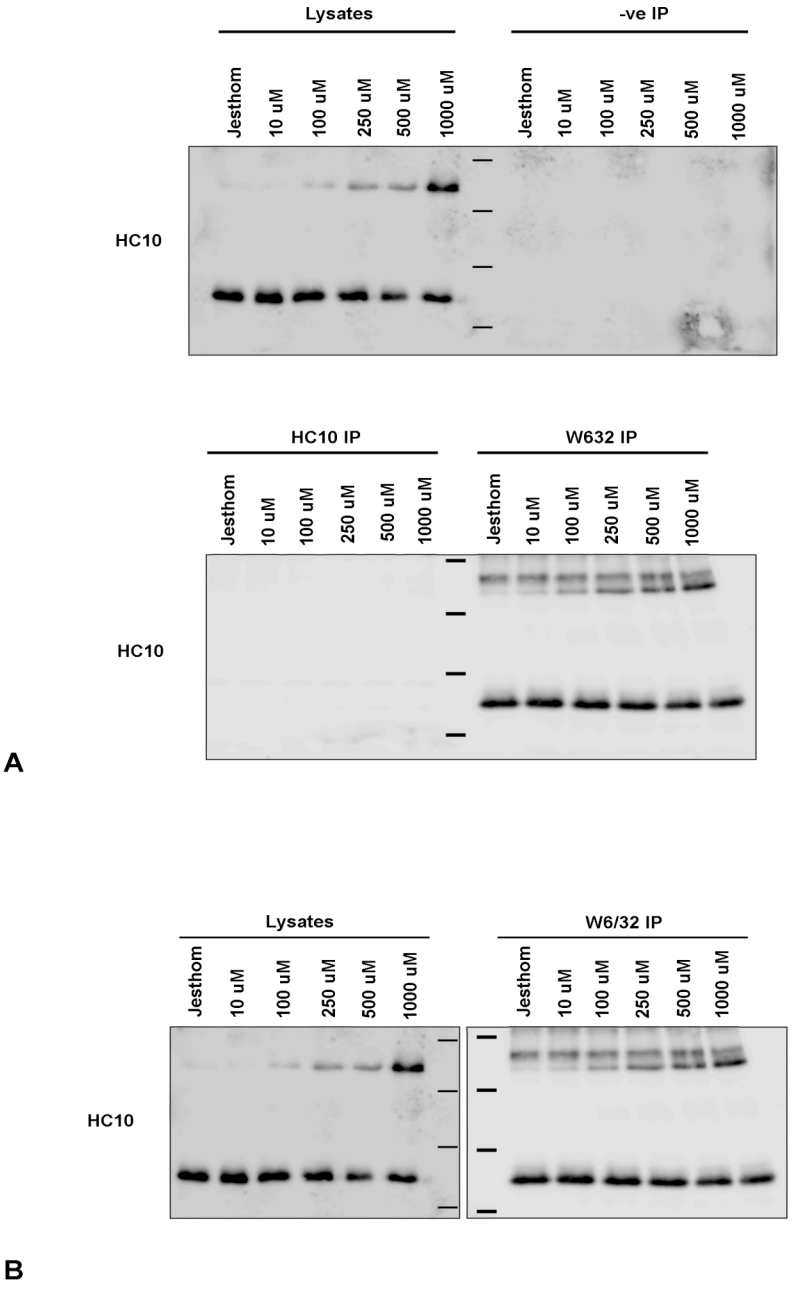


Figure 5.18: Treatment of Jesthom cells with diamide induces the formation of W6/32 reactive dimers. (A) Jesthom cells were treated with diamide in RPMI for 10 minutes at 37 °C. Cells were lysed and lysates were subject to immunoprecipitation with the indicated antibodies. Samples were resolved by 8 % SDS-PAGE before western blotting, membranes were probed with HC10. (B) Cell lysate image aligned with the W6/32 IP image. Results are representative of at least 2 experiments.

Treatment of Jesthom cells with diamide causes an increase in dimers in a concentration dependent manner (Figure 5.18 A, lysates). Immunoprecipitation with HC10 and W6/32 shows that the induced dimers are conformational dimers (W6/32-reactive), rather than misfolded dimers (HC10-reactive) (Figure 5.18 A IP). Aligning the W6/32 IP with the cell lysates image indicate that it is the induced dimer band that is immunoprecipitated with W6/32 (Figure 5.18 B). A non-specific band above the dimer band is also visible in the W6/32 IP image.

Jesthom exosomes have reduced levels of glutathione compared with Jesthom cells.

The data presented so far strongly suggests a model wherein exosomes do not contain levels of GSH that can maintain the cytoplasmic tails of MHC class I molecules in a reduced form, thus permitting the formation of MHC class I dimer structures. It was therefore a priority to directly measure levels of GSH in exosomes in comparison with the cell lysates. To measure the GSH content of exosomes we used a colourimetric glutathione assay kit (Sigma). This assay determines the total GSH (GSH + GSSG) content of a sample. GSH causes reduction of the substrate DTNB to TNB and oxidised GSSG is recycled by glutathione reductase and NADPH. The yellow colour of the product, TNB, is measured. We compared the GSH content of Jesthom cell lysates with exosomes isolated from Jesthom cell supernatants.

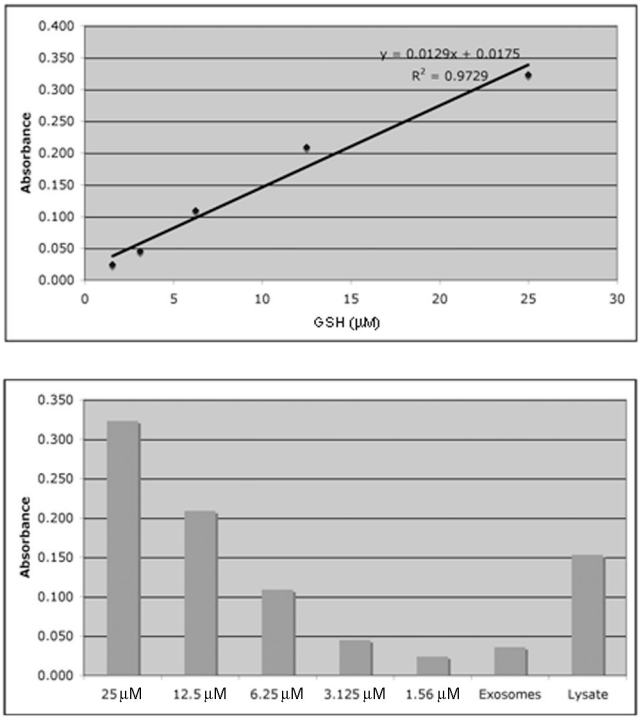


Figure 5.19: Exosomes have lower levels of glutathione compared with whole cells. GSH concentrations were estimated using a colourmetric glutathione assay kit as per manufacturers instructions. Samples were prepared in duplicate and were read on a FLUOstar Optima plate reader at a wavelength of 380 nm. The average of both readings were used to plot the graphs (the exosomes readings were 0.04 and 0.03, and the cell lysate readings were 0.157 and 0.153). Approximately 7 µg of exosomes and cell lysates were measured.

The GSH content of exosomes determined by this assay was significantly lower than in whole cell lysates (Figure 5.19), supporting the idea that cytoplasmic tail dimers are able to form in exosomes because of the lower overall level of GSH.

Exosomes containing dimers can be isolated from peripheral blood cells.

Exosomes have been isolated from many primary cells and biological fluids, proving their existence *in vivo*. However, although MHC class I and class II have been extensively studied in these exosomes, to our knowledge the existence of MHC class I dimers in exosomes has not yet been described.

In order to investigate the presence or absence of dimers in exosomes isolated from peripheral blood, we separated lymphocytes and monocytes from 3 healthy volunteers and matured them in culture. Activated lymphocytes and dendritic cells were compared with unactivated lymphocytes and dendritic cells.

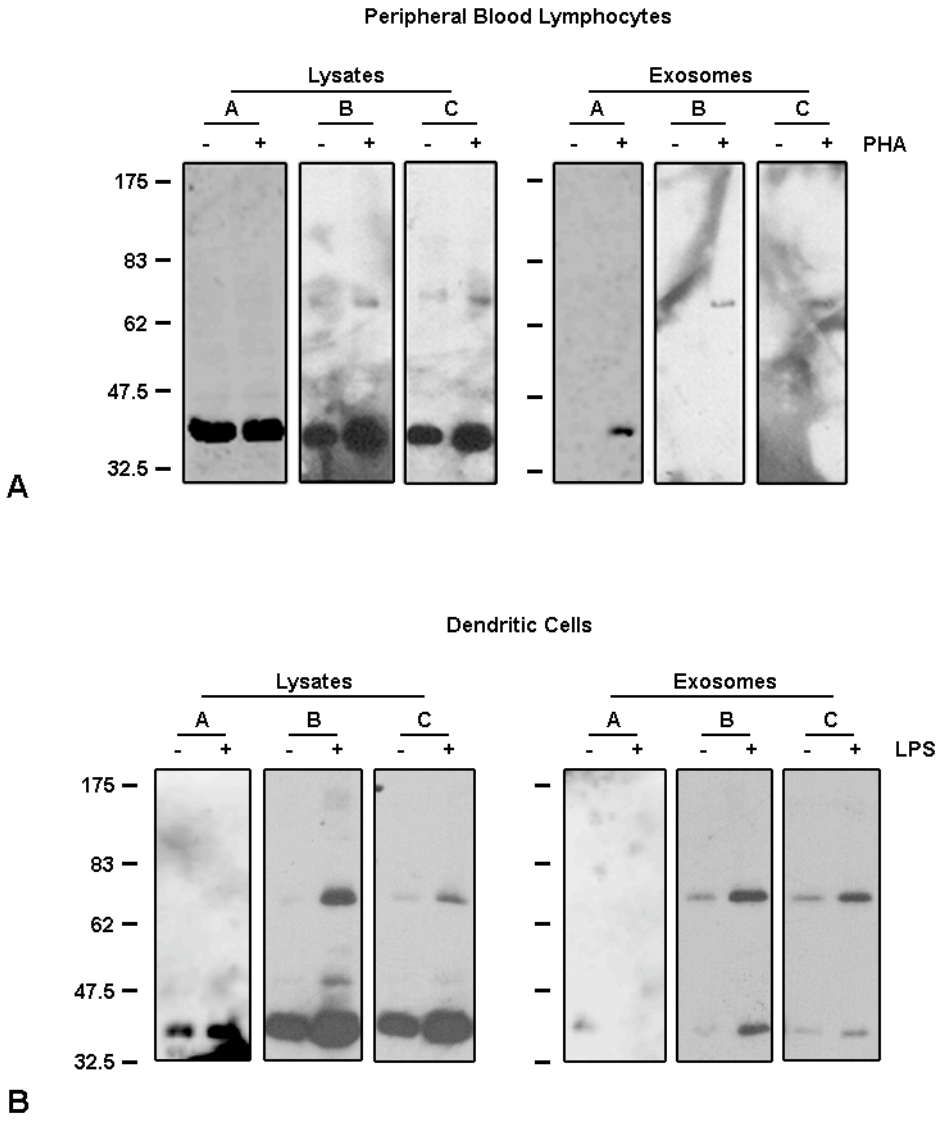


Figure 5.20: Exosomes isolated from PBMCs show differing levels of dimer and monomer expression. PBMCs were isolated from whole blood and cultured for 2 hours to allow monocytes to adhere to the plastic. (A) Lymphocytes, which remained in suspension, were separated from monocytes and either left untreated or treated with PHA for 48 hours. Exosomes were then isolated from the lymphocyte supernatants, samples were resolved by 8 % SDS-PAGE and HC10 western blot. (B) Monocytes were cultured with IL-4 and GM-CSF for 5 days. They were then either left untreated or treated with LPS for 48 hours. Exosomes were isolated from the monocyte supernatants, samples were resolved by 8 % SDS-PAGE and HC10 western blot.

No class I signal was detected from exosomes isolated from unstimulated lymphocytes however exosomes from stimulated lymphocytes show differing patterns depending on the sample (Figure 5.20 A). Sample A contains only monomer in both exosomes (Figure 5.20 A, right panel) and lysates (Figure 5.20 A, left panel). Samples B and C contain small amounts of dimer in the cell lysates, however only dimers are detectable in exosomes isolated from stimulated lymphocytes (Figure 5.20 A).

The pattern for dendritic cells and exosomes isolated from dendritic cells is relatively similar. Dendritic cells from sample A contain no class I dimer band and exosomes isolated from these cells contain only a very small amount of monomer (Figure 5.20 B). Samples B and C contain more monomer than dimer in the dendritic cells (Figure 5.20 B, left panel) but more dimer than monomer in the exosomes (Figure 5.20 B, right panel). The same pattern is seen in stimulated and unstimulated cells however MHC class I is enhanced in both forms following stimulation (Figure 5.20 B). It is clear in this image that there is less class I overall in Sample A compared with Samples B and C so this cannot be ruled out as a possible reason for why we are unable to detect much signal from the exosomes.

These data suggest that some class I alleles are more prone to forming dimers in exosomes than others. As HLA-B27 is well-known to misfold forming dimers we hypothesised that this allele may also be more susceptible to forming dimers in exosomes, and that lymphocytes from Samples B and C may be HLA-B27 positive. To investigate this hypothesis we analysed some of the primary lymphocytes by flow

cytometry using the antibody FITC-B27. Although this antibody is not 100 % specific for HLA-B27, staining would give us an indication of the possibility of these individuals being HLA-B27 positive.

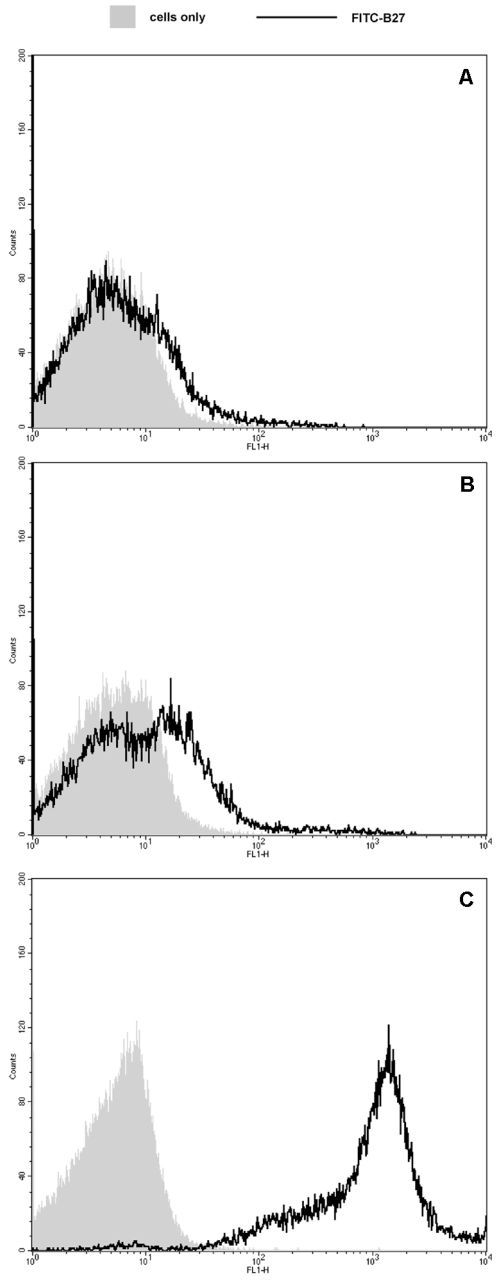


Figure 5.21: FACS analyses of primary lymphocytes. PBMCs were isolated from whole blood and cultured for 2 hours to allow monocytes to adhere to the plastic. Lymphocytes, which remain in suspension, were removed and blocked with 5 % human serum. Cells were stained with FITC-B27 and assessed by flow cytometry.

Analysis of the primary lymphocytes by flow cytometry indicates that Sample C is highly likely to be HLA-B27 positive (Figure 5.21, bottom panel). The result from Sample B is very intriguing. Sample B was found to express dimers in both exosomes isolated from lymphocytes and exosomes isolated from dendritic cells and the lymphocytes show some staining with FITC-B27 (Figure 5.21, middle panel). Sample A had no dimers in either exosomes isolated from lymphocytes or exosomes isolated from dendritic cells and lymphocytes from this sample showed no staining with FITC-B27 (Figure 5.21, top panel).

Although this data is very preliminary, it does suggest that dimers in exosomes may be influenced by the class I allele that is expressed and that possibly HLA-B27 positive individuals may be more susceptible to dimer formation in exosomes.

Exosomes containing dimers can be isolated directly from blood plasma.

Exosomes isolated from primary cells were maintained in cell culture for either 48 hours (lymphocytes) or 7 days (dendritic cells). To try to eliminate the possibility that dimers in exosomes may be forming due to the cells being maintained in cell culture we wanted to try and isolate exosomes from blood plasma directly without having any time in cell culture. A published protocol for isolating exosomes from blood plasma was already available, which involves additional steps over the normal protocol (Caby et al., 2005).

Blood samples were taken from three healthy volunteers, Samples B and C as above (Figures 5.20 and 5.21) and Sample D, a known non-B27 control. Plasma was seperated from whole blood and exosomes were then isolated.

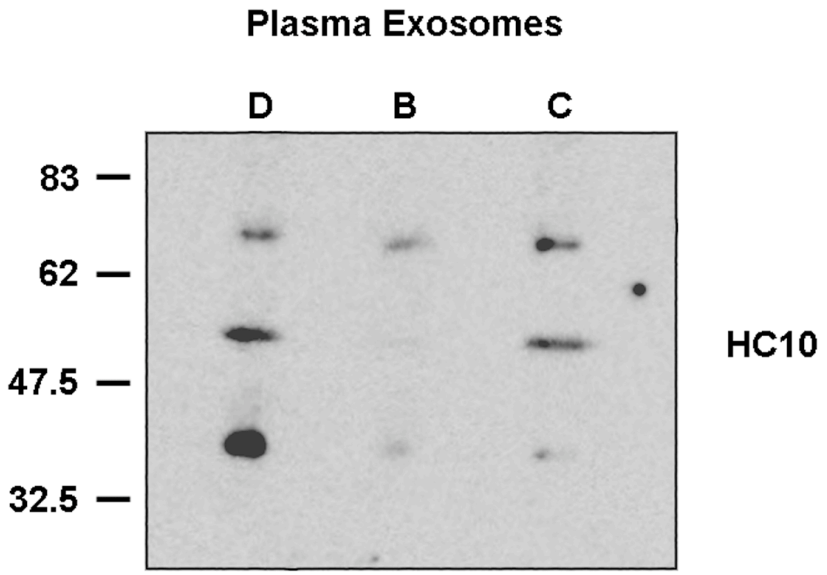


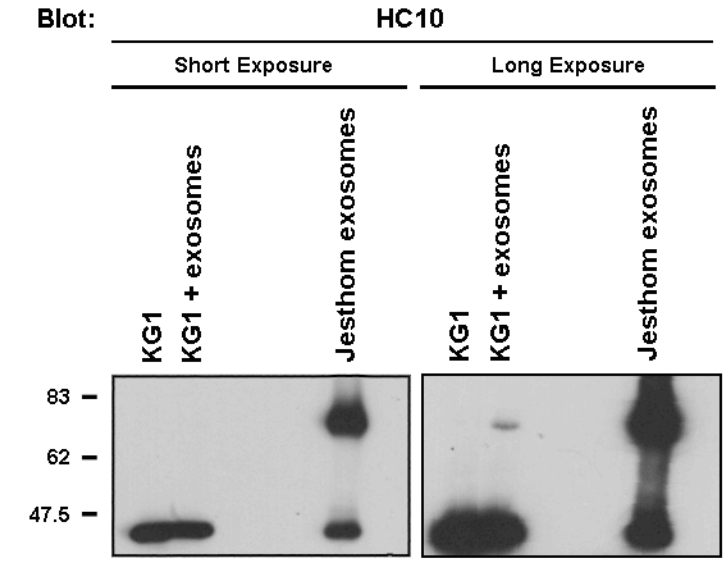
Figure 5.22: Exosomes containing MHC class I dimers are present in plasma. Plasma was separated from whole blood and was subject to centrifugation at 500 x g, 12000 x g, 0.22 μm filtration and ultracentrifugation to isolate exosomes. Samples were resolved by 8 % SDS-PAGE and HC10 western blot. A non-specific band was also identified at around 50 kDa.

Exosomes were isolated from all of the samples, however the monomer:dimer ratio differed among the samples (Figure 5.22). Sample D, the non-B27 control contained predominantly monomer and some dimer whereas samples B and C contained predominantly dimer and only a faint monomer band can be seen in both samples (Figure 5.22).

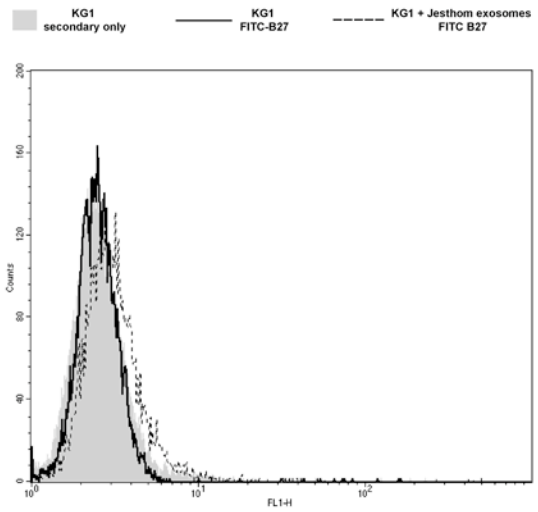
Crucially, this result indicates that exosomes containing MHC class I dimeric complexes are formed *in vivo* and that the monomer to dimer ratio can be different between different people. The physiological relevance of dimers on exosomes has not been proven, however considering the roles exosomes have been implicated in it certainly seems worth studying MHC class I in exosomes in both traditional and non-traditional roles.

Exosomes can transfer MHC class I dimers to KG1-dendritic-like cells.

One of the methods suggested for how exosomes can activate the immune system is through the transfer of their class I molecules to dendritic cells. To test whether it was possible to transfer the exosomal MHC class I dimer structures to other cells, we incubated exosomes isolated from Jesthom cell supernatent with KG1 dendritic-like cells. We analysed KG1 cells by western blotting, flow cytometry and immunofluorescence.



A



B

Figure 5.23: Exosomes can transfer class I molecules to KG1 cells. Exosomes were isolated from Jesthom cell supernatents and resuspended in 0.22 μm filter-sterile PBS. Exosomes were then incubated with stimulated KG1 cells overnight. (A) Cells were lysed and samples resolved by 8 % SDS-PAGE before HC10 western blot. (B) Cells were harvested, stained with FITC-B27 and subject to FACS analysis.

KG1 cells contain their own normal class I molecules but are not HLA-B27 positive and do not normally express dimers. Following incubation with Jesthom exosomes, we were able to detect induction of a dimer band in the KG1 cell lysates, the dimer band runs at the same molecular weight as the dimer band in control Jesthom exosomes (Figure 5.23 A). We were also able to detect faint cell surface staining with FITC-B27 (Figure 5.23 B), an antibody that does not normally stain KG1 cells.

For immunofluorescent staining, KG1 cells grown on coverslips were incubated with exosomes isolated from Jesthom cell supernatent before staining with FITC-B27 (Figure 5.24).

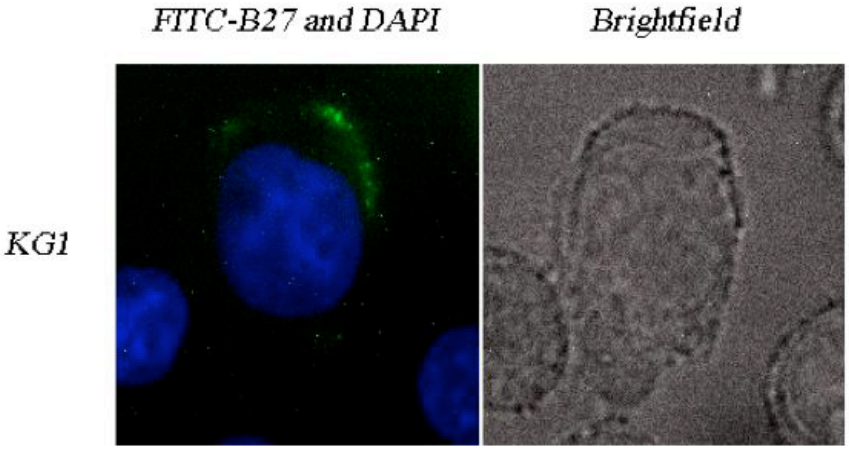


Figure 5.24: Exosomes can transfer class I molecules to KG1 cells. Exosomes were isolated from Jesthom cell supernatents and resuspended in 0.22 μm filter-sterile PBS. Exosomes were then incubated with stimulated KG1 cells, grown on coverslips, overnight. Cells were stained with FITC-B27 and visualised by immunofluorescence microscopy.

The cell in the centre of figure 5.24 shows some punctate staining around its periphery whilst the two cells visible in the right and left hand corners showed no staining at all. It is not possible to distinguish in this image whether the exosomes have bound to the cell surface or have fused with it. However the FITC-B27 staining of some of the cells indicate that HLA-B27 can be transferred from exosomes to KG1 cells.

We were further able to analyse exosomes transfer using a lipid dye. Bodipy ceramide usually stains the golgi apparatus but in excess will also stain other membranes. In an attempt to visualise exosome release from Jesthom cells, we stained Jesthom cells with excess bodipy before immobilising them in agar on coverslips, and re-incubating them at 37 °C with 5 % CO_2 in air for 24 hours.

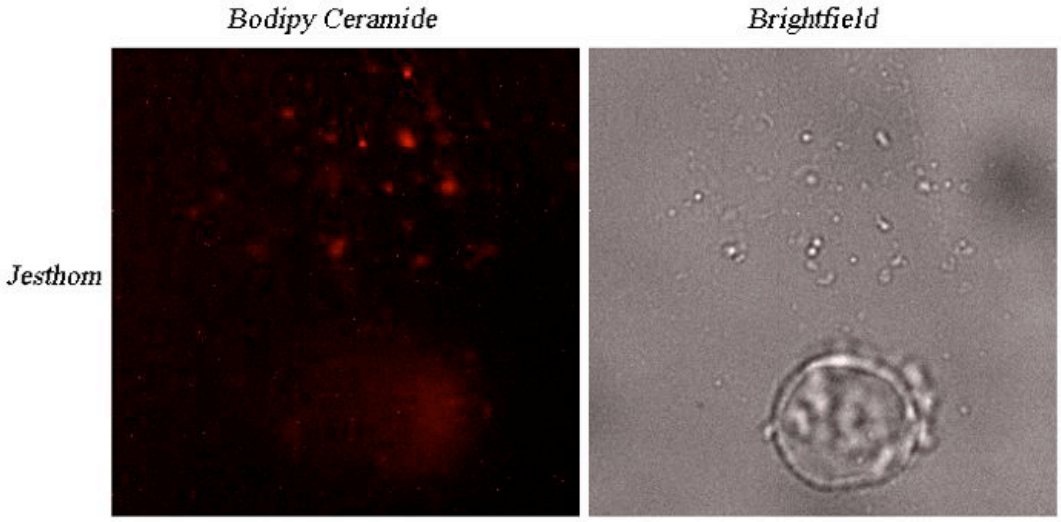


Figure 5.25: Exosome-like vesicles immobilised in agar. Jesthom cells stained with excess bodipy were immobilised in agar on microscope slides covered with a coverslip and incubated at 37 °C with 5 % CO_2 in air, for 24 hours. Cells were visualised by immuno-fluorescence microscopy.

A few of the cells imaged were surrounded by small speckles of Bodipy staining. These speckles usually matched up to areas that were also visible in the brightfield image (Figure 5.24).

To further try to show transfer between exosomes and KG1 cells we isolated exosomes from Jesthom cells that had been stained with excess bodipy. We incubated stimulated KG1 cells with these exosomes for 24 hours.

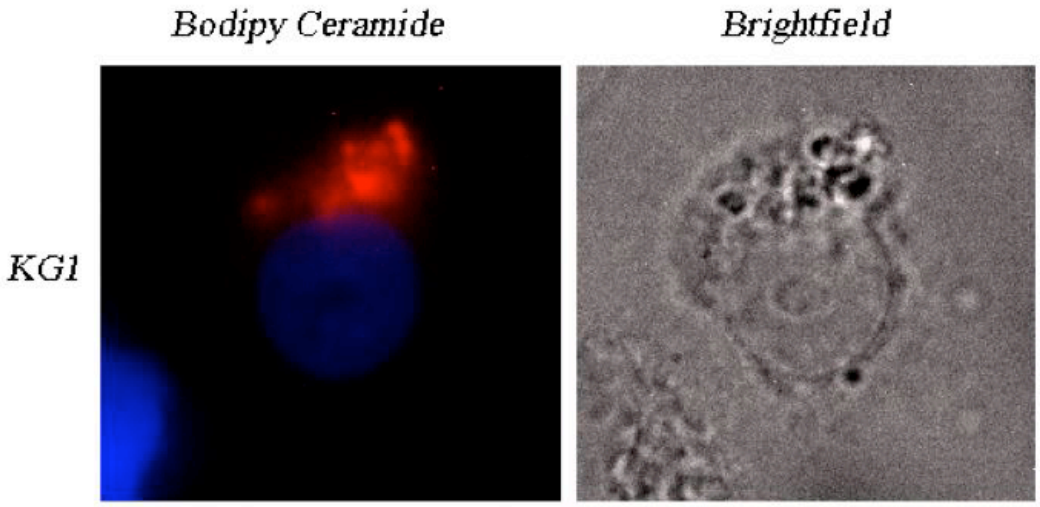


Figure 5.26: Exosomes can transfer staining to unstained KG1 cells. Exosomes isolated from the supernatant of Jesthom cells stained with excess bodipy were incubated with stimulated KG1 cells, grown on coverslips, at 37 °C, 5 % CO_2 in air, for 24 hours. Cells were visualised by immuno-fluorescence microscopy.

Some KG1 cells showed staining with Bodipy although the majority showed no staining at all (Figure 5.26). The regions of bodipy appeared in the brightfield image to consist of small clusters. However, the brightfield image suggests that the cell we imaged is not very healthy looking and this must be taken into consideration when interpreting this result.

Discussion

We have isolated exosomes from the human B cell line, Jesthom, the human dendritic-like cell line KG1, peripheral blood lymphocytes, monocyte-derived dendritic cells, and blood plasma. We confirmed that what we had isolated were classifiable as exosomes by a combination of electron microscopy (Figure 5.1), flow cytometry (Figure 5.5) and western blotting (Figure 5.4). Exosomes showed characteristic 'cup' shaped morphology under electron microscopy and were within the size range of previously published exosomes (Li et al., 2006) (Chaput et al., 2006) (Wubbolts et al., 2003) (Abusamra et al., 2005). Mass spectrometry revealed the presence of MHC class I and class II proteins as well as other known exosome-associated proteins (Figure 5.3) (Mignot et al., 2006). We also identified CD48 and GILT, proteins that had not previously been associated with exosomes. Intriguingly, western blotting revealed that exosomes from Jesthom cells contain enhanced amount of HLA-B27 heavy chain homodimers compared with whole cell lysates.

Exosomes are being extensively studied because of their ability to modulate the immune system, and in the context of HLA-B27 and spondyloarthropathies, HLA-B27 homodimers are being studied to try to determine their role in disease pathogenesis. As work on exosomes continue, their involvement in different aspects of the immune system is becoming clearer. Expression of specific receptors targeting exosomes to different cells of the immune system suggest that exosomes could be yet another way in which the immune system can communicate. The ability of exosomes to interact with immune cells

may put dimeric class I molecules in a prime position to influence and modulate the outcome of these events.

Following the detection of class I dimers in exosomes we investigated whether GILT (γ-interferon inducible lysosomal thiol reductase) could also be detected in the exosomes preparations. GILT is an enzyme that catalyses the reduction of disulphide bonds in the endosomal/lysosomal compartment (Arunachalam et al., 2000), and thus could have contributed to the formation of unfolded MHC class I dimers of the type suggested in the work of Bowness and colleagues (Bird et al., 2003). Although we were able to detect GILT, the levels were very low, and further investigation of the dimers present in exosomes revealed that they were actually essentially all fully folded, suggesting that GILT probably does not influence their formation.

The interaction of exosomes with NK cells has previously been reported (Clayton et al., 2008, Elsner et al., 2007, Gastpar et al., 2005). In relation to this, the discovery of CD48 on the exosomes we have studied is extremely interesting. The expression of CD48 is limited to cells of lymphoid or myeloid origin (Yokoyama et al., 1991), and it is the ligand for the 2B4 receptor (Brown et al., 1998b, Latchman et al., 1998). 2B4 is expressed on NK cells and some T cell subsets and mediates non-MHC mediated cytotoxixity (Valiante and Trinchieri, 1993, Garni-Wagner et al., 1993). 2B4-CD48 interactions are essential for interleukin-2 (IL-2) driven expansion and activation of NK cells (Lee et al., 2006). The cytotoxic activity of NK cells is determined by both the activatory and inhibitory signals that they receive and the usual dominant signal that is

received is inhibitory through the interaction of NK cells and normal MHC class I molecules (reviewed in Bryceson and Long, 2008). However if MHC class I dimers are recognised differently by NK cells, the expression of CD48 may promote NK cell activation.

The detection of novel mixed dimers forming between different class I alleles on exosomes is extremely intriguing. These dimers would also be available for immunosurveilance and may influence immune responses. Whether HLA-B27 is necessary as part of the mixed dimer complex has not been elucidated but does deserve further study.

How these dimer molecules are seen by the immune system is an extremely important question. It has not been fully determined whether exosomes can be seen directly by other cells of the immune system or indirectly through attachment to, or fusion with, an APC. For this reason it was important for us to determine whether our exosomes could interact with, and transfer dimers to, other cells. We took advantage of a dendritic cell-like cell line that was available in our lab and were able to demonstrate the transfer of dimers from exosomes to these cells (Figure 5.23). We were also able to show the potential transfer of lipids from exosomes to KG1 cells using bodipy ceramide stained exosomes, however the brightfield image of the cell shown revealed that the cell imaged was probably not perfectly healthy and this must be taken into consideration (Figure 5.25).

If dimers can influence whether the immune response is inflammatory or suppressive, the transfer of dimers to other cells may be extremely important. Transfer of dimers may influence how the immune system sees a cell that was previously undetected, or ignored.

We have shown that dimers formed in exosomes are fully folded and maintain their association with β_2m and that the formation of these dimers is not dependent on Cys^{67}. We therefore hypothesised that these dimers were forming through an interaction between cysteine residues in the cytoplasmic tail. Indeed results using a cell line expressing HLA-A2 with a cytoplasmic tail truncation are consistent with this theory. Cytoplasmic tail dimers are not unprecedented, mouse class I alleles joined through a cytoplasmic tail cysteine have been previously reported (Capps and Zuniga, 1993) as have human class I dimers (Cresswell and Dawson, 1975), however, it was later shown that, for human class I molecules, these dimers were formed as a product of the isolation process (Springer et al., 1977).

To our knowledge the only previously reported human MHC class I β_2m-containing homodimer to have been reported is the non-classical HLA-G molecule. Interestingly, this molecule contains a truncated cytoplasmic tail and could not therefore form a cytoplasmic tail dimer. However HLA-G is unique amongst class I molecules because of its expression of Cys^{42}. The presence of this cysteine residue allows the formation of fully folded β_2m-associated homodimers (Figure 4.15). It is interesting to suggest that the presence of this cysteine residue may be compensating for the lack of a cytoplasmic tail, allowing these important dimer structures to still be able to form.

We next addressed how and why these dimers apparently readily form in exosomes, but not in cells. One of our initial hypotheses for why dimers formed so readily in exosomes involved the close proximity of such a high concentration of MHC class I molecules. Cholesterol depletion in cells has been shown to result in the clustering of MHC class I molecules at the cell surface (Fooksman et al., 2006). Exosomes are enriched in cholesterol, therefore by depleting exosomes of cholesterol, resulting in the clustering of class I molecules, we hoped to promote dimer formation. Cells, and their exosome-containing supernatants, were treated with the cholesterol depleting agent methyl-β-cyclodextrin (MBC), with complex results (Figures 5.15). When cells and exosome-containing supernatents were incubated with MBC, MHC class I levels were enhanced in exosomes but the monomer to dimer ratio was unaffected. Further blots showed that the enhancement was not solely in class I molecules but also in Alix and Tsg101, important components of the ESCRT pathway involved in MVB formation. Clustering of molecules has been one of the proposed mechanisms for how some proteins enter the MVB pathway, it is therefore possible that by depleting cells of cholesterol we enhanced clustering of proteins at the MVB surface promoting their targeting and inclusion in exosomes.

To remove the effect that treating cells with MBC had on exosome formation, we refined the procedure and isolated exosomes first before treating them with MBC (Figure 5.16). Surprisingly this had no effect on the MHC monomer to dimer ratio implying that the previous result was indeed caused by depleting the cells of cholesterol.

Intracellular GSH is a crucial antioxidant, maintaining an environment that prevents aberrant disulfide bonds from forming. We hypothesised that if GSH levels were lower in exosomes than in cells then this may allow unconventional disulfide bonds to form. This theory presumes that these dimers are bonded through a cysteine exposed to the internal environment of the exosome, of which a cysteine in the cytoplasmic tail would fit with our previous observations. Exosomes form from inward budding of endosomes and very little, if anything, is known about how GSH concentrations are controlled in these vesicles. We hypothesised that increasing the GSH concentration towards cytoplasmic concentrations would prevent these dimers from forming. Indeed, treating exosomes with increasing concentrations of GSH could inhibit dimer formation (Figure 5.17).

As treatment with GSH reduced dimer formation, then logically treatment with an oxidising agent should have the opposite affect. Treatment of Jesthom cells with diamide induced the formation of W6/32 reactive dimers in a concentration dependent manner (Figure 5.18). This result brought to our attention a previous result where the treatment of .220.B8.htpn or .220.B8 cells with diamide had enhanced or induced the formation of dimers in these cell lines respectively (Figure 4.8). This result would suggest that the dimers formed by HLA-B8 in the .220 cell lines are of a similar type to dimers formed in exosomes. From this perspective it would be very interesting to carry out immunoprecipitation experiments on the .220.B8.htpn cell line to confirm this hypothesis. We have shown that treatment of Jesthom cells with diamide induced the formation of W6/32 reactive dimers in cells. However, if we were able to IP W6/32 dimers from the .220.B8.htpn cell line, this would suggest that under some circumstances

these conformational dimers can also be formed in cells without chemical induction, not just exosomes.

To test the hypothesis that GSH levels were lower in exosomes we took advantage of a colourimetric GSH assay kit. We used protein concentration, determined via the Bradford method, as a method to try and measure equal input quantities of lysates from exosomes and cells. The results from this assay clearly show that although exosomes do contain a small quantity of GSH, the concentration in whole cell lysates is much higher (Figure 5.19). These data strongly suggest that MHC class I dimers in exosomes are able to form because of a relative lack of GSH.

The isolation and detection of exosomes containing MHC class I dimers from human plasma is exciting. Foremost, this demonstrates that exosomal MHC class I dimers are not a result of cell culture manipulation, but are in fact produced *in vivo*. This opens up the possibility of direct manipulation of exosomes for *in vivo* applications, since they will not therefore be recognised as totally foreign entities.

Furthermore, the work we carried out on exosomes derived from primary lymphocytes and monocyte-derived dendritic cells shows that the formation of dimers in exosomes is likely to be influenced by which HLA alleles are expressed in any one individual. A survey of the available HLA class I sequences at the IMGT/HLA database (www.ebi.ac.uk) reveals that all known HLA-A alleles possess Cys^{339}, and could thus potentially be involved in forming exosomal dimer structures. In contrast, HLA-B alleles

show dimorphism, wherein they all contain Cys^{308}, but are split amongst their possession of Cys^{325}, with alleles such as HLA-B07, -08, -27, -41, -42 and -44 having it, whereas those without include -B14, -B15, -B35, -B37, -B40 and -B51. Thus each persons HLA haplotype may determine the presence of MHC class I exosomal dimers. In addition, it would be feasible that dimers would form between different alleles, wherein a heterozygous individual with the haplotype HLA-B07 and -B44 could form exosomal B07-B07 dimers, B44-B44 dimers, and B07-B44 dimers. Furthermore, as we have shown here with exosomes from the Jesthom cell line, it is possible also to form dimers between HLA-A and -B molecules, though at a relatively low levels, perhaps due to steric constraints caused by the positions of the cysteines in the cytoplasmic tails. Thus the potential for novel structures to be recognised by the various class I recognising receptors of the immune system would appear to be large. Figure 5.27 shows a schematic representation of the possible MHC class I structures that can be found on cells and exosomes.

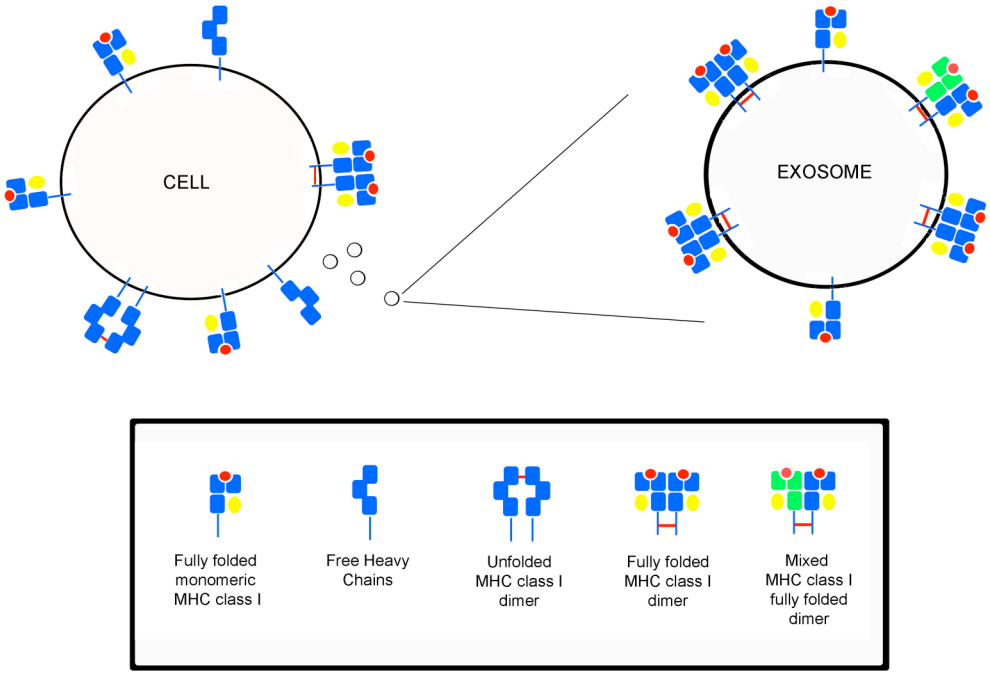


Figure 5.27: Schematic diagram representing MHC class I structures found on cells and exosomes. MHC class I molecules can adopt a number of different confirmations. Exosomes express more fully folded class I dimer than monomer and can also contain novel mixed-allele dimers. Cells in some circumstances can also express fully folded dimers, as well as unfolded dimers, and free heavy chains in addition to the normal fully folded monomer.

Although our work on primary cell derived exosomes is fairly preliminary, an early picture is developing that individuals with HLA-B27 tend to have more MHC class I dimers on their exosomes. It would be very interesting to study exosomes released by primary cells in more detail, in particular a comparison of exosomes from patients with active AS disease compared with non-disease controls. Whether exosomes have any role in the disease process still remains to be determined, but the demonstration of excess

HLA-B27 dimers on exosomes should be of particular interest considering their potential involvement in the disease process.

Certainly the formation of dimers is likely to alter how these class I molecules are seen by the immune system. The interaction of dimeric class I molecules with T cells have previously been reported. In vivo studies have shown that dimeric mouse class I molecules activate T cells and induce a state of unresponsiveness in a peptide specific manner, whereas monomers of the same MHC molecule are unable to generate a response (Abastado et al., 1995), and further reports demonstrating the immunosuppressive capabilities of dimers have also been published (O'Herrin et al., 2001, Fried et al., 2005). These studies used soluble dimeric MHC class I complexes, therefore the results observed following their interaction with T cells was due solely to the MHC class I molecule. Exosomes have been shown to express co-stimulatory molecules, thus the response to a membrane bound MHC class I dimer in the presence of these co-stimulatory molecules may well be different to that of soluble MHC class I dimers and deserves to be investigated.

The dimers we have reported here may also effect how these class I molecules are seen by NK cells. It has recently been demonstrated that the intracellular cysteine residues in the cytoplasmic tail of MHC class I proteins are crucial for extracellular recognition by LILR1 of whole cells (Gruda et al., 2007). Therefore, the formation of disulfide bonds between cytoplasmic cysteine residues may alter how the extracellular domain is recognised by some NK receptors.

The demonstration of novel MHC class I dimers at the surface of exosomes is very intriguing. HLA-B27 dimers have been implicated in the pathogenesis of AS and other spondyloarthropathies, and it has been shown that exosomes can be important modulators of the immune response. We have shown that exosomes containing novel folded B27-B27 dimers and also B27-A2 dimers are released from cell lines maintained in culture, and that exosomes rich in dimeric class I complexes can be isolated from PBMCs and human plasma, demonstrating that these dimers are formed *in vivo*. HLA-B27 has already been shown to have a propensity to misfold forming dimers compared with other alleles, whether this is also the case for dimers in exosomes remains to be determined. Our preliminary data from primary cells indicate that some class I alleles do have more of a tendency to form dimers in exosomes, and the FACs data suggests that one of those alleles is HLA-B27.

Whether HLA-B27 has a tendency to form dimers in exosomes, and how these dimers are seen by the immune system requires further investigation. Considering the immunomodulatory properties reported so far for exosomes it certainly seems important to determine the possible role of HLA-B27 positive exosomes in the disease process. The availability of exosomes from plasma of patients, and also the availability of the transgenic HLA-B27 rat model make the study of exosomes extremely feasible.

Chapter VI: Conclusions and Future Work

The reason for the strong association of HLA-B27 with spondyloarthropathies still remains unresolved, despite over 3 decades of research. Several hypothesies have been put forward to explain this relationship, but so far no concensus on which hypothesis is right has been agreed. The work presented here investigates biochemical features of HLA-B27 in an attempt to provide insights into how it contributes to pathogenesis. In the first chapter of results, I have investigated the role of HLA-B27 in the activation of an ER stress response, leading us to conclude that *in vitro* studies of this potential mechanism are complicated, prone to a number of technical issues, and may therefore not be appropriate for gaining information that would be of significant use when comparing to the real disease scenario.

Secondly, I then also studied the formation of non-B27 dimers and suggest that this may be strongly influenced by the overexpression of class I heavy chain expression.

In the final chapter I have presented data that show that novel fully folded β_2m-associated MHC class I homodimers can be found at enhanced levels in the small immunomodulatory vesicles known as exosomes. We have characterised these exosomes and detected the presence of CD48 and GILT, two proteins that have not previously been reported in exosomes. We have also provided evidence as to how and why these dimers are able to form in exosomes. Intriguingly, the formation of these dimers is not limited to HLA-B27, in fact many MHC class I molecules would have the potential to form such

dimer structures, induced by the possession of cysteine residues in the cytoplasmic tail domain.

The work presented in this thesis provides new data that will contribute to the continued study of HLA-B27 and its strong association with disease.

HLA-B27 and the UPR

Using cell line models we investigated whether the expression of HLA-B27 could activate an ER stress response known as the UPR. To study this we looked at BiP, XBP1 and ATF6, important components in the main pathways of UPR stress activation. The results presented in this chapter suggest that *in vitro* expression models that study the UPR and HLA-B27 should be interpreted with care.

We have demonstrated that BiP levels are increased in cell lines that transiently or stably express both disease-associated, non-disease associated and folding-mutant class I molecules (Figures 3.2, 3.7 & 3.11). The increase in BiP could not be correlated to HLA-B27 expression and in both the HeLa stable and transient expression models, the levels of BiP were higher in the more efficient folding HLA-A2 molecule (Figures 3.2 & 3.7).

Cleavage of ATF6 is stimulated in response to UPR activation. In a previous report it was shown that using a transient expression model of HLA-B27 expression, ATF6 cleavage product could be detected in those cells expressing HLA-B27 (Lemin et al.,

2007). However, using a stable expression model we were unable to detect ATF6 cleavage in response to HLA-B27 expression (Figure 3.10). On very long exposures a faint cleavage band was detected in the HLA-B27 sample but also in the wild-type sample, suggesting that the causative factor was not HLA-B27 (data not shown).

We also looked at XBP1 mRNA, which is constitutively expressed (XBP1u) but, following activation of an UPR, is spliced to produce XBP1s. XBP1 splicing has previously been studied using a transient expression model of HLA-B27 expression, these authors show that transient expression of HLA-B27 does activate the UPR (Lemin et al., 2007). However our results suggest that transient transfection with other class I alleles also results in XBP1 splicing (Figures 3.4 & 3.5). In the stable expression system we were able to detect an increase in XBP1 splicing in the cell line transfected with HLA-B27 (Figures 3.8 & 3.9), however we were unable to show a similar UPR activation signal using BiP expression or ATF6 cleavage (Figures 3.7 & 3.10).

The work presented here indicates that *in vitro* expression systems may not be ideal for studying the effects of HLA-B27 and the UPR. Data obtained from transgenic animal studies or patient samples may be of more relevance than *in vitro* cell-line models in this area of study.

Non-HLA-B27 Dimers

HLA-B27 homodimers have been implicated in the pathogenesis of AS and other spondyloarthropathies. Approximately 90-95 % of patients with AS are HLA-B27 positive, showing that AS can occur in patients that express other class I alleles. If dimers do play an important role in disease development, then studying what conditions influence other class I alleles to form dimers may provide important insights into disease development.

It is, however, reasonably well reported that other MHC class I alleles can form dimers (Capps et al., 1993, Santos et al., 2004). The relationship of these structures to the HLA-B27 versions could be of significant relevance. We have primarily studied the formation of HLA-B8 dimers in the .220 cell line. This same .220 cell line has previously been used to study the formation of HLA-B27 homodimers (Bird et al., 2003). HLA-B8 dimers form in .220 cells that co-express HLA-B8 and human tapasin. The expression of HLA-B8 heavy chains increased when htpn was co-expressed. Dimers could also be detected at the cell surface where they would be available for immunosurveilance.

We were unable to detect dimers in the .220.B8 cell line that were not transfected with tapasin, nor in a .220.B27 cell line expressing low levels of HLA-B27 heavy chain (Figure 4.2). These data implicate the level of expression of the class I heavy chain in dimer formation. In support of this hypothesis we have shown that over-expression of

several other non-disease associated alleles in HEK293 cells resulted in dimer formation (Figure 4.14).

Increased expression of HLA-B27 has already been shown to be important in transgenic animal models, and increased HLA-B27 expression has been detected in HLA-B27$^+$ patient samples compared with HLA-B27$^+$ controls (Taurog et al., 1993, Cauli et al., 2002). The data presented here suggest that under some circumstances, enhanced expression of non-B27 alleles can lead to dimer formation. These non-B27 dimers could conceivably contribute to disease pathogenesis in the same way as HLA-B27 dimers, through altered recognition by immune receptors at the cell surface, or by contributing to ER stress activation.

Studies on HLA-B27 that was mutated to express a cysteine residue at position 42, an HLA-G specific cysteine, suggest that the dimers formed by HLA-B8 may have a more folded structure than previously reported HC10 reactive HLA-B27 dimers (Figure 4.10). We have also demonstrated that incubating .220.B8.htpn cells with diamide, which rapidly depletes GSH, results in an increase in dimer formation, and can induce formation of dimers in the .220.B8 cell line (Figure 4.8). This result is particularly interesting considering the data presented in chapter V. In this work we showed that HLA-B27 dimers in exosomes and Jesthom cells could be influenced by the redox status of the cellular environment. This leads to an interesting question as to whether the dimers detected in .220.B8.htpn cells represent a fully folded β_2m-associated dimer structure, similar to exosome dimers. For this reason it would be extremely interesting to perform

immunopreciptation experiments on .220.B8.htpn cell line to try to ascertain some further information about the structure of these dimers i.e. whether they represent folded of misfolded dimers.

The dimers formed by HLA-B27 and other class I alleles are not necessarily quantitatively or qualitatively the same. Indeed HLA-B27 expresses a cysteine residue at position 67 that has been implicated in HLA-B27 dimer formation and is relatively rare among class I molecules (Allen et al., 1999, Dangoria et al., 2002, Bird et al., 2003). Dimers formed between this residue may be seen differently to dimers formed through other cysteine residues and may be what makes HLA-B27 so strongly associated with AS and other spondyloarthropathies. However, as previously mentioned, approximately 5-10 % of AS patients do not express HLA-B27, indicating that neither HLA-B27, nor the cysteine residue at position 67, are absolutely required for the disease to occur. Any MHC class I dimer structure present at the cell surface could potentially alter how the cell is seen by different immune cells, and any misfolded dimer structure could promote inflammation through ER stress activation. Therefore, studying the formation of non-B27 dimers may provide insights into the triggering processes that lead to the development of disease.

HLA-B27, MHC class I and Exosomes

In this work we have shown that exosomes, isolated from human cell lines, peripheral lymphocytes, monocyte-derived dendritic cells and blood plasma, show an enhanced

level of MHC class I homodimers compared with cell lysates. In particular we have studied HLA-B27 dimers in the context of exosomes, using a cell line naturally expressing HLA-B27 and primary cells derived from both HLA-B27^{+} and HLA-B27^{-} individuals.

We have shown that MHC class I dimers that form in exosomes are fully folded and maintain their association with β_2m ((Figure 5.11). We hypothesise that these dimers are forming through an interaction between cysteine residues in the cytoplasmic tail. Our preliminary data presented here using an HLA-A2 mutant devoid of its cytoplasmic tail supports this hypothesis (Figure 5.14). Work is already underway in our lab to create new HLA-B27 mutants that have cysteine to alanine substitutions at either Cys^{308} or Cys^{325}, or at both positions, in the cytoplasmic tail.

The cysteine residues that are present in MHC class I molecules can be different depending on the allele. For example HLA-A alleles all posses Cys^{339} in the cytoplasmic tail, whereas HLA-B alleles all contain Cys^{308} but are split when it comes to Cys^{325}, with some alleles containing this cysteine residue and some being without. If dimer formation can occur between any cysteine residues in the cytoplasmic tail then potentially all of the HLA-A and -B alleles could form dimers. However, the cysteines involved may influence dimer formation with some residues being more likely to react than others. This may lead to a scenario in which an individuals HLA haplotype would influence their potential class I dimer formation. Indeed the results presented here, from exosomes derived from primary lymphocytes and monocyte-derived dendritic cells, do suggest that

dimer formation can be influenced by the HLA alleles that are expressed. We have also shown that, in exosomes isolated from Jesthom cell supernatants, novel mixed dimers are able to form between an HLA-B27 molecule and an HLA-A2 molecule. These results imply that the potential for novel class I dimer structures is apparently quite large, essentially adding another level of polymorphism to the range of structures that might be recognised by immune system receptors.

Although the work in primary cells that is presented here is preliminary, it does suggest that HLA-B27 is more prone to forming MHC class I dimer structures in exosomes (Figures 5.20, 5.21 & 5.22). To further clarify this point it would be extremely interesting to compare exosomes derived from HLA-B27$^+$ samples with HLA-B27$^-$ controls. It would also be interesting to compare exosomes derived from HLA-B27$^+$ patients compared with HLA-B27$^+$ controls, this would allow us to determine whether the formation of dimers in exosomes can be influenced by disease, or if it is a potential biomarker for disease activity.

The results presented here suggest that dimers in exosomes are able to form because of reduced levels of GSH. GSH is an essential antioxidant within the cell that prevents aberrant disulfide bonds from forming. We have shown that exosomes contain a lower concentration of GSH than whole cells (Figure 5.19) and have also demonstrated that increasing the extracellular GSH concentration can prevent dimers in exosomes from forming (Figure 5.17). In the reverse scenario, incubating Jesthom cells with the oxidising agent diamide was able to induce the formation of folded dimers in a

concentration dependent manner (Figure 5.18). This result indicates that folded dimer structures can also form in cells under certain conditions. Interestingly, the work carried out on .220.B8 and B8.htpn cell lines also indicates that incubating cells with diamide can drive the formation of class I dimers (Figure 4.8). Immunoprecipitations would have to be carried out in order to determine whether the dimers formed in these cell lines are also fully folded.

Another important avenue of work would be to determine whether dimers on exosomes are peptide receptive, and if so, what type of peptides these dimers usually bind. The assumption from the data presented here is that these MHC class I dimers are indeed fully loaded with peptides, because they are recognised by conformational-dependent antibodies. Purification and mass spectrometric analysis of peptides eluted from exosomal MHC class I molecules would be informative. This would be particularly important considering the route taken for exosomes biogenesis. Within the endosome the class I molecules will have been exposed to exogenous peptide antigen, possibly destined for MHC class II molecules, and may in some cases have been able to bind.

It would also be very interesting to determine whether exosomes expressing class I dimers can interact with NK cells, and whether this would lead to an inhibitory or an activatory effect. MHC class I interaction with NK cells is relatively peptide independent and usually results in an inhibitory signal, however if the class I molecule cannot interact with the NK receptor this would result in apoptosis. Previously characterised HLA-B27 homodimers have been shown to interact with different subsets of NK cells when

compared with the HLA-B27 monomer (Allen et al., 2001, Kollnberger et al., 2002). The fully folded class I dimers that we have detected in exosomes may also be differently recognised by NK cell subsets. It is interesting to note that the non-classical class I molecule, HLA-G, exists as a monomer or as fully folded dimers. Of great potential significance, although HLA-G monomers and dimers are recognised by the same receptor, they have been shown to interact with different strengths, with dimers forming a much stronger interaction (Gonen-Gross et al., 2003, Shiroishi et al., 2006, Apps et al., 2007). This suggests that the formation of dimers can influence the interaction between MHC molecules and NK receptors.

To this regard it is also interesting to note that we detected the presence of CD48 on our Jesthom exosome populations. CD48 is the ligand for the 2B4 receptor that is expressed on NK cells and some T cell subsets (Brown et al., 1998b, Latchman et al., 1998, Valiante and Trinchieri, 1993, Garni-Wagner et al., 1993). The interaction between CD48 and 2B4 promotes IL-2 expression, which is important in the expansion and activation of NK cells (Lee et al., 2006). Jesthom cells are an EBV transformed human B cell line and the expression of CD48 in these exosomes may be associated with this fact. However, if class I dimers are not seen in the same way as monomers their expression in exosomes at times of infection may promote and enhance NK cell activity.

To enable further studies to distinguish if a particular detected response was due to class I dimers or class I monomers it would be important to be able to control the formation of dimers in exosomes, resulting in the formation of exosomes containing only dimers or

only monomers. In an attempt to manipulate the formation of dimers in exosomes we incubated cells and exosomes with MBC, a cholesterol-depleting agent that has been shown to cause clustering of class I molecules at the cell surface (Fooksman et al., 2006), however incubation of exosomes, or cells and exosomes, with MBC did not affect dimer formation (Figures 5.15 & 5.16). As we have now demonstrated that the redox environment is important in dimer formation, manipulating these conditions may allow us to produce exosomes that express either only dimers or only monomers. However changing the redox environment is likely to cause global problems within the cell and it would therefore be important to determine that any exosomes produced under these conditions are as we have previously characterised.

Exosomes have been implicated in many aspects of immunomodulation and can have activatory or inhibitory effects. How this occurs has not been fully elucidated. It is proposed that exosomes can have direct effects on other cells of the immune system or that exosomes may bind to, or fuse with, professional antigen presenting cells, having an indirect effect (Admyre et al., 2006, Hwang et al., 2003, Thery et al., 2002a, Denzer et al., 2000). We have been able to demonstrate the transfer of HLA-B27 dimers from exosomes onto KG1 dendritic cell-like cells, and flow cytometry data indicates that exosomes have transferred HLA molecules to the surface of KG1 cells (Figures 5.2 & 5.24). These data suggest that if exosomes do interact with the other cells of the immune system indirectly, dimers can be transferred from exosomes to other cells, making them available for immunosurveillance.

Molecular modelling would allow us to predict a possible structure of the dimers present in exosomes. Results from IP experiments allow us to make some statements about the structure of dimers on exosomes. W6/32 reactivity implies that the $\alpha 2$ domain remains fully folded and ME1 reactivity suggests that the $\alpha 1$ domain also remains intact. However, bonding in the cytoplasmic tail may alter the extracellular domains in some ways. For example, cytoplasmic tail interactions may effect the orientation of the class I molecules at the cell surface. It has previously been demonstrated that class I molecules in the cell membrane adopt a supine orientation (Mitra et al., 2004). Bringing class I molecules together through interactions in the cytoplasmic tail may affect this property, changing how the molecules are recognised by other immune receptors.

A commercial 'class I dimer' reagent is available from BD bioscience, known as DimerX. This structure consists of the extracellular domains of class I molecules fused to the N terminal VH domain of mouse IgG, an immunoglobulin light chain disulphide bonded to the heavy chain, and a non-covalently associated human β_2m molecule.

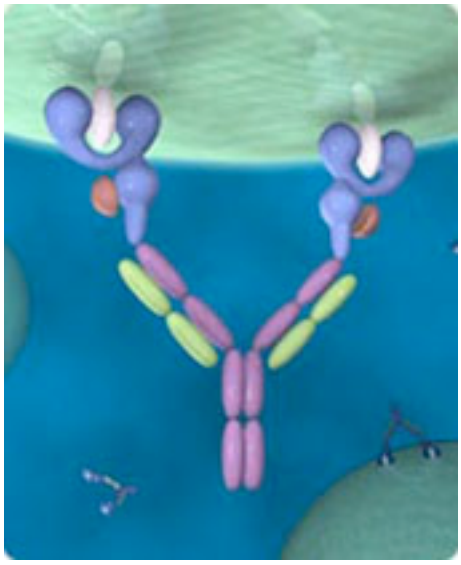


Figure 6.1: Schematic diagram of BD bioscience DimerX molecule. The DimerX molecule is a three-chain complex molecule consisting of a recombinant heavy chain of MHC-Ig fusion chain (blue and purple), an immunoglobulin light chain (yellow) disulphide bonded to the heavy chain, and a non-covalently associated human β_2m molecule (red).

Although in the schematic diagram the class I molecules appear quite far apart this may not represent the physiological structure of this recombinant protein. Indeed the hinge region of the antibody is likely to allow movement of the class I molecules closer together. The use of this reagent may allow us to investigate the function of fully folded dimers. Adaptation of this structure may be possible, perhaps with a further disulfide bridge in the VH chain of the mouse IgG, ensuring the class I molecules are brought closer together. The generation of these reagents results in the fomation of soluble class I dimers that can then be used in numerous experiments. An important caveat of using these molecules is that they lack the cytoplasmic tail, and this may be important for

downstream signalling and should be taken into account if these synthetic class I dimers are used to investigate the function of fully folded class I dimers.

The work presented here on HLA-B27 in exosomes presents a potentially new avenue of research for AS and other spondyloarthropathies. The formation of dimers in exosomes does not necessarily form the basis of a new theory for disease pathogenesis but could in fact support some of the previously proposed theories for disease. For example, if dimers on exosomes are shown to retain the same structure and peptide binding ability as monomeric class I molecules, they could potentially be involved in the arthritogenic peptide hypothesis. To this regard, previous studies have already demonstrated the effect dimeric class I molecules can have on T cell stimulation, and in some cases dimers can elicit a T cell response where a monomeric class I allele cannot (Abastado et al., 1995, O'Herrin et al., 2001, Fried et al., 2005). Alternatively, if these dimer structures are recognised differently to monomeric class I molecules, perhaps by receptors of the NK family lineage, then they may promote disease in the same way as is proposed for misfolded HLA-B27 dimers at the cell surface. Whether HLA-B27 dimers and exosomes are related to disease pathogenesis remains to be determined, but continued study into this area in the future is likely to yield important information about both AS and the potential immunoregulatory role of exosomes.

Chapter VII: References

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