Functional Characterisation of HLA-F

A thesis presented for the degree of Doctor of Philosophy by

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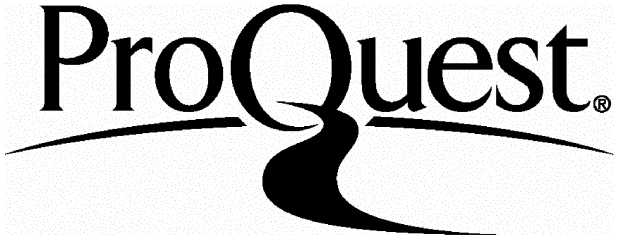

Abstract

The work presented in this thesis is the functional characterisation of HLA-F, a human non-classical MHC class I molecule. HLA-F is thought to have evolved a specific immune function, similar to HLA-E and HLA-G, two other human non-classical class I molecules. HLA-F was first expressed and characterised in mammalian cell lines and bacteria. A prokaryotic system of expression was found to be more useful. Recombinant HLA-F heavy chain and beta-2 microglobulin proteins formed a stable complex when refolded *in vitro* in the absence of synthetic peptide. Furthermore, high-pressure liquid chromatography did not detect any bound peptides following acid elution of the refolded complex. This complex was used as an immunogen to produce a highly specific, high affinity monoclonal antibody (FG1) that was used to study the cell biology and tissue distribution of HLA-F. HLA-F was shown to have a restricted pattern of tissue expression in tonsil, spleen, and thymus. HLA-F could be immunoprecipitated from B cell lines, and from HUT-78, a T cell line. In B cell lines HLA-F was shown to bind TAP, but cell surface expression was only detected when these cells were grown at 26°C. HLA-F tetramers were generated to identify potential receptors for HLA-F. HLA-F tetramer binding could be conferred on non-binding cells by transfection with the inhibitory receptors ILT2 (LIR1) and ILT4 (LIR2). Finally surface plasmon resonance studies demonstrated a direct molecular interaction of HLA-F with ILT2 and ILT4. At present, the function of HLA-F remains unclear however, we now have substantial insight into the biochemistry and intracellular interactions of HLA-F and the cell types, which express and may interact with it.

Acknowledgements

I was extremely pleased when Stephen Powis gave me the opportunity to join him at Guy's Hospital to carry out research on the MHC (Major Histocompatibility Complex). Steve has been successfully involved in the characterisation of new MHC genes. Plans were to use newly available MHC sequences and the sequencing facilities of our laboratory to initiate a study of MHC variability. I started to generate a genomic library and sequencing data on HLA-B flanking regions of different haplotypes, gathered using direct PCR sequencing. In parallel to this study I also embarked on the functional characterisation of a non-classical MHC class I molecule, HLA-F. At Guy's Hospital, I would like to thank both Michelle Goldworthy and Max Wilkinson for their support and help.

September 1997 was a turning point in my PhD studies. I was due to move to the Royal Free Hospital where Steve is now a Professor of renal medicine. However I moved to the IMM in Oxford. I would like to extend my gratitude to Professor John Bell and Professor Andrew McMichael who welcomed me in their laboratories and helped with my project. These laboratories presented all the facilities necessary for the study of a MHC class I protein like HLA-F. From then, I entirely focused on the functional characterisation of HLA-F with the invaluable help of Christopher O'Callaghan. I can not emphasise enough his influence in my research. In a few words I am deeply indebted to him for his assistance, support, encouragement and friendship. I would like to thank those in Oxford for their support. In no particular order, Judy Bastin,

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Publications and Presentation

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Abbreviations

Amino acids

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

General

aa	Amino acid
β2m	Beta-2 microglobulin
BCL	B cell line
cDNA	Complementary deoxyribonucleic acid
CTL	Cytotoxic T lymphocytes
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphospate

DTT	Dithiothreitol
EBV	Epstein-Barr virus
EDTA	Ethylenediamine-tetraacetic acid
ELISA	Enzyme-linked immunoabsorbant assay
FACS	Fluorescence activated cell sorting
HLA	Human leukocyte antigen
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IFN	Interferon
Ig	Immunoglobulin
ILT	Immunoglobulin-like receptor
IPTG	Isopropyl-D-thiogalactopyranoside
ITAM	Immunoreceptor tyrosine-based activating motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LAIR	Leukocyte immunoglobulin-like receptor
KIR	Killer inhibitory receptor
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NK	Natural killer
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TAP	Transporter associated with antigen processing
TCR	T cell receptor

Chapter 1 – The MHC Antigens

1.1 Adaptive immune responses

The vertebrate immune system has evolved complex mechanisms to recognise and eliminate infectious agents. The first line of defence against many common microorganisms is provided by innate immunity, which constitutes a natural barrier and largely involves cells that can engulf microorganisms or phagocytic cells. However, components of the innate immune system cannot always eliminate or recognise infectious organisms. Thus, the vertebrate immune system has evolved an adaptive immune response that provides a high level of specificity and leads to immunological memory. These features are based on the clonal selection and expansion of lymphocytes expressing antigen-specific receptors, each lymphocyte expressing a single receptor which is the result of specific recombination events occurring in the bone marrow to produce B lymphocytes or in the thymus, to generate T lymphocytes.

B lymphocytes can express cell surface receptors called B cell receptors (BCR) and are able to secrete these receptors as antibodies. Antibodies have two different functions that are structurally separated. Antibodies are secreted in the extracellular domain and specifically bind to pathogen molecules. They are responsible for activating systems of destruction of extracellular pathogens and help to contain intracellular pathogens. Thus, it is said that B lymphocytes contribute to humoral immunity.

T lymphocytes also express antigen-specific receptors called T cell receptors (TCR) which are only membrane bound receptors. T cell receptors recognise small peptide fragments bound to the major histocompatibility complex (MHC) molecules at the surface of a cell. Peptides from the cytosol are bound to MHC class I antigens and are recognised by cytotoxic T lymphocytes (CTL) displaying CD8 on their surface. CTL recognise and lyse virus-infected cells or tumour cells. Peptides derived from vesicles bound to MHC class II antigens are recognised by helper T (T_H) lymphocytes displaying CD4 at their surface. Once activated, T_H cells secrete cytokines that regulate other immune effector cells. T_H cells are divided into subsets depending on the cytokines they secret. T_H1 cells release interferon γ and interleukin-2 and control infection of intra-cellular bacteria. T_H2 cells release Interleukins-4 and -5 and control the activation of B cells.

1.2 The major histocompatibility complex (MHC)

A cluster of genes first identified because of their involvement in transplantation histocompatibility was named the major histocompatibility complex (MHC). The MHC is now known to play a key role in the immune response. Situated on the short arm of chromosome 6 in humans, it extends over 4 million base pairs and contains over 200 genes. Organisation of this cluster of genes is similar in the human (HLA) and in the mouse (H-2), and it has been separated into class I, class II and class III regions (Figure 1.1).

In humans, at the centromeric end of the cluster lies the class II region. This region includes genes coding for the α and β chains of the MHC class II molecules HLA-DR, -DP and -DQ. The products of these genes are referred to as class II antigens. Genes associated with antigen processing are also located in this region. The transporter associated with antigen processing (TAP) genes and the tapasin gene are both situated in the class II region. The class III region encodes proteins of the complement system and other proteins involved in different immunological functions such as cytokines. The class I region at the telomeric end of the complex codes for the three classical class I (class Ia) genes. The other component of class I molecules, β2m is encoded on another chromosome (chromosome 15 in humans). The products of theses genes HLA-A, -B, and -C are referred to as class I antigens. The principal feature of MHC genes is their high level of polymorphism. The MHC polymorphism affects the range of peptides that can be bound, the conformation of the bound peptides, and the interaction between the MHC molecule and the T-cell receptor. Thus, the high level of polymorphism has functional significance, and has been selected for during evolution. At the population level, the pressure of selection maintains a wide range of MHC molecules that increase the chance for recognition of a wide range of infectious agents.

A number of class I related genes have also been localised in the MHC. In contrast with classical class I genes, these genes show limited polymorphism and have been classified as non-classical class I (class Ib). The number of MHC class Ib genes varies across species. In humans they are HLA-E, -F and -G. In the mouse a greater number of MHC class Ib genes are physically separated in 3 different clusters called Q, T and M.

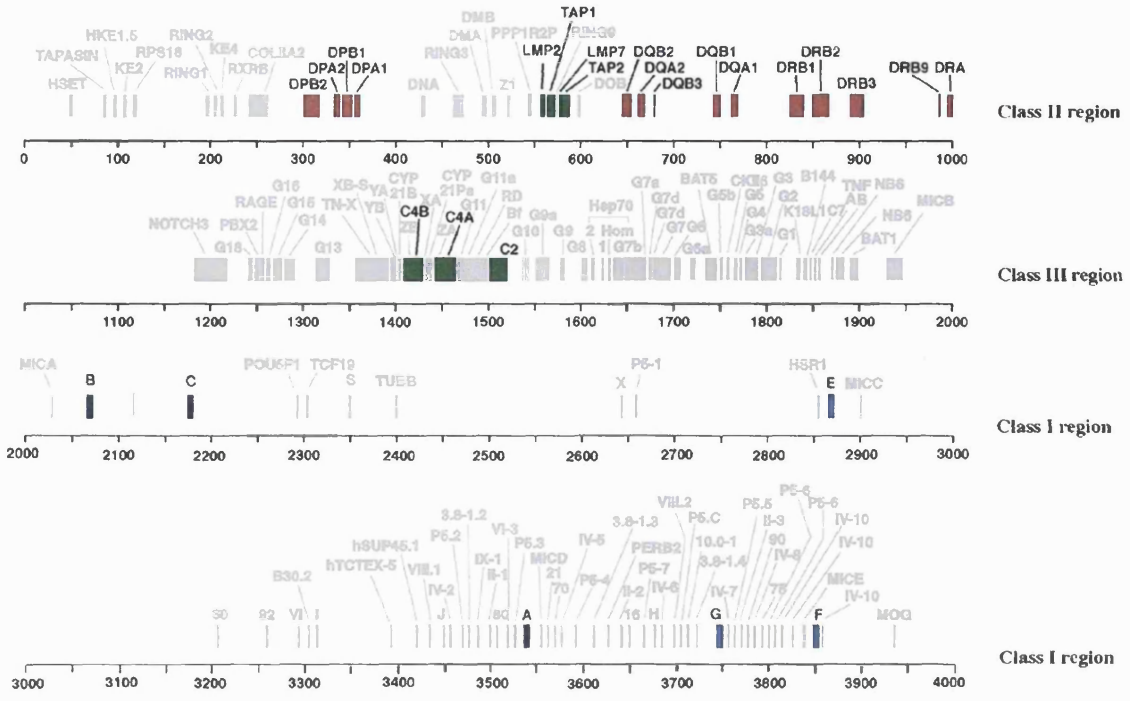

Figure 1. 1 Map of the human MHC region.

Organisation of the MHC in three different areas are illustrated with the distance in thousands of base pairs. Genes involved in the synthesis of class Ia (dark blue), class Ib (light blue), complement proteins and antigen processing (green), and class II (red) are highlighted. See Campbell and Trowsdale, 1993.

1.3 MHC antigen structure and function

The MHC class I, and MHC class II are cell surface glycoprotein molecules that are closely related in overall structure and function. Both class I and class II molecules have four extra-cellular domains, two of which are related to immunoglobulin domains and two of which are involved in the formation of a peptide binding groove.

The two immunoglobulin-like domains are relatively invariant. One of the functions of this part of the structure is to bind invariant co-receptors such as CD8 in the case of class I molecules and CD4 for class II molecules.

For both MHC class I and class II molecules, the two extracellular domains that form a peptide binding groove are composed of two α helices that sit on a β sheet floor made of multiple β strands. The main difference between the class I and class II molecules is that the groove is closed in the former and more open in the latter. Differences between class I and class II structures in addition to different antigen processing pathways, result in the presentation of peptides from different intra-cellular sites to different T cell receptors.

1.3.1 Classical class I antigens

Classical class I molecules are expressed at the cell surface of most nucleated cells and present endogenous peptides to cytotoxic T cells. However, the level of expression is regulated by IFN-γ in certain cell types. The classical class I molecules are made up of three non-covalently associated elements: the heavy chain (45 kDa), β2m (12 kDa) and a peptide of 8-10 amino acids. The mechanism in which cytoplasmic peptides are produced, transported to the endoplasmic reticulum and associate with the class I heterodimer is the antigen processing pathway.

The heavy chain or α chain code for 5 different domains. The two N-terminal domains α1 and α2 are involved in the formation of the peptide-binding groove. α3 is an

immunoglobulin-like domain (Bjorkman et al., 1987). The transmembrane domain anchors the protein in the membrane, and finally there is the cytoplasmic domain. β2m makes contact with the α3 and, to a lesser extent, to the α1 and α2 domains. The α3 domain and β2m have homologous amino acid sequences to the immunoglobulin constant region, and have limited polymorphism. In contrast the α2 and α3 domains are highly polymorphic. These extracellular domains bind to a peptide and interact with the T cell receptor (Bjorkman et al., 1987; Garboczi et al., 1996), whereas the immunoglobluin like domain α3 makes contact with the CD8 molecule (Gao et al., 1997).

The crystal structure of HLA-A2 revealed general information about the MHC class I peptide binding groove (Bjorkman et al., 1987). Structural data permitted the understanding of how a single peptide-binding groove is able to bind with high affinity to a wide range of peptides. The peptide binding groove of MHC class I molecules, composed of two α helices lying above eight anti-parallel β sheets, is closed forcing the binding of peptides restricted to 8-10 amino acids in length in an elongated conformation. The specific binding groove area which interacts with peptides forms pockets which have been named A to F (Garrett et al., 1989). It has been observed that for one allelic form, the peptides eluted from the groove have conserved or semi-conserved residues at definite positions. These residues have been named anchor residues. It seems that side chains of the anchor residues are inserted in specific pockets of the peptide binding groove, providing specificity and strong affinity to the binding site. Different allelic forms would produce pockets lined with different amino acids,

resulting in specific selection pressure and defining anchor residues ensuring the specificity and affinity of the binding. The length of a peptide, combined with the position and nature of the anchor residue is sufficient to determine the binding of a peptide to a class I molecule, irrespective of the other residues present. It is this characteristic which explains the strong affinity of a set of peptides for a single binding site. The T cell receptor recognises the bound peptide and some residues of the class I binding groove. The T cell receptors bind to residues of the MHC class I which are mostly conserved (Garboczi et al., 1996).

1.3.2 Class II antigens

Classical class II molecules present exogenous peptides to CD4 T cells. MHC class II molecules are generally expressed at the cell surface of special cells involved in the immune response, like B lymphocytes and macrophages. Class II expression can also be regulated by cytokines. For example, IFN-γ is able to induce class II expression in cell types that do not constitutively express them.

Class II molecules consist of a non-covalent complex of two chains, α and β. Both chains have two extracellular domains (α1, α2 and β1, β2) and a transmembrane domain. The two domains closest to the membrane α2 and β2, have sequence similarities to immunoglobulin constant regions and have limited polymorphism. Conversely, the α1 and β1 domains which form the binding groove, are highly polymorphic.

1.4 The classical class I pathway

The process by which the cytosolic peptides are displayed by MHC class I molecules at the cell surface can be divided into three steps: the generation of cytosolic peptides by the proteasome and other proteases; the transport of the cytosolic peptides by TAP (transporter associated with antigen processing) into the ER (endoplasmic reticulum); and the formation of the trimeric complex MHC class I heavy chain, β2m and peptide. This complex is then transported to the cell surface for presentation to cytotoxic T cells (Figure 1.2).

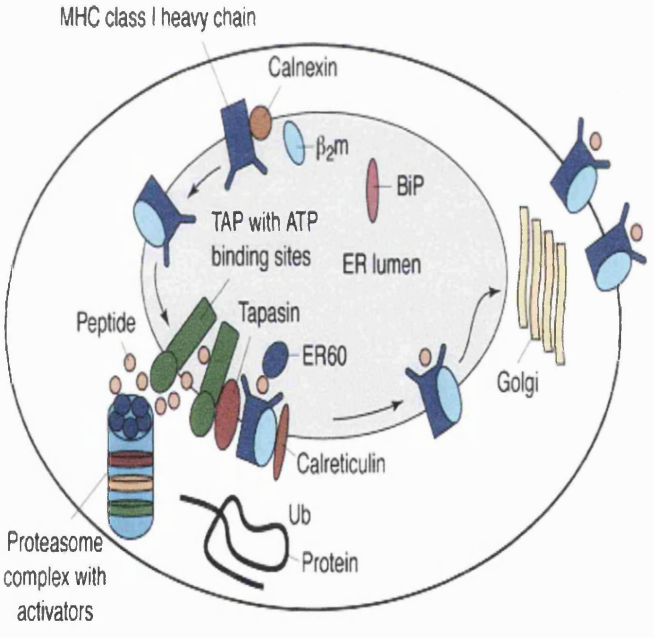

Figure 1. 2 The MHC class I antigen-processing pathway.

BiP, immunoglobulin-binding protein; ER, endoplasmic reticulum; MHC, major histocompatibility complex; TAP, peptide transporter associated with antigen processing; Ub, ubiquitin. See Seliger, Maeurer and Ferrone, 2000.

1.4.1 Production of cytosolic peptides

Proteins are continually synthesised and degraded in cells. In the cytosol, the degradation of a protein by proteases generates peptides. Under normal conditions, class I molecules will display at the cell surface a sample of self-peptides which are recognised by T cell receptors without activating the T cell. Viruses, mutations, oncogenes or cytosolic pathogens result in a quantitative or qualitative modification of the pattern of protein production. This will ultimately provoke a modification of the peptides displayed at the cell surface by class I molecules.

Protease activities are not only localised in the cytosol; they have been located in lysosmes, or in the ER. Under certain conditions, exogenous peptides can be presented by class I molecules. However, class I MHC molecules principally bind peptides generated in the cytosol (Townsend et al., 1986). The origin of class I peptides from the cytosolic compartment is best proved by the existence of specialised transporters which transfer peptide from the cytosol to the ER. Blockage of this transporter molecule affects class I cell surface expression.

In the cytosol, the majority of digestion of proteins into short peptides is performed by the proteasome. This enzyme is composed of subunits forming four stacked rings in a shape of a large cylinder. The protein to be digested is introduced inside the cylinder where the catalytic sites are located. The role of the proteasome in the production of peptides presented by the MHC class I molecules has been shown using specific inhibitors (Harding et al., 1995; Rock et al., 1994). The tagging of a protein with

ubiquitin increases class I presentation. However, ubiquitination of proteins might not be strictly necessary for subsequent binding of resulting peptides to class I molecules. INF-γ induces the production of new proteasome subunits, two of which are LMP2 and LMP7 (Low-Molecular-Mass polypeptides). These units replace constitutively expressed units of the proteasome and modify the specificity of cleavage (Driscoll et al., 1993). The LMP2/LMP7 proteasome preferentially cleaves polypeptides after hydrophobic and basic residues. This modification might favour transport to the endoplasmic reticulum and binding to the class I groove.

1.4.2 TAP transport

After synthesis, the class I heavy chain is directly transferred to the ER, with the domains forming the peptide-binding groove facing the lumen. The principal pool of peptide binding class I molecule is located in the ER. The peptide-class I association forms in the endoplasmic reticulum which is the first possible co-localisation of these molecules (Porgador et al., 1997). Two molecules, TAP-1 and TAP-2 involved in the transport of peptide to the ER were first identified by their homology to ATP binding cassette molecules. The transfection of these two genes in RMA-S mutant cells restores antigen presentation (Powis et al., 1991). TAP-1 and TAP-2 molecules are both necessary for ATP dependant transport of peptides to the ER. Peptides translocated by TAP are usually shorter than 12 residues, although longer peptides can also be transported (Momburg et al., 1994). A wide range of peptide sequences are compatible with TAP transport, but the C-terminal residue has a great importance for TAP

transportation (Neefjes et al., 1995). TAP molecules also have a role in the assembly of the mature class I molecules.

1.4.3 Assembly of MHC class I molecules with peptides

Class I heavy chains bind to calnexin or to the BiP (immunoglobulin-binding protein) shortly after translocation in the ER lumen (Kahn-Perles, Salamero and Jouans, 1994; Rajagopalan and Brenner, 1994). The functions of these two chaperone molecules is not clear, but it has been shown that they do not bind simultaneously to the same class I molecules. In humans, the association to calnexin is interrupted when the heavy chain associates with β2m. The heavy chain-β2m complex then interacts with TAP molecules, tapasin, ER60 and calreticulin to form a multicomplex (Sadasivan et al., 1996). Calreticulin and calnexin have many similarities, but the main difference is their preference of class I molecule conformation. ER60, like calreticulin, appears to promote refolding (Oliver et al., 1997). Tapasin has a central role in the formation of this multicomplex. Tapasin forms a bridge between TAP molecules and the unfolded class I heavy chain-β2m complex. Tapasin also seems to play a role in the peptide editing for class I (Sadasivanet al., 1996; Solheim, Carreno and Hansen, 1997). Peptide binding stabilises the class I heavy chain-β2m complex and releases the trimeric complex formed from the chaperones. The class I molecules are then transported via the Golgi, where they are the subject of glycosylation modifications on their journey to the cell surface. Unfolded class I complexes, unable to properly bind a peptide, are ultimately degraded.

1.5 The class II pathway

Class II molecules bind exogenous peptides resulting from protein degradation in endosomes. The α and β chain polypeptides which form class II molecules are synthesised in the cytosol and transferred in the ER. Chaperone molecules bind to class II molecules in the ER and regulate the refolding and trafficking of these molecules to low-pH, endosomal compartments where they bind peptide. In the ER the α and β chains only associate in the presence of invariant chain (Ii). The invariant chain forms trimers, thus the final product is a nine-subunit complex (Roche, Marks and Cresswell, 1991). Prior to the formation of this complex, each element was previously bound to calnexin; this association being lost upon assembly to the complex. A peptide sequence (CLIP) of the invariant chain binds to the peptide groove of class II molecules, stabilising the class II heterodimers and preventing the binding of peptides within the ER. A second function of the invariant chain is to target class II molecules into the endosomal compartment. In the endocytic pathway, invariant chain is degraded leaving the CLIP peptide in the groove (Avva and Cresswell, 1994). Class II heterodimer which is associated with CLIP, binds to other chaperone molecules such as HLA-DM and HLA-DO. DM is a class II molecule which has evolved to have a chaperone function. It has a peptide editing function (Kropshofer, Hammerling and Vogt, 1999). DM help Class II molecules to dissociate from the CLIP peptide and to bind endosomic peptides. The class II molecules are then transferred to the cell surface where they can be recognised by CD4 T cells. DO is principally expressed in the epithelial cells of the thymus and B cells, but does not appear to bind peptide. DO seems to have inhibitory effects on DM.

1.6 Receptors to MHC class I molecules

The function first associated with classical class I molecules was to present antigenic peptides to cytotoxic T cells. The complex peptide antigen-MHC class I molecule is directly recognised by the T cell receptor and CD8. However, it is now known that CD8 and the T cell receptor are not the only receptors that interact with classical class I molecules. Class I molecules are also able to interact with a variety of Ig-like receptors expressed on different leukocytes. The leukocyte receptor cluster (LRC) on human chromosome 19 contains genes encoding killer cell immunoglobulin-like receptors (KIR), the leukocyte associated immunoglobulin-like receptors and the Immunoglobulin-like transcripts (ILT). The function of these receptors is not yet clear.

Non-classical class I molecules have recently been shown to interact with specific immunoglobulin like receptors. It seemed at first that HLA-E and HLA-G receptors were expressed on NK cells, and it was suggested that these two molecules would regulate NK cell lysis. The direct interaction of HLA-E with NK cell receptors has been demonstrated, but whether HLA-G directly interacts with NK cells remains unclear.

1.6.1 The T-cell antigen receptor (TCR)

T-cell antigen receptors (TCR) are heterodimers of two homologous transmembrane polypeptides linked by a disulphide bond. Two distinct TCR heterodimers, $\alpha\beta$ and $\gamma\delta$ have been identified, both of which associate with CD3, a signal transduction polypeptide complex.

γδ TCRs are able to directly recognise small molecules and intact proteins without the requirement for antigen processing. Recently, the structure of a γδ TCR has been solved identifying structural differences with αβ TCR mainly in the C domains (Allison et al., 2001). αβ TCRs are only able to recognise antigen bound to MHC class I or class II complexes expressed at the cell surface of antigen presenting cells. αβ TCR expressing cells represent 90 to 95% of T cells in humans and mice, and are involved in the T helper and T cytotoxic response.

TCR gene organisation and rearrangement are similar to the immunoglobulin genes. In addition, the structure of the TCR molecules has similarities with the immunoglobulin structure. Analysis of T cell receptor cDNA has shown that the gene is organised with variable (V), joining (J) and constant (C) segments similar to immunoglobulin genes (Hedrick et al., 1984). In humans, the α chain gene is located on chromosome 14 and the β chain gene is located on chromosome 7 (Davis and Bjorkman, 1988). The variable α chain region is the product of a recombination between V and J gene segments during T cell development, which create a VJ exon. The α chain mRNA is the result of the splicing between the VJ exon and the constant exon. The variable β chain region is the result of the recombination of V, D (diversity) and J gene segments which also occurs during T cell development. The VDJ exon formed is subsequently joined to the constant region by splicing of the RNA to produce the β chain. The events responsible for immunoglobulin gene rearrangement also seem to be involved in TCR gene rearrangement. The recombinant events involve two recombinase activating genes

RAG1 and RAG2. In addition, variability is increased at the V, J or D junctions where nucleotides are added or removed.

The first TCR structures confirmed early predictions made on the basis of sequence homology, that the TCR heterodimer and the Fab fragment of immunoglobulins would have similar structures (Garboczi et al., 1996; Garcia, Teyton and Wilson, 1999). Both α and β chains encode for complementary-determining regions CDR1, CDR2 and CDR3. CDR1 and 2 are encoded by the V gene segment and form the periphery of the recognition site. CDR3 encoded by the VJ or VDJ (β chain) gene segments is at the centre of the recognition site. T-cell receptor lies over the top of the MHC-peptide complex in a diagonal orientation. CDR1 and 2 loops interact mainly with the α-helices of the class I molecule. CDR3 loop interacts with the peptide and the MHC class I molecule and is responsible for most of the contact between these molecules.

CD8 is a co-receptor in the binding of TCR molecules to MHC class I molecules. CD8α homodimer binds weakly to an invariant loop situated in the α3 domain of class I molecule (Gao et al., 1997).

1.6.2 KIR

Natural killer (NK) cell cytotoxicity is controlled by the expression of different families of receptors. The families of receptors involved in class I binding usually have inhibitory and activating members. Receptor families involved in the interaction with

specific class I molecules will be presented in the section dedicated to the particular class I molecule.

The killer cell immunoglobulin-like receptor (KIR) family is a novel group of the immunoglobulin super-family of receptors that bind to class Ia molecules (Vales-Gomez, Reyburn and Strominger, 2000). KIR L molecules have a long cytoplasmic tail which contain an inhibitory motif called the immunoreceptor tyrosine-based inhibitory motif (ITIM). KIR S molecules have short cytoplasmic tails and are able to associate with DAP12 molecules that contain an activating motif called immunoreceptor tyrosine-based activation motif (ITAM). The KIR molecules are also different in the number of immunoglobulin domains possessed, either 2 Ig-like domains (2D) or 3 Ig-like domains (3D). HLA-C binds to KIR molecules with 2 Ig-like domains both KIR2DL and KIR2DS, and HLA-B binds to KIR molecules with 3 Ig-like domains, KIR3DL. However, recognition of a particular KIR receptor is also specific to the class I allele. It has been reported that a member of this family, KIR2DL4 would be able to interact with HLA-G but this remains controversial. Furthermore, the cluster of genes coding for KIRs on chromosome 19 is polymorphic and polygenic.

1.6.3 ILT/ LIR

A new family of immunoglobulin-like receptors were identified at the same time by two different groups and have been named differently, ILT (Immunoglobulin like transcript) (Samaridis and Colonna, 1997) and LIR (leukocytes immunoglobulin-like receptors) (Borges and Cosman, 2000). The ILT nomenclature will be used for simplicity. Genes

encoding ILT molecules are grouped on the leukocyte receptor cluster (LRC). ILT molecules have been divided into two groups as a function of their transmembrane and cytoplasmic domains (Figure 1.2). One group have long cytoplasmic domains which contain several inhibitory motifs called tyrosine-based inhibitory motifs (ITIM), they include ILT2, ILT3, ILT4, ILT5 and LIR-8. These ILT molecules mediate inhibition when they bind with their ligands. The second group have short cytoplasmic domains with a charged transmembrane arginine and no obvious signalling motifs, They include ILT1, ILT7, ILT8 and LIR-6. They associate with Fc receptor γ which hold activating motifs called tyrosine-based acticvating motifs (ITAM). These ILT molecules mediate activation of the cells when cross-linked with antibodies. ILT molecules are predominantly expressed in monocytic cells. ILT1 is expressed on subsets of NK cells, whereas ILT2 is expressed on monocytic cells, most B cells, some NK and T cells. It was suspected that ILT molecules, because of their homology with KIR receptors would bind to class I molecules. However, most ILT ligands are unknown. Only ILT2 and ILT4 are able to bind to a wide range of MHC class I molecules, including HLA-G (Allan et al., 1999). Recently it has been shown that ILT2 interacts with the conserved α3 domain of class I molecules (Chapman, Heikeman and Bjorkman, 1999). When ILT2 or ILT4 bind to a class I molecule, these two molecules transduce inhibitory signals (Colonna et al., 1999). In general ILT2 and ILT4 might influence the activation threshold of the cell they are expressed on.

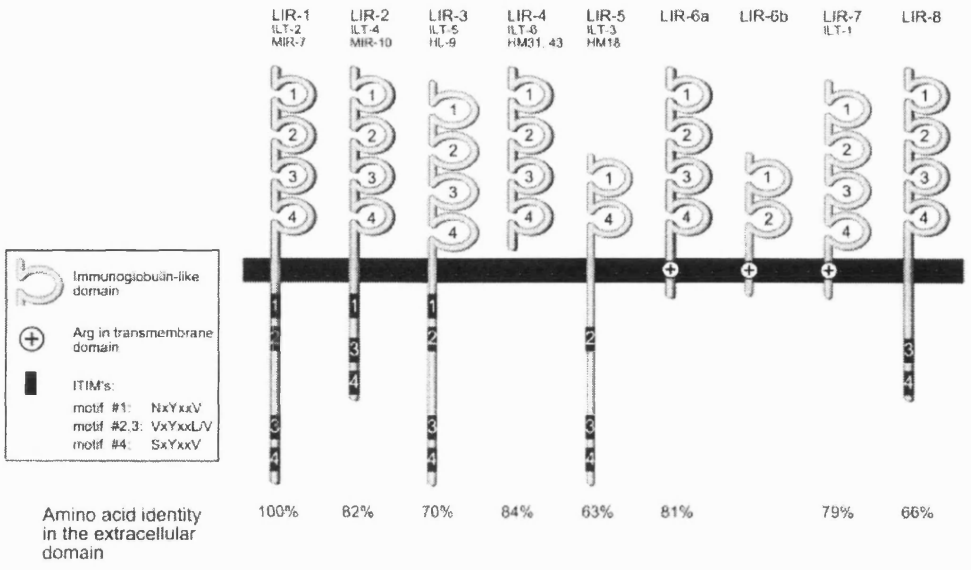

Figure 1. 3 Schematic representation of the ILT/LIRs.

Alternative names for each molecule are indicated. LIR-6a and LIR-6b are the products of alternatively spliced RNAs from the LIR-6 gene. See Borges and Cosman, 2000.

1.6.4 CD94/NKG2

CD94/NKG2 are C-type lectin-like proteins, which are involved in the control of NK cell cytotoxicity and cytokine production. They are heterodimers of CD94 polypeptide, linked with different NKG2 polypeptides. In humans, the genes coding for these polypeptides are clustered on chromosome 12. The inhibition or activation potential of the heterodimers is associated with the presence of different NKG2 polypeptides. NKG2A has ITIM motifs and is responsible for an inhibition signal from the CD94/NKG2A heterodimers. NKG2C is able to associate with DAP12 which holds ITAM motifs, and is responsible for an activator signal from the CD94/NKG2C heterodimer.

Different CD94/NKG2 transfectant cells have been stained with HLA-E and HLA-G tetramers (Allanet al., 1999; Braud et al., 1998a). HLA-E tetramers were able to specifically stain cells transfected with CD94/NKG2A, CD94/NKG2B, or CD94/NKG2C receptors and were also able to stain natural killer cells (NK). However, HLA-G tetramers do not seem to interact with CD94/NKG2 receptors.

Finally protection of 721.221 cells from lysis by NK cells expressing CD94/NKG2A which was first attributed to transfection of class Ia molecules is in fact the direct effect of the interaction of HLA-E with the inhibitory receptor CD94/NKG2A.

1.7 Non-classical class I molecules

HLA-A, -B, and -C molecules were the first HLA molecules to be identified and their structure and function determined. However, other class I like genes were later detected in the human genome using MHC class I probes. Three of these genes, HLA-E, -F and -G have been mapped to the MHC (Geraghty et al., 1992). The MHC class I family has now been divided in two groups; HLA-A, -B and -C are classical class I (or class Ia) and HLA-E, -F and -G are non-classical class I (or class Ib). All class I molecules have high homology, share the same secondary structure, and associate with β2m (Shimizu et al., 1988). Class Ia molecules have a higher level of expression compared to class Ib molecules. The high levels of polymorphism is a characteristic of class Ia molecules and is not observed for class Ib molecules. Finally, class Ia molecules are known to present endogenous peptides to cytotoxic T cells, whereas HLA-E, -F and -G are thought to have evolved specialised functions.

In the mouse, the H-2 complex has many non-classical class I genes, that are physically separated in 3 different clusters called Q, T and M. In contrast with HLA-E, no homologue has yet been identified in the mouse for HLA-F. H2-M3 is of particular interest and its well-defined function is discussed later.

In humans, other class Ia homologues have been characterised, such as CD1 genes, MIC genes and HFE. These molecules have a higher degree of divergence from class Ia genes compared with HLA-E, -F, and -G. Both CD1 and HFE are able to associate with β2m. CD1 molecules present lipid antigen to T cells, HFE does not bind any antigen and has no immunological function. Finally, MICA does not bind β2m or peptide.

1.7.1 HLA-G

HLA-G has a similar gene organisation to the other class Ia genes. The main difference is the presence of a stop codon in exon 6, resulting in most of the cytoplasmic domain not being translated (Geraghty, Koller and Orr, 1987). Compared to class Ia genes, fewer coding polymorphisms have been detected. One or two exons can be spliced out producing 7 differently spliced mRNAs which code for HLA-G1 to -G7 molecules. HLA-G1 is the longest product. HLA-G5, -G6 and -G7 are soluble proteins, as they do not have a transmembrane region (Paul et al., 2000). HLA-G transcription does not seem to be induced by IFN-γ (Gobin et al., 1999). At the maternal-foetal interface HLA-A and HLA-B are not expressed, whereas high levels of expression of HLA-G have been detected. These results first suggested a role for HLA-G in the tolerance of the foetus by the maternal immune system. However, the membrane anchored HLA-G1

molecule does not seem to be necessary for fetal survival (Casro et al., 2000). In addition HLA-G is now known to be expressed in the thymus, epithelial cells, and activated peripheral blood monocytes (Carosella et al., 1999).

HLA-G1 molecules follow the class Ia pathway. Intracellularly, the HLA-G1/β2m complex binds to TAP, and an HLA-G1 mature molecule expressed at the cell surface is composed of the heavy chain non-covalently linked with β2m and a peptide. Trimeric complexes were immunoprecipitated from transfected 721.221G cells and the peptide eluted. This demonstrated that HLA-G was able to bind self peptides. In addition, analysis of the peptide sequenced allowed the determination of the peptide motif (Lee et al., 1995). When the set of peptides eluted from HLA-G and HLA-A2 were compared, it seemed that the peptide diversity obtained for HLA-G was lower than with class Ia. However, the function of HLA-G seems to diverge from class Ia molecules which are known to present viral peptides to cytotoxic T cells.

The function of HLA-G has not been clearly elucidated at this time. It was first demonstrated that a large proportion of decidual NK cells were inhibited on transfection of HLA-G in NK sensitive cells (Chumbley et al., 1994). This inhibition now seems to be indirect. It is known that up-regulation of HLA-E surface expression is a consequence of HLA-G expression. HLA-G provides HLA-E with specific peptides derived from its leader sequence, thus it might be the direct interaction between HLA-E and CD94/NKG2A which mediates the inhibition of lysis by NK cells. Two families of immunoglobulin-like receptors, KIR and ILT/LIR have been shown to bind HLA-G

molecules. However, the interaction between HLA-G and a member of the KIR and CD94/NKG2 family is still controversial. HLA-G tetrameric complexes were able to stain myelomonocitic cells (Allan et al., 1999; Colonna et al., 1997). It was shown that a member of ILT/LIR (immunoglobulin-like transcript/leukocytes immunoglobulin-like receptors) were directly responsible for this staining. Specific anti-ILT4 antibiodies were able to completely inhibit the staining of PBMC by HLA-G tetramer. In the same study, it was shown that HLA-G tetramer could stain cells on transfection with ILT2 or ILT4 molecules. ILT2 and ILT4 interaction with HLA-G was further verified at the molecular level using surface plasmon resonance (personal communication, D Allan).

1.7.2 HLA-E

The 41 kDa HLA-E heavy chain is expressed in most nucleated cells. Like HLA-F, HLA-E has a shorter leader sequence and a shorter cytoplasmic domain compared to class Ia molecules (Figure 3.1). HLA-E and HLA-F in transfected 721.221 cells are not exported to the cell surface as the other class I molecules (Shimizu et al., 1988). Rapid advances were made in HLA-E studies when the mouse non-classical class I molecule Qa-1 and HLA-E were identified as homologue genes on the basis of sequence homologies (Connolly et al., 1993). Both Qa-1 and HLA-E have identical substitutions at position 143 and 147 when compared to other class I molecules. It was determined that Qa-1b specifically bound to peptides derived from mouse class Ia molecules leader sequence, and that the mechanism was TAP dependent (Aldrich et al., 1994). At the cell surface, Qa1-b interacts with both types of TCR and a receptor on NK cells.

By analogy with Qa-1, HLA-E was studied for its capacity to bind peptides derived from human class I leader sequences. It was determined that HLA-E was able to bind to peptides derived from HLA-A, -B, -C, and HLA-G leader sequences, with the primary anchor residues located at position 2 and 4 (Braud, Jones and McMichael, 1997). With shorter leader sequences, HLA-E and HLA-F expression does not generate peptides able to stabilise HLA-E. The extra-cellular domains of HLA-E showed a general structural analogy to class Ia molecules. However, the HLA-E binding groove has evolved to bind a very restricted number of peptides imposing a high level of selectivity at each position of the peptide. *In vivo*, HLA-E cell surface expression is dependent on the expression of human class Ia molecules possessing the binding peptide to HLA-E and of TAP (Braud et al., 1998b).

To identify potential ligands to HLA-E when expressed at the cell surface, HLA-E tetrameric complexes were generated and used to stain PBMC. CD56, CD16 positive cells (NK cells) and a subset of T cells were stained by HLA-E tetramers (Braudet al., 1998a). Additionally, PBMC staining by HLA-E tetramers could be blocked by anti-CD94 antibodies, suggesting that the CD94/NKG2 receptors may be involved. Furthermore, HLA-E tetramers specifically stained cells transfected with CD94/NKG2A, CD94/NKG2B, or CD94/NKG2C receptors. NK cell immunoglobulin-like receptors (KIR) do not appear to be involved in the staining of NK cells by HLA-E tetramers. Finally, protection of 721.221 cells from lysis by NK cells expressing CD94/NKG2A, that was first attributed to transfection of class Ia molecules, is in fact

the direct effect of the interaction of HLA-E with the inhibitory receptor CD94/NKG2A.

More recently it has been suggested that HCMV can escape immuno-surveillance by NK cells by up-regulating HLA-E cell surface expression by providing a peptide able to bind to HLA-E (Tomasec et al., 2000).

1.7.3 Human CD1

Human CD1 is a family of 5 non-polymorphic genes, that have been divided into two groups (Park and Bendelac, 2000). Firstly, CD1a, CD1b, CD1c and CD1e genes are present in humans but not in rats and mice. The second group is represented by CD1-d and is present in rats and mice. CD1 molecules only show 30 % homology to class Ia genes but still associate with β2m and have an immune function. They present microbial and self-lipid antigens to T cells. It as been suggested that the CD1 binding groove would accommodate the lipid tails of the antigen (Zeng et al., 1997). CD1 isotype of the first group are mainly expressed on antigen presenting cells. CD1 molecules have separate pathways for antigen presentation and do not follow the same pathways as class I molecules (Sugita et al., 1999). A model has been established in which CD1 isotypes have evolved to target different endosomal compartments, where they would bind different lipids. It has been shown that on injection of analogues of mycobacterial hexose-1-phosphoisoprenoids there was T cell proliferation in patients infected with *Mycobacterium tuberculosis* (Moody et al., 2000).

1.7.4 H2-M3

H2-M3 is a mouse non-classical class I molecule which was first associated with the presentation of a maternally transmitted antigen (Mta). The Mta was identified as an N-formylated peptide derived from a subunit of NADH dehydrogenase (Loveland et al., 1990). H2-M3 is now known to specifically present N-formylated peptides derived from mitochondrial and prokaryotic sources (Shawar et al., 1991). N-formylated peptide, able to bind H2-M3 have been identified as part of the proteins generated by the intracellular bacteria *Listeria monocytogenes*. H2-M3 has a much higher affinity for N-formylated peptide compared with non-formylated ones. Thus, it seems that H2-M3 has evolved to bind N-formylated peptides because only prokaryotes and mitochondria initiate protein synthesis with an N-formylated methionine. H2-M3 is only expressed at very low levels on the cell surface of B cells (Chiu et al., 1999). Cell surface expression is not induced by culture of B cells at low temperature, but can be induced by the presence of N-formylated peptide (Chiu et al., 1999). Addition of specific N-formylated peptides results in the detection of H2-M3 at the surface of B cells, T cells, macrophages and dendritc cells extracted from the spleen. However, this result was only true for some peptides such as LemA and Fr38. It seems that the cell surface expression of H2-M3 upon activation by N-formylated peptide LemA and Fr38 is TAP dependant (Chiu et al., 1999). In contrast with these results, the protection against *Listeria monocytogenes* by different H2-M3 restricted CD8+ T cells clone does not seem to be totally TAP dependent.

1.7.5 HLA-F

HLA-F was cloned and characterised by Daniel Geraghty (Geraghty et al., 1990). This gene is located on chromosome 6 at the telomeric end of the human MHC. Only limited coding polymorphism has been identified (Geraghty et al., 1990; Kunishima et al., 1999; Lury, Epstein and Holmes, 1990) and a HLA-F null allele has been reported in the Japanese population (Uchigiri et al., 1997). Across species, HLA-F is highly conserved in the human and chimpanzee differing by only three residues in the extracellular domain (Otting and Bontrop, 1993). It has been suggested that HLA-F may represent a basic ancestral gene from which the other human class I molecules have evolved (Shiina et al., 1999).

Sequence comparisons of HLA-F with other class I molecules suggest that it has a similar basic molecular structure to the other class I MHC molecules and their homologues. HLA-F conserves many features such as the cysteines required for the class I MHC disulphide bonds, consistent with a similar mature protein conformation. The HLA-F α3 domain is highly conserved, consistent with its involvement in the interaction with β2m. The HLA-F α1 domain contains the conserved N-linked glycosylation site at the Asn 86. The position and length of the trans-membrane region are similar to those of the class Ia proteins, making it likely to be a membrane protein. However, analysis of the protein sequence in comparison with class Ia molecules also suggests that HLA-F may have more significant differences from the standard class Ia sequences than the other class Ib molecules, HLA-E and HLA-G. Among the 10 highly conserved residues pointing to the antigen binding site of class Ia molecules (Met 5- Tyr

7- Phe 22- Gly 26- Tyr 59- Tyr 84- Trp 143- Lys 146- Tyr 159-Tyr 171). HLA-F differs at 5 of these residues (positions 5, 22, 26, 84 and 146) compared to only two differences at positions 5 and 143 in HLA-E and one difference at position 143 in HLA-G (Geraghty et al., 1990).

Studies of the HLA-F transcript suggest a low level of expression, and a specific pattern of expression. RNase protection assays detected higher levels of HLA-F in B lymphoblastoid cell lines and resting T cells (Geraghty et al., 1990). HLA-F was also detected in foetal liver (Houlihan et al., 1992), and skin. HLA-F transcript was undetected in some T cell lines, fibroblasts, and adult liver (Geraghty et al., 1990; Houlihan et al., 1992; Lury, Epstein and Holmes, 1990). HLA-F seems to be the only class Ib promoter with a functional IFN-stimulated response element (ISRE) to IFN-γ (Gobin et al., 1999), but NF-κB does not appear to induce transactivation of any class Ib gene including HLA-F (Gobin et al., 1998).

Shimizu et al have demonstrated that β2m could be immunoprecipitated with the HLA-F heavy chain from HLA-F transfected 721.221 cells. However, no cell surface expression was observed and they did not determine if HLA-F was associated with a peptide (Shimizu et al., 1988). These results differ from the results obtained with class Ia and HLA-G. It is now known that it is the lack of peptide provided by the class I leader sequence which was responsible for the absence of HLA-E at the cell surface of 721.221 cells (Braud et al., 1998a). There are no published functional studies of the HLA-F molecule and its function remains unknown.

1.8 Aims of this research

Although HLA-F was identified at the same time as HLA-E and HLA-G, to date there is less published data for HLA-F and little is known of its function. HLA-F is likely to have an immunological role because of its location within the MHC and its homology to other class I molecules. The hypothetical role of HLA-F in the immune system makes the study of this molecule in health and disease of great scientific interest.

The research presented here is an extensive study of the HLA-F molecule in an attempt to understand its function. A series of experimental approaches, including biochemical, immunohistochemical, and functional were designed. HLA-F was first expressed, purified and analysed using a prokaryotic expression system. The recombinant protein produced was used as an immunogen to generate a high-affinity, specific monoclonal antibody. Using this antibody, the cellular biology of HLA-F and its tissue distribution were studied. Finally HLA-F tetramers were generated and used to identify potential ligands for HLA-F.

Chapter 2 - Materials and Methods

2.1 Molecular biology

2. 1.1 Cloning strategy

The cloning of portions of HLA-F cDNA in different plasmids was based on PCR technology. Primers were designed to specifically amplify defined cDNA sequences and insert the PCR fragment generated in a plasmid. Each primer had a restriction endonuclease cleavage site added at the 5' end of the specific sequence necessary for the PCR amplification. In this way the PCR product produced has a restriction endonuclease cleavage site at each extremity. After restriction endonuclease cleavage and purification of the PCR product, the amplified DNA fragment was ready to be ligated into prepared plasmid. The ligation reaction was then transfected into competent bacteria to generate clones.

The choice of the restriction endonuclease cleavage enzymes were mainly dictated by the presence of cleavage sites at specific positions in the plasmid, and by the absence of these sites in the insert to be cloned.

2.1.2 Quantitative determination of DNA by Spectophotometry

Optical density (OD) of dilutions of DNA solutions were measured at 260 nm in a quartz chamber. At this wavelength, an OD of 1 corresponds to 50 μg/ml of double-stranded DNA. The ratio 'OD 260 nm/OD 280 nm' provides an estimate of the purity of nucleic acid. A Ratio of 1.8 to 2 indicates adequate DNA preparation.

2.1.3 Polymerase chain reaction (PCR)

Primers were designed for their specificity by analysing their alignment to homologues, and their quality confirmed by Tm and heterodimer formation. The cloning strategy required a restriction endonuclease cleavage site present in the plasmid polylinker and not present in the insert. Pfu polymerase (Stratagen, USA) was used when the PCR product was cloned. This is a high fidelity enzyme due to a 3' to 5' exonuclease proof-reading activity.

50 to 200 ng of plasmid or 100 to 500 ng of cDNA (template), 2.5 U of Pfu, 200 μM of each dNTP (A, T, C and G), 20 pmole of each primer (forward and reverse primers), 1 x reaction buffer (BioLabs, USA) were used in a standard reaction. The 10 x reaction Buffer composition was 100mM KCl, 100mM $(NH_4)_2SO_2$, 200 mM Tris-CL (pH 8.75), 20 mM $MgSO_4$, 1% Triton X-100, 1000 μg/ml BSA.

Reactions were performed in a Perkin Elmer or Hybaid thermal cycler machine. A three cycle programme was used for each PCR. The first cycle was a denaturation cycle at 95°C for 5 minutes. It was followed by 30 to 35 amplification cycles, composed of a denaturation step at 95°C for 1 minute, an annealing step at 50°C for 1 minute and a polymerisation step at 72°C for 3 minutes. The last cycle was an elongation cycle at 72°C for 10 minutes.

The annealing step was optimised as a function of features of the primers, and the polymerisation time optimised as a function of the length of the product to amplify. Results from the PCRs were analysed on agarose gels.

2.1.4 Agarose gel electrophoresis

Agarose, a linear polymer was melted in the presence of TBE (45 mM Tris-Borate, 1 mM EDTA), an electrophoresis buffer. A microwave was used to bring the mixture to boiling. The resulting transparent solution obtained was cooled down to 60°C before the addition of ethidium bromide to a final concentration of 0.5 μg/ml. The percentage of agarose w/v was adjusted (from 0.8% to 2%) as a function of the predicted size of the DNA to be analysed. Gels were poured into a mould and set at room temperature. Once set, the electrophoresis buffer (TBE) was added until it just covered the gel. DNA samples were mixed with agarose gel loading buffer (including bromophenol blue dye) and loaded into wells. Minigels were run at 50 to 100 V. Due to TBE having a neutral pH, the negatively charged DNA runs towards the anode. The gels were examined under ultraviolet light and photographed.

DNA samples (PCR products or digested vector) were compared to a molecular size ladder to estimate the size of the DNA species present in the samples. Different standard markers were used (manufacturer supplied, BioLabs, USA).

2.1.5 Bacterial culture

Sterile conditions were used for the culture of the bacteria. *E.coli* bacteria were grown in Luria-Bertani (LB) medium (10 g Bactotryptone, 5 g Yeast Extract, 10 g NaCl per litre) or low salt LB (10 g Bactotryptone, 5 g Yeast Extract, 5 g NaCl per litre). Alternatively, terrific broth was used for which 12 g bactotryptone, 24 g yeast extract, 4 ml glycerol were mixed per 900 ml, autoclaved, cooled to 60°C or less following which 100 ml of sterile solution (0.17 M KH_2PO_4, 0.72 M K_2HPO_4) was added. LB mixtures

were adjusted to pH 7.0 with NaOH before sterilization by autoclaving. Prior to autoclaving, 15 g of bacto-agar were added to each litre of liquid media for the production of LB agar plates. When antibiotic (ampicillin, kanamycin) selection was needed, these thermolabile substances (sterilised by filtration) were added only when the media was below 45°C. Ampicillin and kanamycin were added to a final concentration of 100 μg/ml. Media containing agar were poured into plates and stored at 4°C. Before use, the plates were stabilised at room temperature and dried.

Blue /white selection plates were spread with 50 μl of 2% IPTG in water, and 105 μl of 2% x GAL in dimethyl formamide.

2.1.6 Preparation of competent bacteria and frozen bacterial stocks

Bacteria have to be treated to become competent to DNA transformation. Frozen stocks of the desired *E.coli* strain were streaked out onto an agar plate of LB medium, and incubated overnight at 37°C. Following incubation, a single colony was transfered to 500mls of LB medium in a 2 litre flask. The cells were grown at 37 °C with agitation to an optical density of 0.6 O.D at 600 nm (mid-log phase). Cells were recovered by centrifugation at 3000 rpm for 20 minutes at 4°C using a Beckman J-6B centrifuge and resuspended in 100mls of sterile ice cold buffer 1 (30 mM KO Acetate, 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$, 15% v/v glycerol). Cells were pelletted by centrifugation and resuspended in 20 mls of ice cold buffer 2 (10 mM Na-MOPS pH 7.0, 75 mM $CaCl_2$, 10 mM KCl, 15% v/v glycerol). Finally cells were aliquoted at 200 μl per eppendorf tube and stored at -80°C. 50 μl of these competent cells were used for a single transformation.

To freeze bacteria, 20 % v/v of sterile glycerol was mixed to bacterial cultures in their mid-log phase and stored at -80°C.

2.1.7 Transformation of bacteria with plasmids

Frozen competent cells were taken out of the -80°C freezer and thawed on ice. 50 ng of plasmid DNA, or half the volume of a ligation was pipetted into an eppendorf tube, and cooled on ice for 5 minutes. 50 µl of competent bacteria were mixed with the DNA sample and left on ice for 20 minutes. The bacteria were then heat shocked for 2 minutes at 42°C, and incubated on ice for a further 2 minutes. They were finally plated out onto agar plates containing the appropriate selection media, and incubated overnight at 37°C.

2.1.8 Small scale preparation of plasmid from transformed bacteria

Single colonies were used to inoculate 2 ml of LB medium supplemented with the appropriate antibiotic. Cultures were then incubated overnight at 37°C on a shaking platform. Cells were pelleted in a Heraeus Biofuge 13 microcentrifuge at 13000 rpm for 2 minutes. Plasmid DNA was purified from the cell pellet using the QIAprep Spin Miniprep Kit (QIAGEN, Netherlands). Cells were first resuspended in the resuspension buffer, then lysed in a buffer containing NaOH, SDS and RNase A. Neutralisation buffer was finally added to adjust the pH and salt concentrations. At this stage plasmid DNA is in solution, and the insoluble material was removed by centrifugation at 13000 rpm for 10 minutes. Plasmids were purified with QIAprep columns and eluted in TE buffer pH 8.

2.1.9 Large scale preparation of plasmid from transformed bacteria

Single colonies of transformed bacteria were used to inoculate 1 litre of terrific broth medium, supplemented with the appropriate antibiotic. Cultures were then incubated overnight at 37°C on a shaking platform. The expanded cultures were spun down in 1 litre flasks with a Beckman J-6B microcentrifuge at 3000 rpm for 30 minutes. The pelleted cells were first resuspended in 100 mls of resuspension buffer (50 mM Tris pH 7.4, 10 mM EDTA), followed by 100 mls of lysis buffer (200 mM NaOH, 1% SDS). Finally 100 mls of neutralisation buffer (2.55 M potassium acetate) was added. High-molecular-weight DNA and bacterial debris were removed by filtration, and the DNA were precipitated with a 0.6 volume of isopropanol. The nucleic acid pellet obtained after centrifugation at 3000 rpm for 15 minutes was resuspended in 10 ml of Tris EDTA buffer at pH 7.4 and plasmid DNA purified on a CsCl gradient in the presence of ethidium bromide.

The plasmid DNA solution was warmed to dissolve 12 g of caesium chloride (1 g of CsCl per ml). Once completely dissolved, 1 ml of ethidium bromide (10 mg/ml) was mixed to the DNA-CsCl solution and left in the dark at room temperature for 30 minutes. The protein precipitate which forms with the ethidium bromide was spun down for 10 minutes at 2000 rpm in a Beckman GS-6R centrifuge (Bekman, USA). The clear ethidium bromide solution was transferred to quick-seal tubes, and centrifuged for 48 hours at 48000 rpm in a Beckman Ti 70 rotor. Two bands were visible in the centre of the gradient, the lower band consisting of closed circular plasmid DNA that was collected. Ethidium bromide was removed from the DNA with

1-butanol saturated with water, and CsCl removed by DNA precipitation in isopropanol. Finally, DNA was resuspended in TE at pH 8 and stored at -20 °C.

2.1.10 Restriction endonuclease digestion

Reaction buffers were supplied by the manufacturers (BioLabs, USA). When double digests were performed, the reaction buffer was chosen according to the manufacturers indications (BioLabs catalog). In general, 2 μg of DNA plasmid from miniprep or maxiprep was digested in a total volume of 50 μl; a maximum of 10 % (v/v) enzyme was used. Digests were performed at 37°C for 2 to 12 hours and analyzed with agarose gel electrophoresis.

2.1.11 Extraction of DNA fragments from agarose gel

Digested vectors and PCR products were purified from agarose gel using a QIAquick gel extraction kit (QIAGEN, UK). Agarose gel electrophoreses were run in TBE buffer. The band to be purified was identified under UV light and excised from the agarose gel. The gel fragment was placed in an eppendorf tube and the agarose solubilized at 50°C for 10 minutes in the QG buffer (from kit). The samples were then purified using a QIAquick column and eluted in EB buffer (10 mM Tris-Cl, pH 8.5).

2.1.12 Purification of PCR products

PCR products and digested PCR products were gel purified; this was particularly useful when the PCR product was not very clean and allowed a product of a given size to be specifically purified. Alternatively, clean PCR products were phenol–chlorophorm extracted and the aqueous phase passed over a sepharose G50 column equilibrated in

TE buffer at pH 8.0. The column was made of a 1ml syringe in which the outlet was blocked with a glass bead. G-50 suspension was poured into the syringe and the buffer spun out in a 15 ml falcon tube at 2000 rpm for 2 minutes in a benchtop Beckman GS-6R centrifuge. The sample was eluted by centrifugation at 2000 rpm for 2 seconds. At the end, PCR product was recovered in TE buffer and purified from DNA fragments less than 80 bp, nucleotides and polymerase enzyme.

2.1.13 Plasmid preparation for cloning

Typically, 2 µg of plasmid DNA preparation were cleaved by restriction endonuclease enzymes in a final volume of 50 µl. After a 2 hour incubation period at 37°C the sample was equilibrated to room temperature and 1 µl (1U) of calf alkaline phosphatase (Boehringer Mannheim, UK) was added. After five minutes incubation the digested and phosphorylated sample was phenol:chlorophorm extracted and the DNA fragment was gel purified . The vector was then the ready for ligation.

2.1.14 PCR product preparation for cloning

PCR products were analysed on agarose gel and gel purification performed. 200 ng of the purified samples were then digested in 50 µl at 37 °C using appropriate buffers and enzymes. After this digestion, the insert was either gel purified or phenol:chlorophorm extracted followed by a G-50 column purification. The insert was then ready for ligation.

2.1.15 DNA ligation

Ligations were performed in a total volume of 10 µl, using 1 µl of bacteriophage T4 DNA ligase and 1 µl of 10 x ligase buffer. In general, 100 ng of plasmid DNA were mixed with 10 x the molar amount of insert. Reactions were incubated at room temperature for 2 hours or more. The ligation control consisted of a ligation in the absence of inserts. 5 µl of the ligation mixture were added to 50 µl of competent *E.coli* bacteria (strain XL1Blue) for transformation.

2.1.16 DNA sequencing

The coding regions of HLA-F or HLA-E were sequenced using the enzymatic method of Sanger et al. 1977. Sequencing reactions were performed using the BigDye Terminator Cycle Sequencing Ready Reaction (Perkin-Elmer,UK) and were analyzed with the ABI PRISM 310 automatic sequencer.

2.2 General protein techniques

2.2.1 Protein quantitation

Protein concentration was determined with the BIO-RAD Protein assay based on a Bradford dye-binding procedure (BIO-RAD, Germany). The dye reagent was first diluted to 1/5 in water. Generally, 10 µl of different protein dilutions were mixed with 990 µl of the diluted reagent, and the optical density measured at 595 nm. Typically, at this wavelength 1 µg of BSA in 1 ml of the BIO-RAD diluted reagent has an optical density of 0.05. Alternatively, the coefficient of extinction was calculated from the protein sequence (ExPASy-http://www.expasy.ch/) and the concentration calculated from a spectrophotometer reading at 280 nm.

2.2.2 SDS-polyacrylamide gel electophoresis (SDS-PAGE)

SDS-Polyacrylamide gels are composed of chains of polymerised acrylamide in a buffer containing 0.1% of SDS (Laemmli 1970). mini-PROTEAN II electrophoresis cells (BIO-RAD, Germany) were used to run 12 or 15% acrylamide gels. The solution to prepare these gels were made with 2.3 mls of water, 5 mls of 30% acrylamide (37.5:1), 2.5 mls of 1.5 M Tris pH 8.8, 100 µl of 10% SDS. 100 µl of 10 % APS and 4 µl of TEMED were added to polymerise the gel. On top of this, a stacking gel (2.7 mls water, 0.67 mls of acrylamide (35.5:1), 0.5 ml 1 M Tris pH 6.8, 40 µl 10% SDS) was polymerised with 40 µl of 10% APS and 4 µl of TEMED. 5 x non-reducing SDS-PAGE loading buffer was made from 50 mls 1M Tris pH 6.8, 30 g SDS, 1 g bromophenol blue made up to 250 mls with water. To make a reducing loading buffer, 100 µl of β-2 mercaptoethanol was added to 300 µl of 5 x non reducing buffer. 10 x Tris-Glycine SDS-PAGE running buffer was made from 75 g Tris base, 470 g glycine, and 25 g of SDS made up to 2.5 litres with milli-Q water. Biorad minigels were run at 200 V until the bromophenol blue reached the bottom of the gel.

After the run, gels were simultaneously fixed and stained in a coomassie brilliant blue dye buffer (1.25 g coomassie brilliant blue R250, 250 ml water, 250 ml methanol, 50 ml acetic acid) and de-stained in the same solution without the dye.

2.2.3 Isoelectric focusing gel

Loading buffer was made from 28.5 g of urea, 1 ml of NP-40, 1ml of ampholines (pH 3.5-9.5), 2.5mls of β-2 mercaptoethanol made up to 50 mls with dH_20. The gel was made from 33g of urea mixed under running hot water with 13.2 mls of dH_2O, 12 mls

of 10% NP-40, 9 mls of acrylimide (30:1.6), and degassed for 10 minutes. After degassing, 2.4 mls of ampholines at pH 5-7, 0.6ml of ampholines at pH 3.5-9.5 were added and then 120 µl of 10 % APS, 60 µl of TEMED were mixed to polymerise the gel. Gels were poured into Hoefer SE 600 plates separated by 1 mm spacers and left to set for 2 hours. Plates were fixed in a Hoefer SE 600 tank, top buffer and lower buffer were respectively made of 2 g of NaOH in 1 litre dH_2O, and 4.9 ml of orthoposphoric acid in 3.5 litres of H_2O. Gels were run for 14 hours at 880V (Maximum voltage 920 V, maximum power 8 V, maximum current 12 mA).

2.2.4 Testing of prokaryotic protein expression

The prokaryotic system of expression used was driven by T7 promotors. Plasmids were transformed into the *E.coli* strain BL21pLys. Single colonies were picked to inoculate 2 mls of LB containing the appropriate antibiotics in a universal container. The cultures were grown at 37 °C on a shaker until the absorbence at 600 nm reached 0.5. At this point a pre-induction sample was collected, then the expression was induced with a final concentration of 0.5 mM IPTG. Expression samples were collected 3 hours post-induction. 100 µl of each of the samples were spun down, and the cells resuspended in 20 µl 1 x SDS-PAGE reducing loading buffer. 10 µl of the pre-induction sample, and 20 µl of the post-induction sample was loaded and run on 15 % SDS-PAGE. Expression was optimised with the use of different media, or by inducing protein expression at different optical density of the cultures.

2.2.5 Large scale protein expression

Generally, large scale protein expressions were performed in low salt LB supplemented with the relevant antibiotics. Plasmids were transformed into *E.coli* strain BL21 pLys. Single colonies were picked to inoculate 2 mls of media in a universal container. Containers were shaken for 4 to 6 hours at 37°C; 2 ml were then used to inoculate a 1 litre flask containing 200 mls of media left standing overnight in a hot room (37 °C). The overnight culture was split to inoculate four, 2 litre flasks containing 1 litre of media, which were shaken at 230 rpm at 37 °C until the culture reached a 0.5 optical density value. At this point, protein expression was induced with IPTG at a final concentration of 0.5 mM, and the culture continued for a further 3 hours. Cells were then spun out for 30 minutes at 4°C in a Bekman J-6B centrifuge. The supernatant was discarded and the pellets were resuspended in 50 ml of PBS and stored overnight at -20°C.

2.2.6 Inclusion body preparation

Bacteria stock obtained after large scale protein expressions were thawed and sonicated on ice using a 1 cm diameter probe attached to a Misonix XL2020 (Misonix New York, USA) sonicator. Ten 30 second pulses were performed with 2 minute intervals to prevent heating of the sample. Insoluble material was spun down at 15,000 rpm in a Beckman J2-21centrifuge with a JA20 rotor. Inclusion bodies present in the insoluble material were washed three times. A wash consisted of the pellet homogenisation in a detergent solution (0.5% triton, 50 mM Tris pH 8.0, 100 mM NaCl, 0.1% sodium azide) followed by a centrifugation at 13,000 rpm. After three washes the pellet was finally resuspended in 50 mM Tris pH 8.0, 100 mM NaCl, and centifuged again. The

purified inclusion body preparation was solubilised in urea solution (8 M urea, 50 mM Mes pH 6.8, 0.1 mM EDTA, 0.1 mM DTT) with overnight agitation at 4 °C. Insoluble material was spun down for 20 minutes at 13,000 rpm. The protein concentration of the urea solution was determined using a Bradford dye-binding assay (BIO-RAD, CA, USA), and the solubilised protein preparation was stored in aliquots of 1 ml at -80 °C. Purity of the protein preparation was estimated by SDS-PAGE analysis.

2.2.7 Protein refolding

The refolding solution generally used was composed of 700 mM L-Arginine, 100 mM Tris pH 8.0, 0.1 mM PMSF, and a redox couple was generated with 5 mM reduced glutathione and 0.5 mM oxidized glutathione. This solution was stirred with a magnetic stir bar and equilibrated at 4°C. Urea solubilised proteins were individually diluted in a universal container before it was added to the whole refolding solution. β2-microglobulin was added 2 hours prior to HLA-F heavy chain, solubilised in a guanidine solution and the refolding solution stirred for a further 48 hours. Proteins were then concentrated in Amicon stir cells holding a 10 kDa cut-off filtration membrane.

2.2.8 Gel filtration chromatography

Pharmacia FPLC system (Upsalla, Sweden) was used to perform gel filtration chromatography. Samples of 8 to 10 ml were injected on Pharmacia 26/60 Superdex 75 or 26/60 Superdex 200 columns and run at 4 ml/minute in 20 mM Tris pH 8.0, 50 mM NaCl buffer. Protein elution was monitored by spectrophotometer readings at 280 nm. Fractions of 5 ml corresponding to the different peaks were collected.

2.2.9 Ion exchange chromatography

Ion exchange chromatography was performed on a BioCAD/SPRINT perfusion chromatography system (Perseptive Biosystem Inc., MA, USA). The anion exchange column used was the POROS 10HQ column (Perseptive Biosystems). Samples in 20 mM tris pH 8.0, 50 mM NaCl were loaded in the column equilibrated with the same buffer. The elution was performed with a 1 M NaCl gradient. Protein elution was monitored by spectrophotometer readings at 280 nm, and 0.5 ml samples were collected.

2.2.10 Hydrophobic interaction chromatography

Hydrophobic interaction chromatography was performed on a BioCAD/SPRINT perfusion chromatography system (Perseptive Biosystem Inc., MA, USA). The correctly sized fractions from gel filtration chromatography were pooled, buffer exchanged into 20 mM phosphate buffer pH 8.0, 1.5 M ammonium sulphate and injected onto a POROS 20 ET column (Perseptive Biosystems). Proteins were eluted with a gradient of ammonium sulphate falling to 0mM. Protein elution was monitored by spectrophotometer readings at 280 nm, and 0.5 ml samples were collected.

2.2.11 Peptide elution

HLA-F/ β2-microglobulin complex was refolded *in vitro* and purified by gel filtration chromatography. As a positive control, recombinant HLA-A2 and β2-microglobulin proteins were refolded *in vitro* in the presence of a HLA-A2 restricted EBV epitope (GLCTLVAML). Complexes were purified by gel filtration chromatography. Both purified complexes were concentrated over a 10 kDa exclusion filtration membrane

(Amicon, USA) and acetic acid added to a final concentration of 10%. The samples were passed through a 3 kDa exclusion filtration membrane to isolate small molecules (Amicon, USA) and fractionated on a C-8 HPLC column (Rainin Instrument company Inc, USA). Elution was performed with a 0 to 80% acetonitrile gradient in 0.1% trifluoro-acetic acid over 15 minutes at 0.5 ml/minute. Peptide elution was monitored at 220 nm with a Gilson UV detector model 116.

2.3 Production of enzymatic biotinylated HLA-F complex

Enzymatique biotinylation was performed with recombinant Bir A enzyme produced by Dr Chris O'Callaghan 1999 (O'Callaghan C et al., 1999). The concentrated refolding buffer of HLA-F with biotinylated tag was exchanged into a Bir A buffer (100 mM Tris pH 7.5, 20 mM Nacl, 5 mM $MgCl_2$) using PD-10 column (Pharmacia, Sweden). The sample was then concentrated in 2 mls using a centriprep with a 10 kDa exclusion filtration membrane (Amicon, USA). 10 µl of Bir A enzyme (5 µM), 100 µl of 100 mM ATP, 10µl of 100 mM biotin, and protease inhibitors (0.1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin) were added to the 2 ml sample. The biotinylation reaction was incubated overnight at room temperature. Following incubation, unbound biotin was removed by gel filtration chromatography.

2.4 Surface plasmon resonance

SPR binding studies were performed at 25 °C on a BIAcore 2000 (BIAcore AB St Alban, UK) in HBS buffer (150 mM NaCl, 3 mM EDTA, 10 mM HEPES pH 7.4).

Strepatavidin and class I molecules were covalently coupled onto research Grade CM5 sensor chips (BIAcore AB) using the BIAcore AB amine coupling kit.

2. 5 Immunological methods

2.5.1 Monoclonal antibody production

Female BALB/c mice which were transgenic for both HLA-B27 and human β2-microglobulin (Weiss et al., 1990) were immunized subcutaneously with 50 μg of recombinant monomeric HLA-F protein on day 1, day 14 and day 35. The recombinant proteins were emulsified in complete Freund's adjuvant for the first injection, and emulsified in incomplete Freund's adjuvant for the next two injections. On day 65 a further 50 μg of protein without adjuvant was administered by an intraperitoneal injection. Spleens were harvested on day 70 and the spleen cells fused to PS-NS1/ag 4.1 (NS-1) cells (when not stated otherwise) by the method of Koller and Milstein (1975). Partner cell lines NS-1 and NS-0 cells were provided by the Department of Cellular Science, University of Oxford. Spleenocytes and NS-1 cells were washed in RPMI 1640 free medium and counted. Spleenocytes were then mixed with NS-1 at a ratio 4:1 or 10:1 and centrifuged at 300 g for 10 minutes. The tube was gently tapped to disrupt the pellet and 1.5 ml of PEG/DMSO (Sigma, UK) was added dropwise to the cell pellet in 1 minute. RPMI was then added dropwise, first 3 ml for two and half minutes, then 7 ml for 5 minutes. Cells were centrifuged at 300 g for 10 minutes, gently resuspended in R10 and plated into microtitre wells (100 μl per well) for cell culture. After 24 hours of culture, 100 μl of R10 supplemented with 2 x HAT (Sigma, UK) were added to each well.

2.5.2 Antibody purification

Hybridomas were grown until they died in R10 containing only 5% foetal calf serum. 250 ml to 1 litre of hybridoma supernatant was filtered and ran at 10 ml/minute through a protein A column (POROS 20A) using a BioCAD/SPRINT perfusion chromatography system (Perseptive Biosystem Inc., MA, USA). Antibodies were eluted with 5 ml of 0.1 M glycine pH 2.8 in fraction of 0.5 ml which were quickly neutralised with 50 µl of Tris 1 M pH 8.0. Antibody isotypes were determined using the isoStrip kit (Boehringer Mannheim, UK).

2.5.3 ELISA (Enzyme-linked immunoabsorbant assay)

ELISAs were performed to screen the production of monoclonal antibodies. HLA-F recombinant proteins were immobilised on Nunc Maxisorp microtitre plates (Nage Nunc International, USA) and blocked overnight at 4°C with PBS (Phosphate buffer saline), 1% BSA, 0.5% Tween® 20. 50 µl of culture supernatant were transferred to a washed ELISA well and incubated for 1 hour. The second antibody layer was a HRP conjugated goat anti-mouse IgG Fc (PIERCE, USA). After washes, BM blue POD substrate (Boehringer Mannheim, UK) was used as a developing reagent and optical density read at 450 nm.

To test the reactivity of w6/32 on class I recombinant protein, w6/32 were incubated in Nunc microtitre plates 2 to 3 hours at 37°C and the plates were blocked overnight with PBS (Phosphate buffer saline) 1% BSA, 0.5% Tween® 20. After 6 washes, dilutions of recombinant proteins were incubated for 1 hour at room temperature. Plates were washed six times before incubation with a rabbit anti-human β2m (DAKO, Denmark)

for 20 minutes at room temperature. The second antibody, an alkaline phosphatase-conjugated goat-anti-rabbit Ig, was also incubated for 20 minutes at room temperature. The ELISA was developed with a colorimetric reagent for alkaline phosphatase. The reading at an appropriate wavelength can give a relative measurement of the amount of refolded protein compared with a positive control.

2.5.4 Immunoblotting

SDS-PAGEs were run in BIO-RAD minigels, and proteins electotransfered to a Hybond-c super nitrocellulose membrane (Amersham, UK) in transfer buffer (20 mM Tris pH 8.3, 150 mM Glycine). A Hoefer semiPhor transfer chamber was run for 1 hour at 70 mA (voltage limit 50 V). Membranes were blocked in PBS (2% gelatine , 0.5% Tween® 20), stained and washed in PBS (0.5% gelatine, 0.05% Tween® 20). HRP anti-mouse (DAKO, Denmark) at 1/2000 was used as a second antibody layer. Detection was performed with the ECL kit (Amersham Pharmacia, UK).

2.5.5 Immunoprecipitation and coimmunoprecipitation

Pellets of 10 million metabolically labelled cells were resuspended in 500 µl lysis buffer (20 mM Tris pH 7.6, 10 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40) with 250 mg of Mega 9 and protein inhibitors (500 µl of 0.5 M iodoacetamide, 500 µl of 0.2 M PMSF in 50 ml of lysis buffer) and incubated for 20 minutes at 4°C. Nuclei were then spun down at 13,000 rpm for 5 minutes in a bench-top centrifuge, and the lysate pre-cleared twice with pansorbin for 30 minutes at 4°C. Pansorbin was spun down and the supernatant mixed with purified antibody to a concentration of 10-15 µg/ml . After 1 hour incubation, 100 µl of protein A sepharose (Sigma, UK) was added and incubated

for a further hour. The protein A beads were then washed 4 times in a wash buffer (20 mM Tris pH 7.6, 10 mM EDTA , 450 mM NaCl, 0.5% Nonidet P-40) and stored as a dry pellet at -20°C. The concentration of salt was increased in the washing buffer to decrease non-specific binding.

For coimmunoprecipitation the method described above was used, however the lysis buffer was made of 50 mM Tris pH 7.6, 150 mM NaCl, 1% digitonin, protein inhibitors, and the wash buffer made of 50 mM Tris pH 7.6, 150 mM NaCl, 0.5% digitonin. Immunoprecipitated proteins were analysed by one-dimensional isoelectic focusing electrophoresis (1D-IEF) or SDS-PAGE followed by autoradiography.

2.5.6 Immunochemistry

Normal post-mortem tissues were obtained from the Histopathology Department of the John Radcliffe Hospital, (Oxford, UK). Tissues were snapped frozen in liquid nitrogen, then cut with a cryostat. Cell lines were cytospined five minutes at 350 rpm. Slides were dried for 6 to 18 h, fixed in acetone at room temperature for 10 min, air dried and stored at -20°C. For staining, the primary antibody FG1 was incubated at a concentration of 30 μg/ml for 30 minutes at room temperature. The second antibody, a goat anti mouse HRP (DAKO, Denmark), was incubated at 1/50 dilution at room temperature 30 minutes. The substrate used was Diamenobenzidine (Sigma, UK). Biological samples were contrastained with hematoxyilin. Adherent cell lines were grown in a Lab-Tek chamber slide system (Nage Nunc International, USA), and suspension cell lines were cytospined. Cells were then fixed for 10 minutes in acetone

at room temperature. Cells were finally stained directly or indirectly with fluorchrome or HRP conjugated antibodies.

2.6 Cellular methods

2.6.1 Separation of peripheral blood leukocytes

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood. Fresh peripheral venous blood was mixed with heparin (Leo Laboratories, UK). Two volumes of blood were diluted in 1 volume of R 10 medium (RPMI ; Gibco, USA), supplemented with 10% foetal calf serum). 14 ml of diluted blood was carefully layered on top of 6 ml Lymphoprep Ficoll-Hypaque (Nycomed, Scandinavia) and centrifuged at 1400 rpm in a bench top swinging bucket centrifuge for 25 minutes without braking. The buffy coat was harvested, and washed twice in R10.

2.6.2 General cell culture

Eukaryotic cells were grown in R10; (RPMI ; Gibco, USA), supplemented with 10% foetal calf serum (Gibco, USA), 50 U/ml penicillin (Sigma, UK), 50 U/ml streptomycin (Sigma, UK), 2 mM glutamine, (Flow Laboratories) or Dulbecco's Modified Eagle Medium (Gibco, USA) supplemented as for RPMI. Cells were incubated in a 37 °C humidified incubator with 5.5% CO_2. Non-adherent cells were split, or spun down and resuspended in fresh media. Adherent cells were washed in serum free RPMI and incubated in 5 mM EDTA, 0.5% porcine tyrosine (Sigma, UK) for 5 minutes to resuspend them. Cells were then washed and resuspended in fresh media.

2.6.3 Eukaryotic cell stock

Eukaryotic cells were frozen in a solution made of 10% dimethyl sulfoxide (DMSO) in foetal calf serum. Cell pellets were resuspended in the freeze mix to a concentration of two to four million cells/ml and aliquoted in freezing tubes. Tubes were then quickly put into a freezing container (Nalagen, UK) and transfered to a -70°C freezer. After 24 hours the tubes were stored in liquid nitrogen. Frozen cells were thawed in a water-bath at 37°C for 5 minutes and slowly diluted to 20 ml of the appropriate medium. Cells were then spun at 1200 rpm in a bench-top centrifuge and washed. Cell viability was determined by incorporation of tryptan blue dye (Sigma, UK) and counted on a slide heamacytometer.

2.6.4 IFN treatment

Typically, adherent cell lines and suspension cell lines were cultured in the presence of 500 U/ml of IFN gamma (Sigma, UK) for 48 hours. The treatment was tested on U937 and Hela cells by monitoring class I expression with HC10 and w6/32 monoclonal antibodies.

2.6.5 DNA transfecction by calcium phosphate precipitation

Kidney fibroblast cell line 293T and Hela cells were grown until 50% confluence. Plasmid DNA were mixed with 1 volume of 0.25 M $CaCl_2$ to which 1 volume of 2 x HEBS was added drop by drop under agitation. The solution was left standing for 5 minutes and then dropped onto the cell monolayer. Cells were then grown overnight. Typically, 40 µl of plasmid DNA was precipitated in 2 ml to transfect one 15 cm^2 plate.

2 x HEBS (280 mM NaCl, 50 mM HEPES, 2.8 mM Na_2HPO_4) was adjusted to pH 7.0 with 1 M NaOH and sterilised by filtration.

2.6.6 Metabolic labelling

Cells were spun at 1400 rpm in a bench-top centrifuge and washed twice in PBS. The cell pellets were then resuspended in methionine-free R10 supplemented to 10 mM Hepes pH 7.6 to a concentration of 20 million cells/ml and incubated for 1 hour at 37°C with gentle agitation every 20 minutes. 100 μCi of ^{35}S-Methionine (Amersham, UK) per 10 million cells were then added and the cells incubated for a further hour at 37°C for different durations as a function of the experiments. Metabolic labelling was stopped by addition of 20 ml cold PBS. Cells were then spun down at 1400 rpm in a bench-top centrifuge and unincorporated radioactive methionine supernatant disposed of in a radioactive sink.

2.6.7 Pulse chase experiments

Metabolic labelling was carried out as described previously. The metabolic labelling pulse was generally for 15 minutes. Chases were performed by adding R10 supplemented with 400 mM methionine, 10 mM hepes pH 7.6. The chase time indicated for each experiment was variable.

2.6.8 Iodination of cell surface proteins

10 million cells were washed twice in PBS and resuspended in 1 ml PBS. Cell surface proteins were labelled with 500 μC of I^{125} at room temperature for 15 minutes and gently agitated every 5 minutes. Cells were then washed twice in 2 mM NaI PBS and

resuspended in lysis buffer used for immunoprecipitation (20 mM Tris pH 7.6, 10 mM EDTA, 100 mM NaCl, 0.5% Nonidet P-40) supplemented with protein inhibitors (500 µl of iodoacetamide, 500µl of 0.2 M PMSF) and 250 mg of Mega 9 per 50 ml of lysis buffer.

2.6.9 Flow cytometry analysis

Cell lines and PBMC were stained with antibodies or tetramers and analysed by flow cytometry using standard protocols. Fresh cells were washed in PBA (1 mg/ml BSA, 0.1% azide in PBS) and incubated with 1 µg of antibodies for 40 minutes at 4°C in approximately 100 µl. For intracellular FACS staining, the washed cells were incubated 15 minutes at room temperature in FACS permeabilisation solution (Becton Dickinson, USA) prior to incubation with the first antibody. Cells were then washed twice in cold PBA. The second antibody was diluted in line with the manufacturers indications and incubated for 40 minutes at 4°C. Cells were finally washed and fixed in 1% formaldehyde in PBS. Tetramer staining was performed at 4°C for 40 minutes in PBS. PBMC were stained on ice immediately after Ficoll-Hypaque separation. Tetramers were used at a concentration corresponding to about 10 µg per ml of HLA-F heavy chain. Cells were analysed on a FACSscan (Becton Dickinson, USA) and the data analysed using the CellQuest software. Lymphocytes were gated according to size (forward scatter) and density (side scatter).

Chapter 3 - Production and Analysis of Recombinant HLA-F Molecules

3.1 Introduction

The human leukocyte antigen F gene was originally identified based on its DNA sequence homology with other class I genes. Geraghty et al. cloned a 5.4 kb Hind III fragment from a HLA deletion mutant cell line 721.144 which was still able to hybridise to a class I cDNA probe (Geraghty et al., 1990). Sequence alignment of this fragment identified a gene with strong homology to the HLA-A2 gene. This gene was first designated HLA-5.4, then subsequently renamed HLA-F.

HLA-F has a low level of polymorphism and is classified as a MHC non classical class I molecule (class Ib), as are HLA-E and HLA-G. HLA-F has two known coding polymorphisms, one at position 1243 and one at position 1640 nucleotides numbered according to Geraghty et al. 1990. The first polymorphism is a synonymous substitution (67Ala[GCC] to Ala[GCG] (Shimizu et al., 1988). The second polymorphism is a nonsense mutation at position 118 Y (Uchigiri et al., 1997). These observations were made in the Japanese population, and there is not any published data on HLA-F polymorphism in other ethnic groups. The work presented in this thesis was based on the sequence published by D. Geraghty (Geraghty et al., 1990).

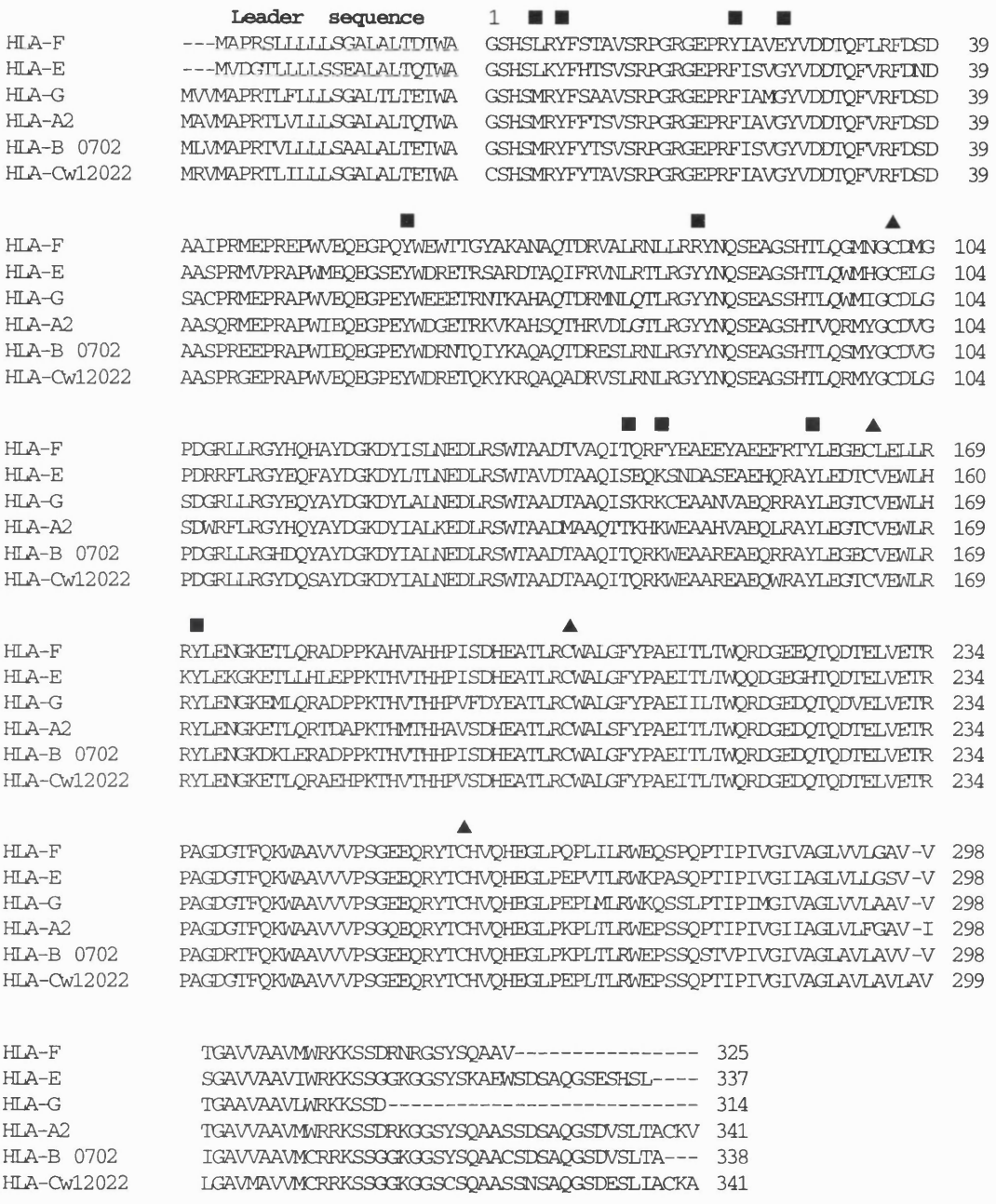

Figure 3. 1 Comparison of HLA class I precursors.

■ Conserved site among MHC class I proteins.

▲ Conserved cysteine in all MHC class I proteins.

HLA-F (P30511), HLA-E (P13747, HLA-G (P17693), HLA-A2 (P01892), HLA-B (P01889), HLA-C (I72113) were taken from swissprot GenBank data libraries.

HLA-F has an overall gene structure similar to class Ia genes with three exceptions. The 3' untranslated region of HLA-F transcripts diverge from other class I genes, the sequence belonging to a different and uncharacterised gene family (Geraghty et al., 1990). In addition, two features ultimately modify the protein translated when compared with class I proteins. HLA-F gene translation, like that of HLA-E starts at what corresponds to the second methionine (ATG) unlike the other MHC class I genes. This results in the expression of a shorter leader sequence for these two molecules (Figure 3.1). Secondly a mutation in the 3' splice site of intron 6 suggests that HLA-F mRNA lacks exon 7 which results in the expression of a protein with a shorter cytoplasmic domain (Figure 3.1).

The low levels of mRNA transcripts *in vivo* and the fact that no cell surface expression was obtained in transfected cells, have hindered the investigation of the HLA-F molecule (Geraghty et al., 1990; Shimizu et al., 1988). For these reasons, the production of recombinant proteins was undertaken. This chapter describes the production of the HLA-F protein using prokaryotic and eukaryotic expression systems. Bacteria were chosen as a robust system to produce significant quantities of HLA-F and β2m recombinant proteins. It was then necessary to refold these two proteins together and to purify the correctly refolded complex. A second strategy involved expression in mammalian cells of the HLA-F heavy chain tagged with motifs that enable detection and purification. Mammalian systems of expression usually result in production of smaller amounts of protein compared to bacterial systems of expression. However, the

benefits of expression in mammalian cells is the production of membrane integrated proteins which should sustain better post-traductional modifications.

3.2 Cloning and expression of HLA-F recombinant protein in a bacteria

Gene recombination in bacterial systems of expression can result in the production of hundreds of milligrams of protein per litre of culture. However, high levels of expression usually leads to the formation of inclusion bodies; thus it is necessary to be able to refold the protein produced. Garboczi et al. have shown that the HLA-A2 ectodomain could be refolded *in vitro* from recombinant proteins produced in bacteria (Garboczi, Hung and Wiley, 1992). Analyses of the HLA-F protein sequence showed 81% similarity with HLA-A2. The standard cysteine pairs in the α2 and α3 domains of class I molecules are present in identical positions in the HLA-F molecule and the position and length of the trans-membrane regions are similar to the other class Ia proteins, making it likely to be a membrane protein. Finally, β2m can be immunoprecipitated with HLA-F heavy chain from HLA-F transfected 721.221 cells (Shimizu et al., 1988). All these data suggest that HLA-F recombinant proteins could be refolded like HLA-A2. However, class I molecule production is most efficient when their ligand or peptide antigen is added to the refolding mix (Garboczi, Hung and Wiley, 1992; O'Callaghan et al., 1998; Reid et al., 1996). Although a peptide ligand has not yet been identified for HLA-F we decided to proceed with the production of HLA-F recombinant proteins. HLA-F cDNA was inserted into a cloning vector and the HLA-F ectodomain was subcloned in different expression vectors.

HLA-F specific primers F-L5' and F-U3' (Table 3.1, Appendix 1) were designed to amplify HLA-F cDNA by polymerase chain reactions (PCR) and to clone its products into a cloning vector, PCR-Script (Stratagene, USA). HLA-F transcripts had previously been detected in most lymphoid tissues and in B cell lines (Geraghty et al., 1990). A cDNA library (pGADIO 3100) made from an EBV transformed B cell was used as a template. One clone (F0b2) had an identical sequence to the published sequence (Geraghty et al., 1990), thus confirming the presence of the HLA-F transcripts in B cells. Furthermore, as predicted from the analysis of the genomic sequence that shows a point mutation at a splicing site in the seventh exon, this exon was spliced out of the transcript. A DNA maxi prep of this clone was produced and the stock used as a template in other experiments.

At the same time, HLA-F specific primers 1F5' and 1F3' (Table 3.1, Appendix 1) were designed to clone HLA-F extra-cellular domains (1-275) into the T7 vector of expression pET-30b (Novagen,UK). The same B cell cDNA library was used as a template for the PCR. Out of 7 clones sequenced, two had identical sequences to the published sequence (Geraghty et al., 1990). However the five other clones had a modification which would have changed the serine (TCT) position 252 to a proline (CCT). It has not been determined if this modification was a genuine polymorphism or the result of the methodology used.

pET-30b expression vector (Novagen, UK) with the correct HLA-F extra-cellular domains was transfected into *E coli* strain BL21 (DE3) pLys (Novagen,UK) for

recombinant protein expression. Bacteria were grown in the appropriate selection media and expression induced with 0.5 mM IPTG (Isopropyl β-d-thiogalactopryanoside) (Sigma, UK) until the culture reached an absorbance of 0.5 at 600 nm. Three hours after induction, bacterial cells were lysed in SDS-PAGE loading buffer and the expression product analysed on SDS-PAGE. No expression was observed. Different conditions were tested including different induction protocols, different culture media, and different *E.coli* strains. However, using the C terminal His tag attached to the protein expressed by the pET-30b vector, it was possible to detect protein expression after a purification step. In these conditions a band of the correct molecular size (32.8 kDa) was detected on SDS-PAGE in the insoluble fraction of the bacterial lysate. HLA-F recombinant protein was expressed but could not be directly detected on SDS-PAGE because of the very low level of expression.

Low levels of expression can be the results of unfavourable secondary structures formed at the 5'end of the insert. To reduce predicted secondary structures of HLA-F mRNA, the effect of different synonymous mutations introduced into the 5' end of the insert were analysed with the Lasergene program (DNASTAR Inc, USA). Mismatch polymerase chain reaction using 2F5' and 1F3' primers (Table 3.1, Appendix 1) were performed to produce a new construct. Using this construct it was possible to directly observe expression of recombinant protein on SDS-PAGE. The protein produced from HLA-F cloned in pET-30b vector had a His tag at the C terminus and was called F-His.

Subsequently the HLA-F ectodomain was cloned in pGMT7 (Reid et al., 1996), another T7 vector which does not lead to the production of a C terminus His tag protein using 5'primers with same altered sequences. The three 3' primers were BLF1 3' insert (1-280 aa), BLF2 3' insert (1-278 aa), BLF3 3' insert (1-276 aa) (Table 3.1, Appendix 1). All constructs were sequenced and correct clones stocked. The proteins produced from these constructs were named F1, F2 and F3. F1 expression analysed on SDS-PAGE is presented in Figure 3.2. β2-microglobulin light chain was expressed from a pHN1 plasmid (a gift from D.N. Garboczi and D.C. Wiley, Harvard, USA). β2-microglobulin was also expressed as an insoluble protein.

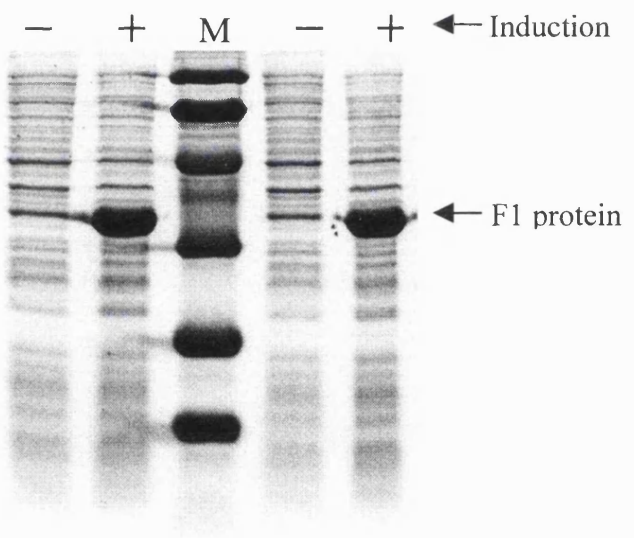

Figure 3. 2 Expression of HLA-F recombinant protein (F1) in *E.coli.*

Bacterial cells form two different clones were lysed in reducing SDS-PAGE loading buffer, and samples loaded on a 15% polyacrylamide gel. (+) induction of HLA-F expression with IPTG, (–) no induction. (M) is the molecular size marker (97.4, 66.2, 45, 31, 21.5, 14.4 kDa from top to bottom). Protein expression is represented by the presence of a strong band at the expected molecular weight of F1 (32.3 kDa) in the (+) lane.

Construct name	vector	Primers	HLA-F insert	Protein name
F0b2	PCR-Script	F-L5' F-U3'	Leader To Untranslated	Non applicable
F1cl1	pET-30b	1F5' 1F3'	1-275 aa and C-ter His tag	No expression
F1cl2	pET-30b	**2F5'** 1F3'	1-275 aa and C-ter His tag	F-His
FBL1	pGMT7	**FBL5'** FBL13'	1-280 aa	F1
FBL2	pGMT7	**FBL5'** FBL23'	1-278 aa	F2
FBL3	pGMT7	**FBL5'** FBL33'	1-276 aa	F3

Table 3. 1 HLA-F plasmids.

This table is a summary of the HLA-F cloning and expression vector. "1" does not correspond to the first methionine but the predicted first amino acid of the mature protein, a glycine. More details on the production of these constructs are presented in Appendix 1. Primers with synonymous mutations are in bold.

3.3 Large scale production and refolding of HLA-F and β2m recombinant proteins

Transfected bacteria were grown in four to six litres of media and induced for protein expression. Bacteria from large scale protein expression were subsequently lysed by

sonication. The insoluble fraction was washed in detergent (Triton X100) buffer to remove lipids. The remaining material, composed mainly of inclusion bodies was solubilised by overnight incubation at 4°C in urea solution. Protein concentration was determined using the BIO-RAD protein assay, and recombinant protein purity visualised by SDS-PAGE. An average of 120 mg of protein per litre of culture was produced for HLA-F heavy chain and an average of 300 mg per litre of culture was produced for the light chain, β2m.

HLA-F has previously been shown to associate with β2m in a transfected cell line (Shimizu et al., 1988), but it has not been determined if this complex bound peptide or if it was even capable of binding any peptide. Refolding of HLA-F/β2m complex was attempted *in vitro* using the conditions previously determined for HLA-E (O'Callaghan et al., 1998) without addition of any synthetic peptides. 20 mg of urea solubilised β2m was added by slow dilution to 500 ml of 400 mM L-Arginine, 100 mM Tris pH 8.0, 2 mM EDTA, 5 mM reduced glutathione, 0.5mM oxidised glutathione, 0.1 mM PMSF (phenylmethyl-sulphonyl fluoride). β2m was allowed to equilibrate with stirring for 2 hours at 4°C. 40 mg of urea solubilised heavy chain (F-his) was then added by slow dilution to the refolding mix. In initial experiments there was significant precipitation when the heavy chain was added. This was overcome by adding 5 mM DTT to the urea solubilised protein, followed by addition of one volume of guanidine buffer (6M guanidine-hydrochloride, 10 mM sodium acetate pH 5.5, 10 mM EDTA) and by using 700 mM L-Arginine in the refolding buffer. After a 48 hour incubation at 4°C the

refolding mixture was concentrated under nitrogen using a 10 kDa exclusion filtration membrane (Amicon, USA).

3.4 Purification of HLA-F/β2m complex of recombinant proteins

The HLA-F/β2m complex was first purified by gel filtration chromatography in 20mM Tris pH 8.0, 50 mM NaCl using a Superdex 75 column (Pharmacia Uppsala, Sweden). Gel filtration chromatography of the concentrated refolded protein (Figure 3.3) identified the presence of a product of the approximate predicted size (44 kDa) for a 1:1 complex of HLA-F (32.3 kDa) and β2-microglobulin (11.7 kDa), and a peak of the expected molecular size for β2m (11.7 kDa). Peak 2 eluted 6 ml earlier than ovalbumin (a 43 kDa protein marker) and 10 ml after chymotrypsinogen A (a 67 kDa protein marker). SDS-PAGE analysis in reducing conditions (Figure 3.4) confirmed the presence of HLA-F and β2-microglobulin in an approximately equimolar ratio in the peak with the expected size for the 1:1 complex. No visible contaminant band was observed.

To further characterise the protein complex purified by gel filtration chromatography, this sample was analysed by anion exchange chromatography. Gel filtration chromatography fractions corresponding to the complex were pooled and run on anion exchange chromatography with a POROS 10 HQ column using a BioCAD/SPRINT perfusion chromatography system (Perseptive Biosystem Inc., USA).

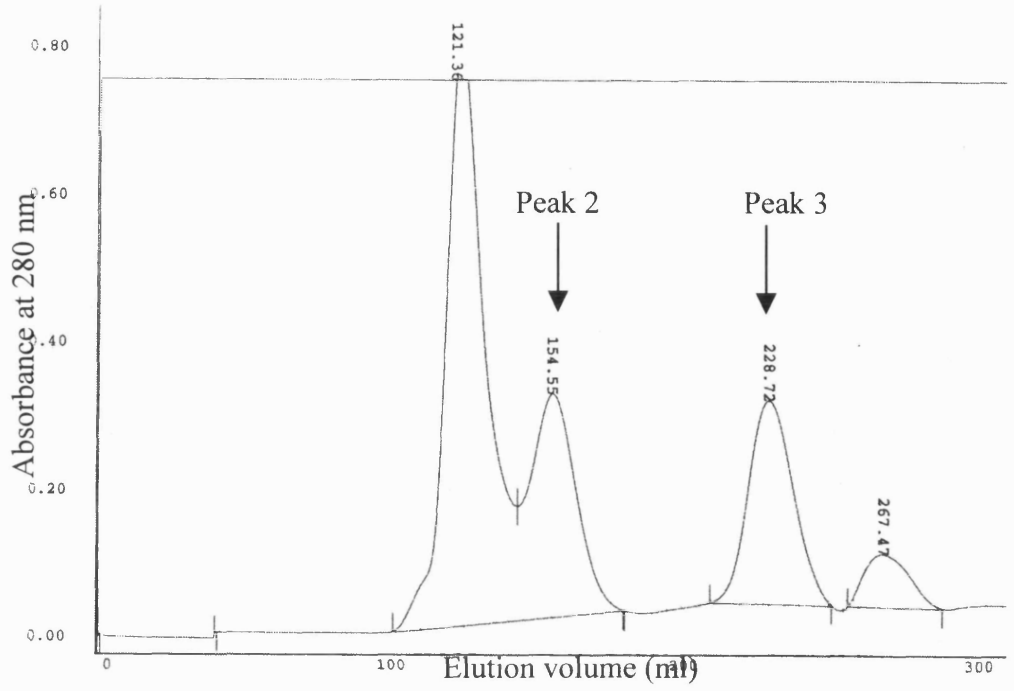

Figure 3. 3 Gel purification of HLA-F complex.

Gel filtration profile of concentrated refolding mix loaded on to a Superdex 75 column. The first peak elutes at 110 ml, and consists of soluble aggregates. Peak 2 elutes at the approximate predicted size for a 1:1 complex of HLA-F (44 kDa). Peak 3 is of the expected molecular size for β2m (11.7 kDa).

Samples were loaded in 20 mM tris pH 8, 50 mM NaCl and eluted with a 1 M NaCl gradient, a method previously used to purify classical class I molecules. The first experiments involved the use of F-His which has a 6 histidine tag at the C-terminus of the protein. In these conditions, most of the protein eluted as aggregates, and no complexes were identified after analysis of the fractions containing proteins on SDS-PAGE. The first modification made to the protocol was to use F1, F2, and F3 heavy

chain proteins rather than the 6 *his* tag protein, F-His. Again, no protein complexes were purified after anion exchange chromatography when these different truncated HLA-F heavy chains were used.

Different refolding buffers were tested to refold F1 with β2m. Multiple variations of L-arginine concentration and different redox potentials were tried. However, no single homogeneous peak could be purified after anion exchange chromatography. Thus, other purification techniques were investigated. Cation exchange chromatography was used and different pH's tested for both anion and cation chromatography. However, the complex F1/β2m could not be purified in all conditions tested. Hydrophobic interaction chromatography was then attempted as a further alternative purification technique.

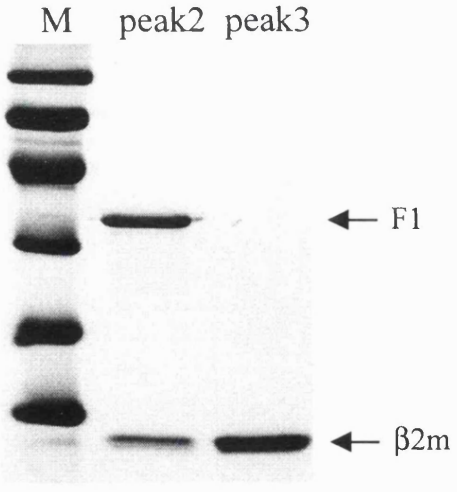

Figure 3.4 SDS-PAGE analysis of peaks identified by gel filtration chromatography.

Reducing SDS-PAGE confirmed the presence of both HLA-F heavy chain (F1) and β2m in the second peak, and β2m protein in the third peak. (M) is the molecular size marker (97.4, 66.2, 45, 31, 21.5, 14.4 kDa from top to bottom).

Correctly sized fractions from gel filtration chromatography were pooled. This sample was then tested for its solubility in different concentration of ammonium sulphate. The sample was buffer exchanged into 20 mM phosphate buffer pH8.0, 1.5 M ammonium sulphate and injected onto a POROS 20 ET column (Perseptive Biosystems). Proteins were eluted with a gradient of ammonium sulphate falling to 0 mM (Figure 3.5). Non-reducing SDS-PAGE analysis of the proteins present in each peak was performed (Figure 3.6). Two clear bands corresponding to HLA-F and β2m were observed in the first peak (P1) and second peak (P2). The third peak (P3) showed the presence of high molecular weight proteins.

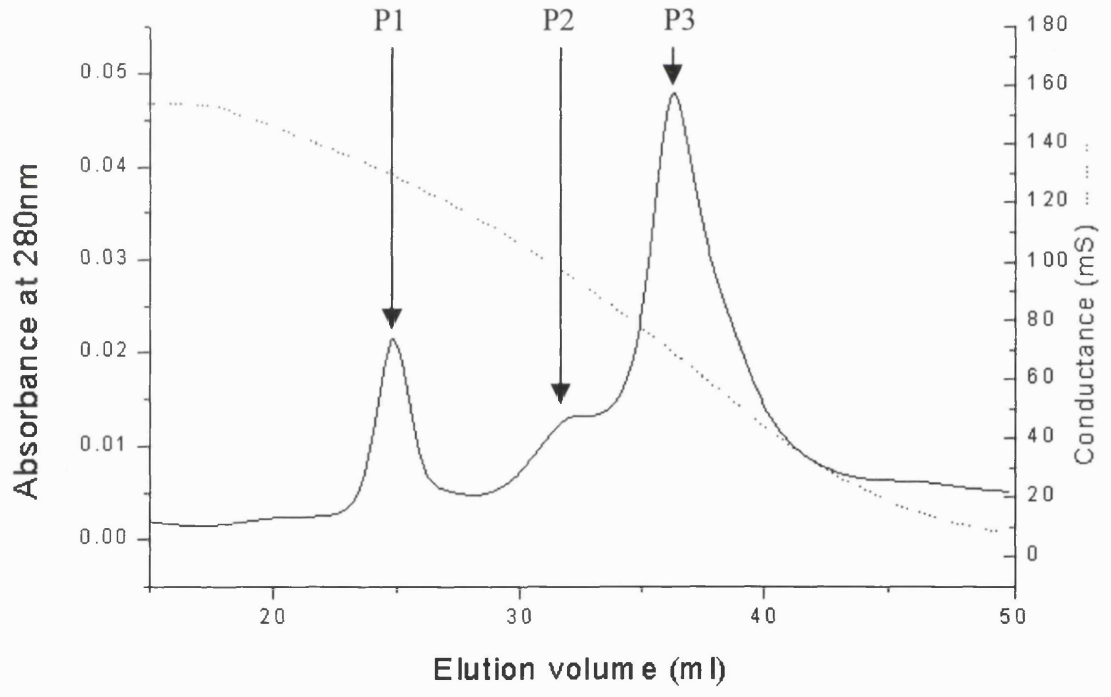

Figure 3. 5 hydrophobic interaction chromatography purification.
Hydrophobic interaction chromatogram of HLA-F/β2m complex sample purified by gel filtration. Proteins were eluted in a gradient of ammonium sulphate (dotted line). Three peaks (P1,P2 and P3) were identified and samples analysed on SDS-PAGE.

When analysed by reducing SDS-PAGE, these proteins were identified as aggregates of HLA-F and β2m.

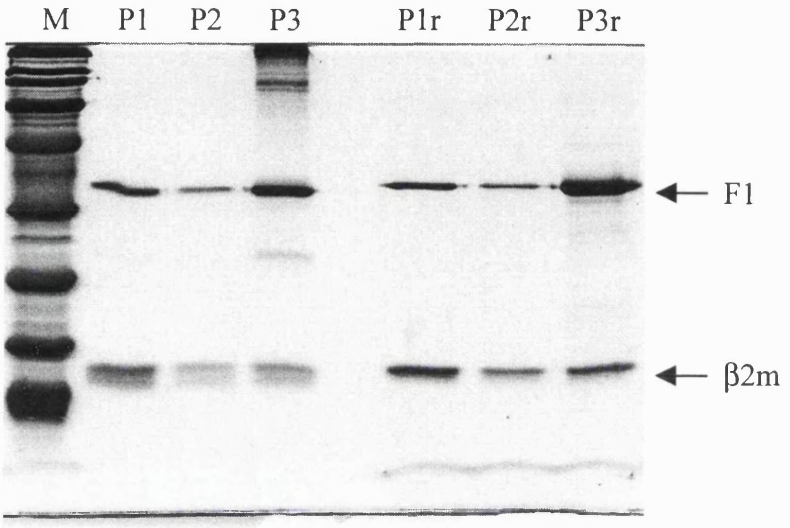

Figure 3. 6 SDS-PAGE analysis of the peaks identified by hydrophobic interaction Chromatography.

Samples were analysed in non reducing conditions (P1, P2, and P3) and in reducing conditions (P2r, P2r, and P3r) on a 15% acrylamide gel. The gel confirmed the presence of both HLA-F and β2m proteins in the three peaks. (M) is the molecular size marker (200, 116.2, 97.4, 66.2, 45, 31, 21.5, 14.4, 6.5 kDa from top to bottom).

3.5 Refolded state of HLA-F/β2m complex

Identified HLA-F/β2m complex, fractionated by gel filtration chromatography and hydrophobic interaction chromatography, were tested for their reactivity with w6/32. w6/32 is a conformation-specific antibody with broad reactivity against correctly folded HLA-A, -B and -C molecules (Brodsky et al., 1979). More recently w6/32 was shown to recognise the HLA-F molecule from transfected cells (Shimizu et al., 1988). w6/32

was tested for its reactivity to HLA-F and HLA-B7 recombinant proteins by ELISA. This conformational antibody has shown similar reactivity to HLA-F and HLA-B7 (data not shown).

HLA-F recombinant proteins purified by gel filtration were analysed by gel filtration for dissociation or aggregation after incubation at 37°C, another indication of the stability of the refolded molecule. The complex was stable after 30 minutes and 3 hours incubation at 37°C, although after 3 hours some soluble aggregate had begun to form (Figure 3.7).

3.6 Peptide elution

HLA-F and β2m recombinant proteins were able to form a complex when refolded in vitro without the addition of synthetic peptides. However, it was important to determine if a peptide was associated with the complex during the production process. HLA-F and β2m recombinant proteins were produced in bacteria and it was possible that bacterial peptides may have been carried over to the refolding buffer. HLA-F was submitted to acid elution and the eluants analysed by HPLC (Figure 3.8). HLA-F/β2m complexes purified by gel filtration chromatography were concentrated over a 10k Da exclusion filtration membrane (Amicon, USA). Acetic acid was then added to a final concentration of 10%. The sample was filtered through a 3 kDa exclusion filtration membrane (Amicon, USA) and the eluant analysed by HPLC. Analysis of HLA-F elution samples at 220 nm showed there was no elution of any peptide.

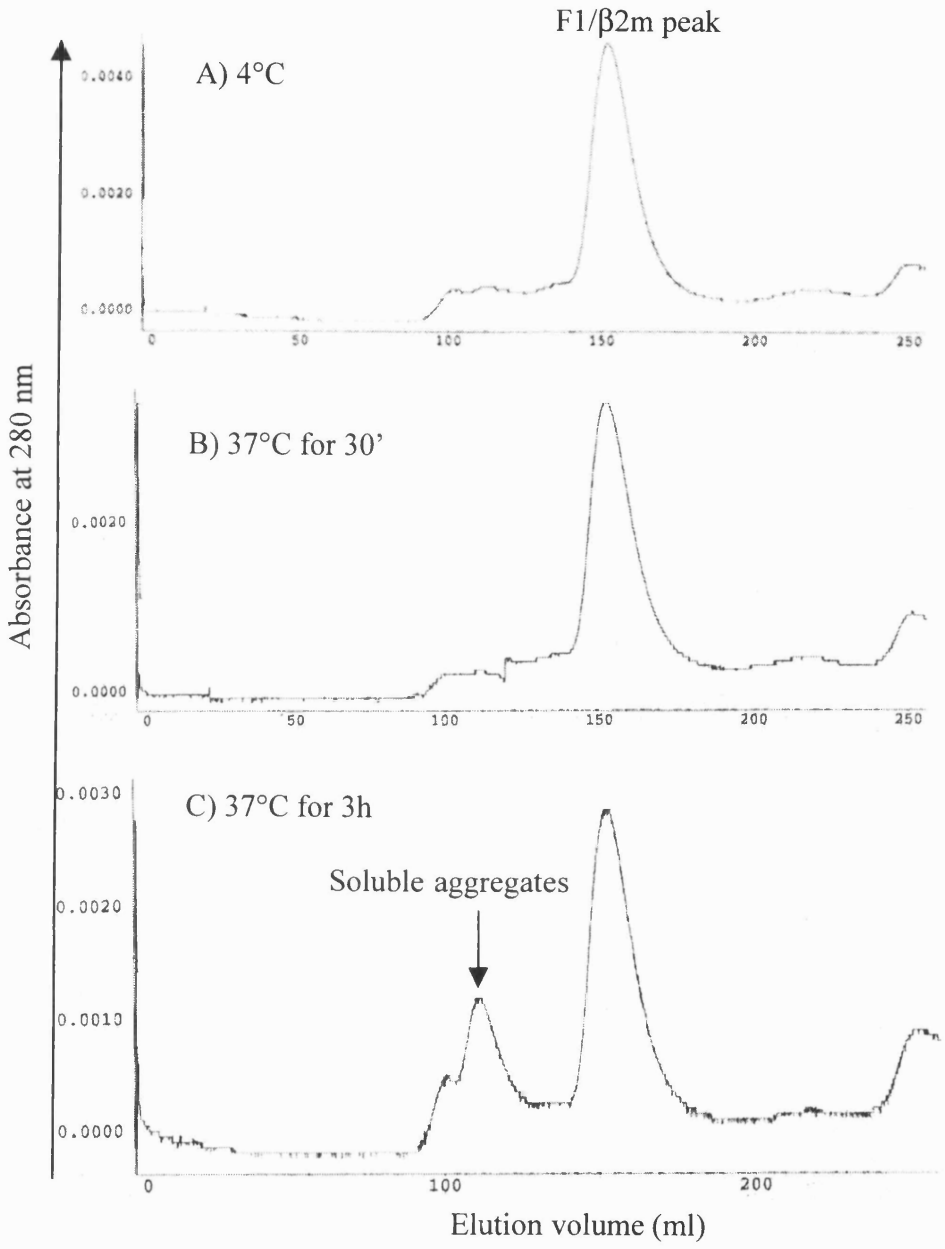

Figure 3. 7 Recombinant proteins HLA-F/β2m complex stability at 37 °C.

HLA-F/β2m complex of recombinant proteins purified by gel filtration were incubated for 30 minutes (B), and 3 hours (C) at 37°C or stored at 4°C (A) and run on gel filtration chromatography. Gel filtration chromatograph demonstrated that the HLA-F recombinant protein is stable at 37 °C.

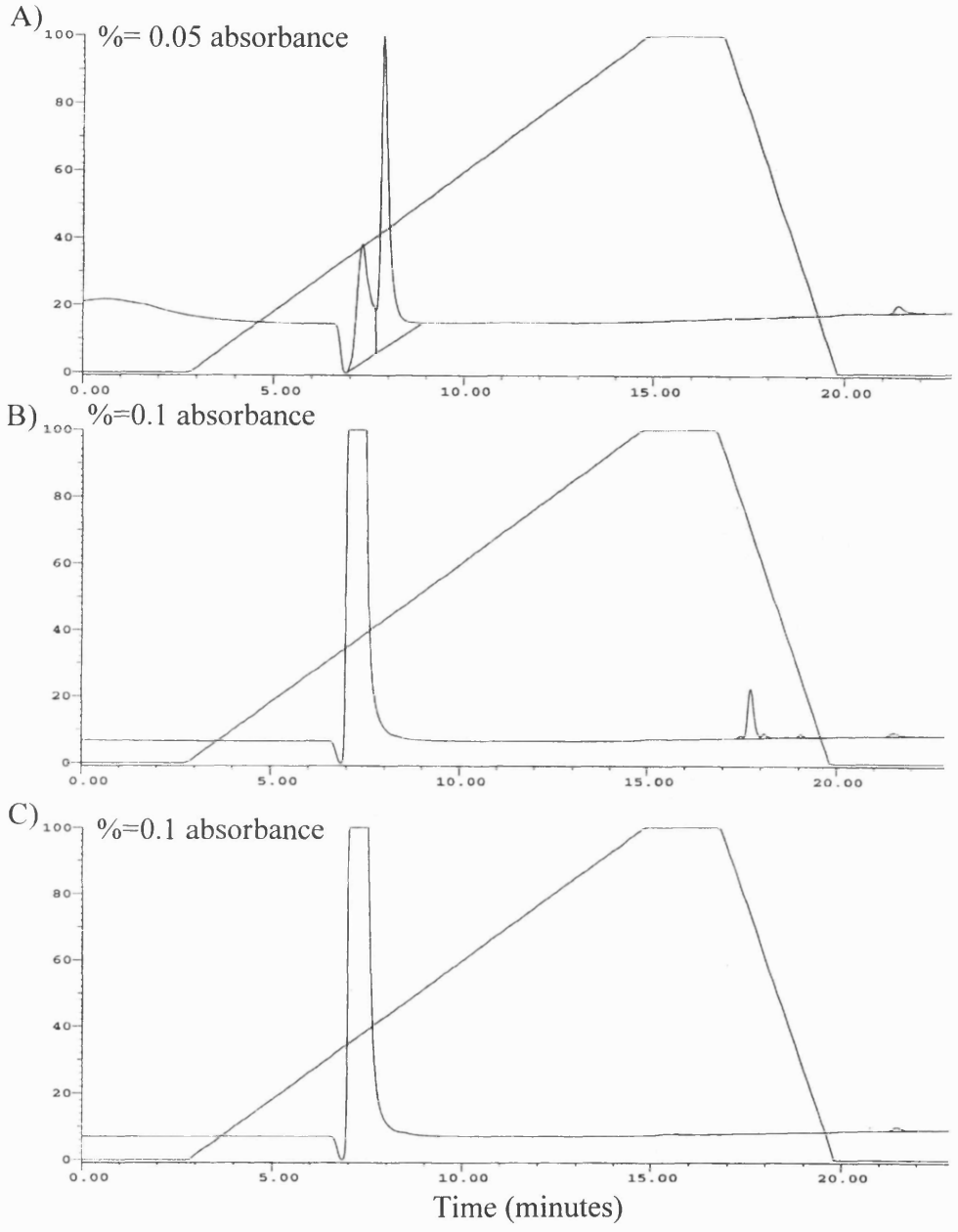

Figure 3. 8 No detectable peptide was eluted from HLA-F recombinant molecule.

Purified recombinant protein complexes of HLA-A2 (B), and HLA-F (C) were submitted to acid elution. HLA-F (A) was not submitted to acid elution. Samples were analysed on HPLC reversed phase chromatography. Peptides were detected at 220 nm. Peptide detected at 220 nm was observed in the HLA-A2 eluant but not in the HLA-F eluant.

Identical readings were obtained for the negative control (no acidification). Finally, a distinct peak was observed at 220nm when the same elution experiment was carried out on purified recombinant HLA-A2 complex obtained from the refolding of HLA-A2, β2m and GLCTLVAML (an EBV peptide).

3.7 Expression of HLA-F in mammalian cell lines

Cell surface of HLA-F was described as undetectable when the molecule was previously expressed in an HLA null mutant cell line (Shimizu et al., 1988). However, this experiment was hindered by the lack of a specific HLA-F antibody. To widen the type of cell line that could be analysed in the absence of specific anti HLA-F monoclonal antibodies, an expression system was used which added a tag to allow detection of the recombinant protein.

The full-length open reading frame of HLA-F heavy chain cDNA was amplified by PCR from the F0b2 plasmid and cloned in pcDNA 3 (Invitrogen, Netherlands) mammalian vectors of expression. pcDNA 3 vectors induce a high level of expression in mammalian cells from a CMV promoter. In addition these vectors have a SV40 origin of replication site which allows replication of the plasmids in mammalian cells containing the SV40 T antigen, such has 293T cells. The PCR product resulting from PCR using CO50 and CO51 primers was cloned in pcDNA 3.0, and the construct designated as F (Table 3.1). The PCR product resulting of the PCR using CO48 and CO49 primers was cloned in pcDNA 3.1a and designated F-Myc/His. pcDNA 3.1 is a eukaryotic expression vector which adds a Myc and His tag at the C-terminus of the

protein expressed (Table 3.1). All the constructs were sequenced and correct clones stocked. β2m cloned in pcDNA 3.0 was a gift from Dr C O'Callaghan.

Constructs name	primers	Vector	Protein domain
F-Myc/His	CO50 CO51	pcDNA 3.1a	Complete ORF + Myc and His tag
F	CO48 CO49	pcDNA 3.0	Complete ORF
β2m	-	pcDNA 3.0	Complete ORF

Table 3.2 HLA-F constructs for mammalian cell expression.

ORF: open reading frame. More details on the production of these constructs are presented in Appendix 1.

Expression of HLA-F was first tested in 293T, a human kidney fibroblast cell line. 293 T cells were co-transfected with F-Myc/His and β2m constructs using the calcium phosphate precipitation method. Expression was observed by immuoprecipitation (Figure 3.9) but also by Western blot and intra-cellular FACS staining using 9E10, an anti-Myc antibody (data not shown). 9E10 immunoprecipitated HLA-F recombinant protein, but only limited amounts of β2m was associated with this recombinant protein (Figure 3.9).

A nickel column was also used to purify the F-Myc/His protein from a transfected cell lysate. Results of this purification were analysed by SDS-PAGE and western blot showing that little HLA-F protein was purified. Furthermore, there was heavy contamination (data not shown).

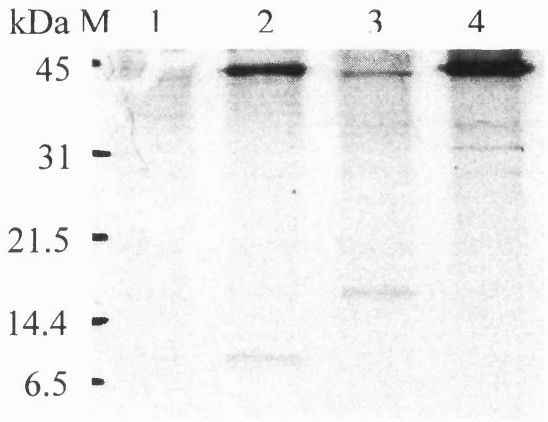

Figure 3. 9 Immunoprecipitation of HLA-F with a myc antibody.

Untransfected (lane 1 and 3), or F-Myc/His, β2m co-transfected (lane 2 and 4) 293 T cells were metabolically labelled. Class I molecules were immunoprecipitate with w6/32 antibody (lane 1 and 2). Proteins with a C-Myc epitope were immunoprecipitated with 9E10 antibody (lane 3 and 4). The majority of F-Myc/His protein was not associated with β2m.

293T, Hela, and COS cells were co-transfected by F-Myc/His and β2m using the calcium phosphate precipitation method. No visible increase of w6/32 cell surface staining was observed on the cell surface of transfected 293T compared with untransfected cells when analysed by flow cytometry. Anti-myc antibody was used to investigate cellular localisation of transiently transfected cells by immunofluorescence. The second antibody was FITC conjugated anti-mouse. Observation with a fluorescent microscope suggested that HLA-F recombinant protein expression was mainly intracellular (Figure 3.10).

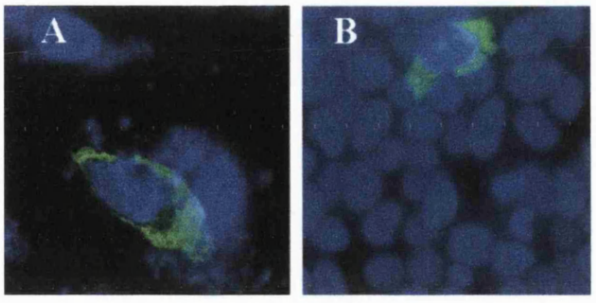

Figure 3. 10 Cytoplasmic localisation of F-Myc/His proteins expressed in Hela and 293T.

Hela (A) and 293T (B) cell lines transiently transfected with F-Myc/His and β2m were fixed in acetone. HLA-F recombinant protein were detected with a 9E10, an anti-Myc monoclonal antibody, and revealed in green with a FITC conjugated antibodies (DAKO, UK). Nuclei were revealed in blue using DAPI reagent. The recombinant HLA-F protein pattern of expression is cytoplasmic.

The T cell line Jurkat and B cell lines 744.221 and NAN-BCL were transfected with the F-Myc/His construct and treated with neomycin to produce stable transfectants. B cell lines were difficult to transform, and died in the selection media. Jurkat transfected cells were successfully growth in the selection media, but no expression could be detected by intracellular staining or Western blot when analysed using an anti-Myc antibody.

3.8 Discussion

The MHC seems to have arisen from a series of duplications and subsequent recombinations, which lead to the formation of many pseudogenes (Shiina et al., 1999). However, there are reasons to believe HLA-F is not a pseudogene. The HLA-F

sequence has not accumulated nonsense mutations maintaining a gene organisation similar to other class I genes. The gene has been conserved during evolution in primates (Otting and Bontrop, 1993). The transcript is expressed in a variety of tissues (Geraghty et al., 1990). Finally transfection of the HLA-F gene in human cells result in the production of the HLA-F heavy chain that co-precipitates with β2m (Shimizu et al., 1988). This data suggests that the HLA-F molecule is functional.

HLA-F was cloned from a B cell line, confirming the expression of HLA-F as a transcript in these cells. It was also verified from the sequence of this clone that exon 7 is spliced out, as was predicted from the genomic analysis. Finally, we observed that one third of the clones obtained had a mutation at position 252 compared to the sequence published by Geraghty (Geraghty et al., 1990). It was not determined if this modification was a polymorphism.

Our first approach to the production of soluble HLA-F molecule was to use proteins synthesised in *E.coli*. The HLA-F heavy chain ectodomain and β2m could be expressed in bacteria, although modifications of the N terminal codon used was necessary for a high level of HLA-F expression. Human classical and non-classical class I recombinant proteins have been refolded *in vitro* (Garboczi, Hung and Wiley, 1992; O'Callaghan et al., 1998; Reid et al., 1996). Biochemical study of HLA-F, has shown that, *in vitro*, HLA-F heavy chain protein and β2m could combine to a 1:1 molar stoichiometry to form a complex. This complex was of a homogeneous molecular size when purified by size exclusion gel filtration. HLA-F/β2m complexes could be further purified by

hydrophobic interaction chromatography. Purification of HLA-F by different chromatographic methods indicated that the complex refolded is homogenous in size and hydrophobicity.

The recombinant protein complex HLA-F/β2m produced is reactive with w6/32, a conformation-sensitive monoclonal antibody (Parham, Barnstable and Bodmer, 1979). This suggests that the protein generated shares conformation determinants with HLA-F proteins expressed in mammalian cell lines, which can also be immunoprecipitated with w6/32 (Shimizu et al., 1988). Finally, HLA-F can assemble and remain stable at 37°C for 30 minutes without apparent modifications. These results together suggest that conformation of the proteins produced are stable and likely to be correctly refolded.

In vitro refolding of HLA-E, HLA-A2 and HLA-B27 class I molecules has been shown to be most efficient in the presence on their ligands, to refold under the conditions used (Garboczi, Hung and Wiley, 1992; O'Callaghan et al., 1998; Reid et al., 1996). In the absence of information on HLA-F ligand(s), HLA-F/β2m complexes were formed in the absence of synthetic peptide, but we were unable to demonstrate the presence of any peptides when the complex was subjected to acid elution and high-pressure liquid chromatography. Although, this experiment should detect a limited number of peptide forms, however a high heterogeneity in bound peptide would not have been detected using this method. Furthermore, the monitoring method of HPLC eluted samples was specific to amino acids and may not have detected other ligands such as lipids or sugars. The ability of HLA-F to refold in the absence of synthetic peptides *in vitro* cannot be

taken as evidence that HLA-F assembly does not require peptides *in vivo*. The recombinant HLA-F was produced in *E. coli* and there may be small, undetected fragments of molecules present during refolding which could stabilise the recombinant protein in the absence of a synthetic peptide. It is also possible that, *in vitro*, there may be a sufficient degree of stability in the absence of peptide to allow the formation of a complex, whereas in the endoplasmic reticulum failure to load peptides may direct HLA-F towards the degradative pathway, as occurs with HLA-E.

The second approach used for the production of HLA-F molecule was the expression of a tagged HLA-F molecule in human cell lines. The presence of a tag potentially overcomes the absence of an antibody specific to tagged HLA-F. It was possible to detect high levels of HLA-F in transiently transfected 293T cells. However, in these cell lines HLA-F was predominantly not associated with β2m. It is possible that the C-terminal tag modifies HLA-F behaviour. It is also possible that the high level of expression and the cell line used are responsible for this behaviour. At this stage, it seemed likely that most of the HLA-F molecules expressed in 293T cells were not in the correct conformation. Furthermore, the use of the His tag to purify the molecule from cell lysate was not efficient. These results could have differed with stable transfectants expressing lower level of HLA-F. However, in the absence of a specific monoclonal antibody it has not been possible to detect HLA-F from stable transfected cells grown in the selection media.

The production of a recombinant HLA-F molecule, the main goal of the first part of this study was achieved. HLA-F-β2m complex was produced *in vitro* without the addition of synthetic peptides, and peptides were not detected after acid elution. It now seems appropriate to use the HLA-F recombinant proteins to generate specific monoclonal antibodies and this is described in Chapter 4.

Chapter 4 - Production and Characterisation of anti-HLA-F Antibodies

4.1 Introduction

Before antibodies were available to study protein expression, the study of HLA-F transcripts might have shed light on the function of the molecule. For example, HLA-G transcripts were mainly detected in foetal trophoblast tissues. Thus, HLA-G was thought to be involved in antigen presentation at the foetal-maternal interface. However the pattern of expression of HLA-F transcripts was shown to be expressed in most cell types studied similarly to class Ia, and was not restricted as HLA-G (Wei and Orr, 1990).

Analysis of the HLA-F gene sequence suggested that it encoded a class I protein (Geraghty et al., 1990). Limited polymorphism and a specific pattern of expression of the transcript classified HLA-F as a non-classical class I molecule (Geraghty et al., 1990). HLA-E and HLA-G, two other non-classical class I molecules encoded in the MHC seem to have immunological functions (O'Callaghan and Bell, 1998). However, other human Class I homologues have been identified which have either immunological or non-immunological functions such as HFE. The hypothesis of an immunological role for HLA-F was proposed because of its location within the MHC, its pattern of expression and its homology to other class I molecules. This hypothesis was mainly based on the HLA-F gene sequence and transcript studies.

To characterise HLA-F function, direct studies of HLA-F protein are necessary. When this investigation began, data on the HLA-F protein were very limited. HLA-F heavy chain was shown to be associated with β2m, but HLA-F protein was not detected at the cell surface (Shimizu et al., 1988). This study took advantage of the 721.221 cell line, which has an HLA-A, -B, and -C null phenotype. w6/32, an antibody with broad specificity to MHC class I molecules was used to detect HLA-F in HLA-F transfected 721.221 cells. No specific antibodies to HLA-F were available and the lack of reagents to detect and purify HLA-F was an important factor in the slow progress made over the last 11 years. To extend our investigation, we therefore planned to generate specific anti HLA-F antibodies using the reagents described in Chapter 3.

4.2 Production of polyclonal antibodies

Truncated HLA-F heavy chain (amino acids 1-280 of the mature protein), refolded *in vitro* with β2m, was purified by gel filtration as described in Chapter 3. The protein was dialysed against PBS and used as an immunogen for the production of monoclonal and polyclonal antibodies.

A female New Zealand white rabbit was first immunised with recombinant monomeric HLA-F protein. Immunisation was tested by ELISA using the immunogen as substrate. A last boost was performed 14 days before the animal was bled out. Dilutions of serum were tested by ELISA on HLA-F, HLA-A2 and HLA-B7 recombinant proteins. At equivalent dilution, signal for HLA-F was twice as strong as that for HLA-A2, or HLA-B7. The serum also gave a strong background when used on western blot of HLA-F

transfected 293 T cell lysate. From these experiments it appeared that, as expected, the polyclonal antibodies generated were not specific and demonstrated significant cross-reactivity with other class I homologues.Thus it was decided to generate monoclonal antibodies.

4.3 Monclonal antibody production strategy

Hybridomas were produced by the fusion of myeloma cells with splenocytes following the method described by Köhler and Milstein (1975). The production and screening of hybridomas was adapted to optimise the formation of specific anti-HLA-F monoclonal antibodies, which would discriminate against MHC class I homologues. The strategy adopted is presented in Figure 4.1.

Anti-HLA-F antibodies were raised and screened against refolded HLA-F recombinant protein samples purified by gel filtration as described in Chapter 3. To limit the number of monoclonal antibodies generated which cross-reacted with other human MHC class I homologues, the animals immunised were female BALB/c mice transgenic for both HLA-B27 and human β2m (Weiss et al., 1990). Antibodies produced were tested for their reactivity to HLA-A2 and HLA-B7 recombinant proteins as a first cross-reactivity control.

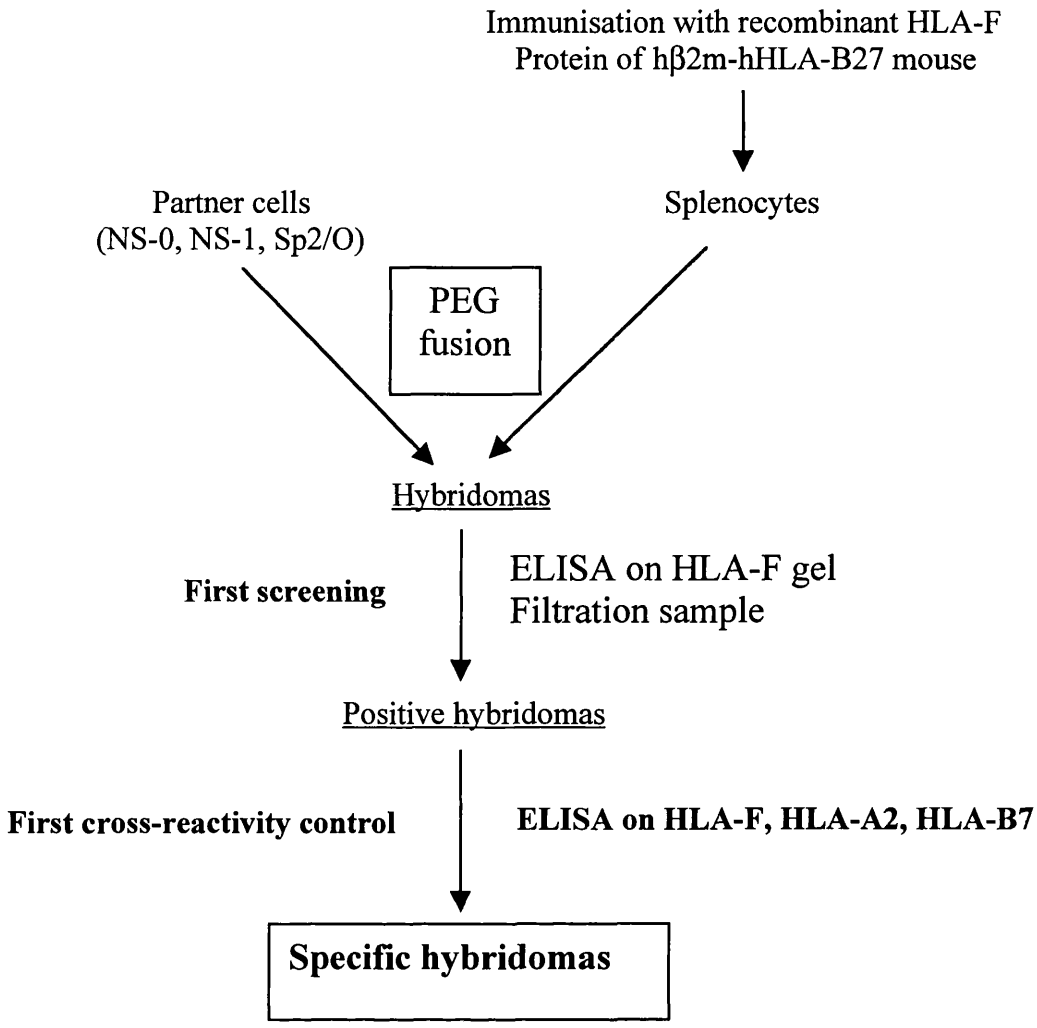

Figure 4. 1 Strategy adopted for the production of specific antibodies against HLA-F.

4.3.1 Fusion 1

NS-0 cells were fused with splenocytes of the best-responding mouse in a 1/10 ratio. The fusion was dispensed over 7 plates (96 wells/plates). 121 wells containing hybridomas were counted. Of these 121 wells, 10 were positive when culture supernatants were tested on an HLA-F recombinant protein by ELISA. The hybridomas

from these 10 wells were expended in 24 wells/plates and the supernatant tested on a batch of other HLA recombinant proteins as a first cross-reactivity screening. Three hybridomas quickly lost their expression. Screening results of the 7 hybridomas left are presented in Figure 4.2.

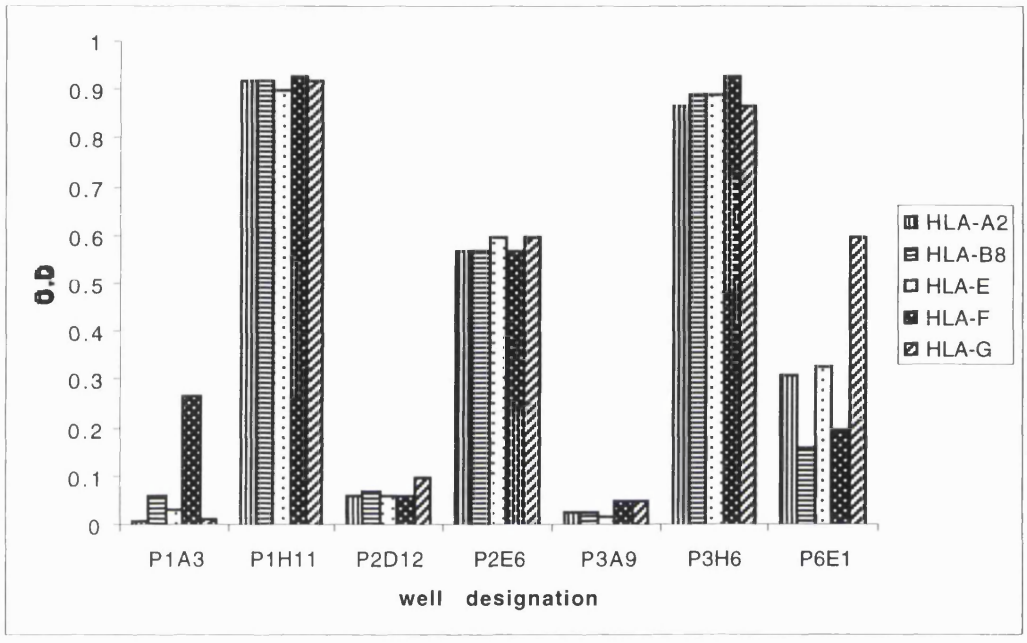

Figure 4. 2 Screening results of fusion 1.

HLA-A2, -B8, -E, -F and -G recombinant proteins were absorbed on ELISA plates and specific antibodies to each HLA molecule detected using an anti mouse Ig HRP conjugated as second antibody. O.D values presented were deducted from the negative control O.D.

In parallel to the ELISA screening, individual well culture supernatants were also tested on HLA-F transfected human cells. As presented in Chapter 3 staining of F-Myc/His transfected 293T cells with a Myc antibody has shown an intracellular expression of the

recombinant protein. 293 T cells transiently transfected with HLA-F-Myc/His and β2m or β2m alone were permeabilised and incubated with culture supernantant. Cell staining by an anti-mouse Ig FITC conjugate was then analysed by flow cytometry. The comparison of results obtained with ELISA and intra-cellular flow cytometry staining are presented in Table 4.1. Intra-cellular flow cytometry results from two supernatants are presented in Figure 4.3.

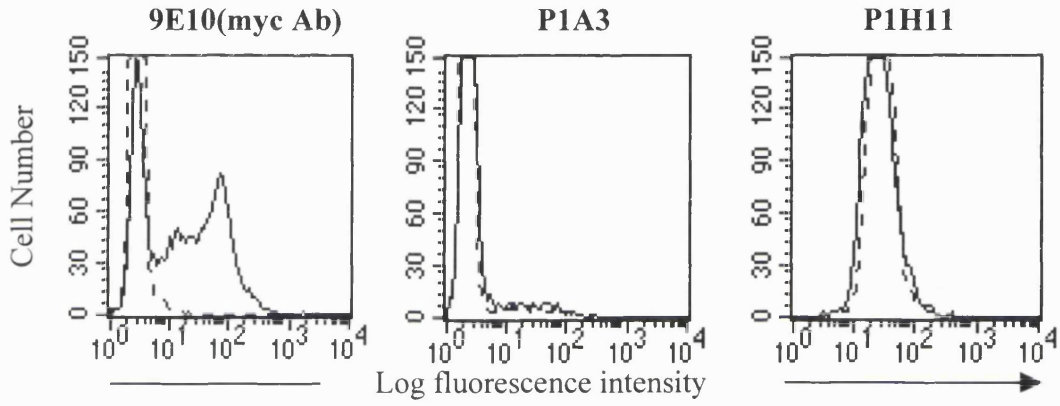

Figure 4. 3 Example cell staining on HLA-F transfected 293T cells.

Transiently transfected cells with HLA-F-myc-HIS and β2m (line) or β2m alone (dashed line) were first permeabilised and fixed before incubation with the first antibody (in bold). The second antibody used was a rabbit anti mouse FITC conjugated antibody. P1A3 was negative, and P1H11 stained all the cells.

The loss of positive hybridoma clones during the first stage of their growth is common. Our first fusion was no exception. Culture supernatants from well P1A3, and P3A9 became negative. P1A3 supernatant was the only specific hybridoma when tested on ELISA. P2D12 when grown further had a stronger signal on ELISA. P2D12, P3A9, P3H6, and P6E1were cloned and the isotype determined. Furthermore, all the antibodies

produced were of IgM isotype. This initial experiment was deemed unsuccessful and new fusions were designed to increase the efficiency of the fusion and to specifically select for Ig G antibodies.

Well designation	ELISA HLA-F	Cross-reactivity Tested by ELISA	Intra-cellular flow cytometry
P1A3	++	−	−
P1H11	++	+	+ on all cells
P2D12	+	+	−
P2E6	++	+	−
P3A9	+	+	−
P3H6	++	+	−
P6E1	++	+	−

Table 4. 1 Comparisons of the results of two screening methods.

4.3.2 Fusion 2

NS-1 cells were used as partners for the second fusion with a ratio of 1 to 4 splenocytes. Four times more wells containing hybridomas were counted (483). Culture supernatants of hybridomas containing wells were screened by ELISA on recombinant HLA-F protein, but this time the secondary antibody used was an anti mouse IgG (PIERCE, UK).

To evaluate another screening strategy, one randomly selected plate was screened using immuno-fluorescence. pcDNa 3.1 F-Myc/His transfected 293 T cells were again used. To make the screening more practical, cells were successively grown, transfected, fixed,

permeabilised and stained in the same 96 well plate. Each well was incubated with individual culture supernatant. The second antibody used was anti-mouse FITC conjugated (DAKO, UK). Positive clones were not detected by fluorescent microscopy, apart from the positive control.

From the ELISA screening 11 wells were positive when these wells were expanded and the culture supernatant was tested for cross-reactivity with HLA-A2 and HLA-B7 recombinant proteins, only four wells with the strongest signal remained positive to HLA-F (Figure 4.4). Of these 4 positives, only P8F4 and P8E6 did not cross-react. Hybridomas from the P8F4 well died. Hybridomas from well P8E6 were cloned and positive hybridomas frozen.

4.3.3 Fusion 3

The Sp2O cell line was used as partner cells. This cell line produces IL-6 which improves the yield of hybridoma. Only 7 wells containing hybridoma were counted. Culture supernatant of these wells was negative when tested on HLA-F recombinant protein by ELISA.

4.3.4 Fusion 4

The protocol used for the fourth fusion was identical to the protocol used in the second fusion. 620 wells contained hybridomas. All wells were screen by ELISA, and two culture supernatants were positive. Only the supernatant coming from the F4P7G1 well

was negative when tested by ELISA on HLA-A2 and HLA-B7 recombinant protein (Figure 4.4). F4P7G1 hybridomas were cloned and positive clones frozen.

4.3.5 Summary of fusions

Table 4.2 summarises the results obtained from the four fusions. Only the specific antibodies, FE6 and FG1 were identified. These monoclonal antibodies were selected for further characterisation.

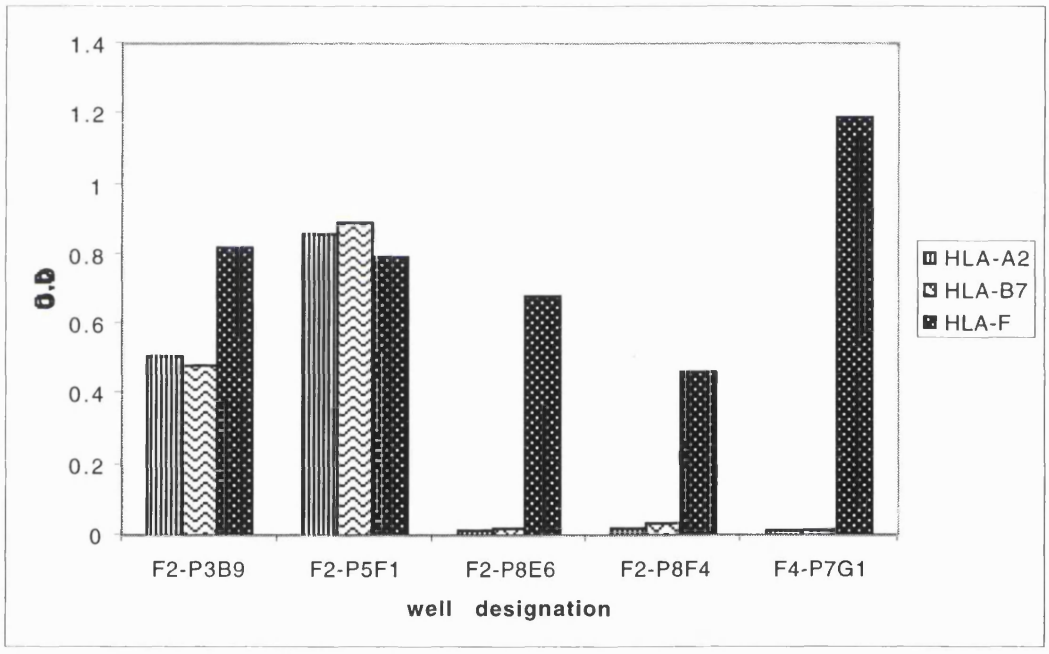

Figure 4. 4 Cross-reactivity of HLA-F positive hybridomas from fusion 2 and 4.

HLA-F recombinant proteins were absorbed on ELISA plates and specific antibodies to HLA-F detected using an anti-mouse IgG HRP conjugated. O.D values presented are deducted from the negative control O.D.

	Fusion 1	Fusion 2	Fusion 3	Fusion 4
Fusion partners	NS-0	NS-1	Sp2/O	NS-1
Ratio Partner cells / Spleenocytes	1/10	1/4	1/4	1/4
Wells Containing Hybridomas	121	483	7	620
Screening Techniques Tested	FACS	Imuno-staining FACS	_	_
ELISA Second Antibody	Anti-mouse Ig (DAKO)	Anti-mouse IgG (PIERCE)	Anti-mouse IgG (PIERCE)	Anti-mouse IgG (PIERCE)
Number of Hybridomas Produced	121	483	7	620
Positive Hybridomas	10	11	0	2
Selected Hybridomas	0	F2P8E6	0	F4P7G1
Ab (Isotype)	_	FE6 (Ig G1)	_	FG1 (Ig G2b)

Table 4. 2 Summary of the monoclonal antibody production.

Out of the four fusions performed and 1231 wells containing hybridomas, only 2 specific monoclonal antibodies have been produced FE6 and FG1.

4.4 Large scale production of monoclonal antibodies

Hybridomas from selected wells were cloned by limited dilution, and aliquots frozen in liquid nitrogen as soon as possible. The antibody from the F2-P8E6 hybridoma was named FE6, and antibody from the F4-P7G1 hybridoma was named FG1. Hybridoma clones were progressively grown until the culture volume of R10 reached four litres. At this point it was incubated at 37°C until cell death. Antibodies were purified by affinity chromatography performed on a BioCAD/SPRINT perfusion chromatography system (Perseptive Biosystem Inc., USA) with a POROS 20A column (Perseptive Biosystem Inc., USA). Typically, 5 to 10 mg of antibody were produced per litre of media, with high purity when analysed by SDS-PAGE. 1 mg of FG1 was FITC conjugated with Fluoro Tag FITC Conjugation Kit (Sigma, UK).

4.5 Characterisation of FE6 and FG1 monoclonal antibodies

FE6 and FG1 were characterised to determine their use in the study of HLA-F. The isotypes of each clone were determined, FE6 was Ig G1-Kappa and F4P7G1 was Ig G2b-Kappa. Both monoclonal antibodies were then tested for their ability to detect HLA-F by western blot, intra-cellular FACS staining and immunoprecipitation.

4.5.1 Western blotting

Western blot combines the resolution of gel electrophoresis with the specificity of immunochemical detection. It can be used to determine the presence and quantity of an antigen. 9E10, FE6 and FG1 antibodies were used to detect HLA-F tagged with a myc epitope on western blot (Figure 4.5). 9E10 could detect a single band at the expected

molecular weight (44 kDa) for HLA-F myc tagged from 293T transfected cells, and not from mock transfected cells. Cell lysates of these cells were use to make strips of membranes on which FE6 and FG1 antibodies were tested. FE6 was able to detect a single band at the expected molecular weight for HLA-F myc tagged, with nothing visible in the control lane. FG1 was able to detect faint bands, two of which were present in the negative control lane, the third which corresponded to HLA-F expected molecular weight. This experiment demonstrated that FE6 antibody was able to specifically detect the denatured form of HLA-F when expressed at high levels in 293T cells. In contrast, FG1 was not able to detect HLA-F efficiently in its denatured form, on nitro-cellulose membranes.

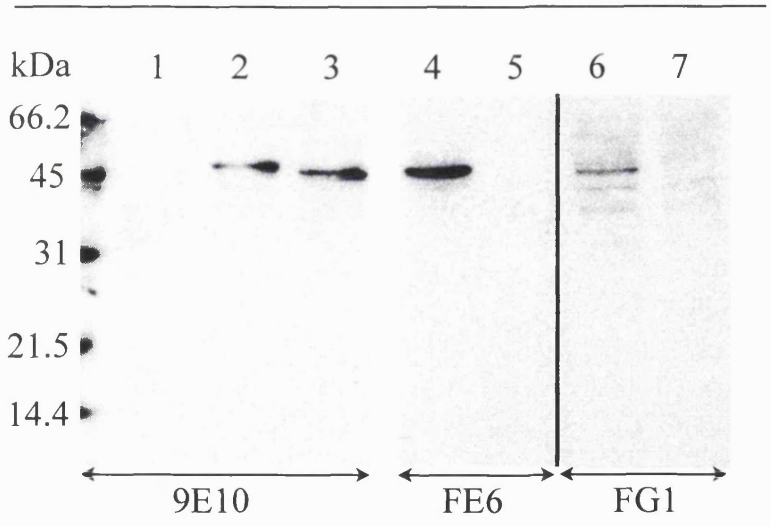

Figure 4. 5 Detection of HLA-F by Western blot.

Cell lysates of 293 T cells transiently transfected with respectively, β2m alone (lanes 1, 5 and 7), β2m and myc tagged HLA-E (lane 2), β2m and myc tagged HLA-F (lanes 3, 4 and 6) were run on two SDS-PAGE. Proteins were transfered to a membrane. 9E10 was used to detect the myc epitope (lanes 1, 2 and 3). FE6 (lanes 4 and 5), and FG1 (lanes 6 and 7) were tested for HLA-F detection. The second antibody was an anti mouse HRP (DAKO, UK). Only FE6 clearly detect HLA-F on Western blot.

4.5.2 Cell staining

Labelled antibodies can be used to detect the presence or absence of an antigen in cells or tissues, and can also determine the sub cellular localisation of an antigen. Staining of F-Myc/His transfected 293T cells with a Myc antibody showed an intracellular expression of the recombinant protein (Chapter 3). To test the ability of the anti-HLA-F monoclonal antibodies to detect the HLA-F molecule in mammalian cells, we again used 293 T transfected cells. 293 T cells transiently transfected with HLA-F-Myc/His were permeabilised and fixed at the same time. These cells were incubated with FE6, FG1, and 9E10 (myc) antibodies or isotype control. The second antibody used for detection was an FITC conjugated anti-mouse. Staining was analysed by flow cytometry (Figure 4.6). 9E10 antibody detected transfected cells. No cross reactivity was detected when FE6 and FG1 were used to stain mock transfected cells compared to the isotype control. However, FE6 and FG1 stained a population of transfected cells. Both antibodies (FG1 and FE6) could specifically detect HLA-F protein in transfected cells by intracellular flow cytometry staining. HLA-F recombinant protein staining with 9E10 antibody was of higher intensity and involved a higher number of cells compared with the staining with FE6 and FG1. When FG1 and FE6 were also used for tissue section staining, only FG1 was able to specifically stain frozen tissue sections but not paraffin fixed tissue sections.

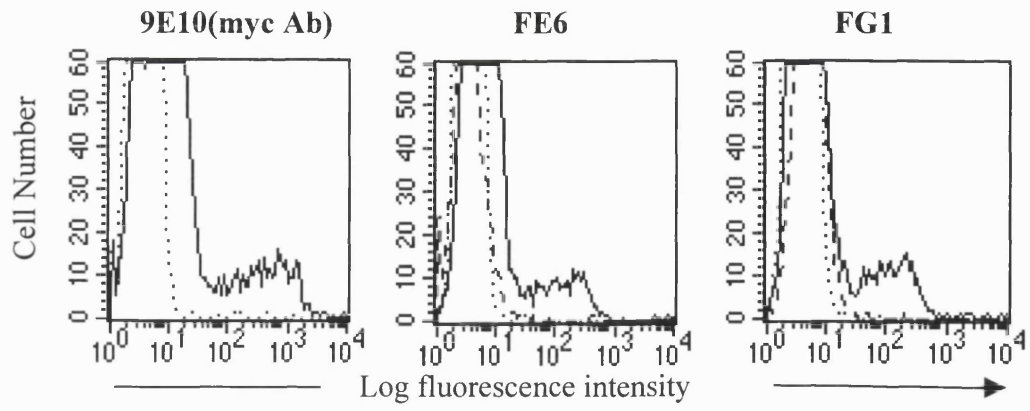

Figure 4. 6 FE6 and FG1 specifically detect HLA-F in transfected 293T cells.

Transiently transfected cells with β2m (dashed line) or HLA-F-myc-HIS (line) were first permeabilised with saponin before incubation with the first antibody (in bold). The second antibody used was a rabbit anti-mouse FITC conjugated antibody (DAKO). Isotype DAKO control was also used to stain transfected cells (dotted line)

4.5.3 Immunoprecipitation

Immunoprecipitation is a useful immunochemical method. When coupled with gel electrophoresis techniques, immunoprecipitation can determine the presence of post-translational modifications and interaction with ligands that are difficult to determine using other techniques. FE6 and FG1antibodies were used to immunoprecipitate HLA-F from untransfected and transfected cell lines (Figure 4.7) One dimensional iso-electric focusing of immunoprecipitates from 293T cells transfected with HLA-F identified two strong bands in transfected cells, but not in mock transfected cells. A further band was also observed migrating at the pI of β2m. These two bands, or doublets, were present after immunoprecipitation from LCL 721.221 cells with FG1 or w6/32 antibodies. In

the same conditions doublets have previously been reported with other class I proteins, but there is not any proven explanation for this phenomenon. To confirm further the identity of the precipitated protein, HLA-F was tagged with a Myc epitope sequence, which appropriately altered the migration of the two HLA-F heavy chain bands. Unlike w6/32, FG1 did not immunoprecipitate HLA-E.

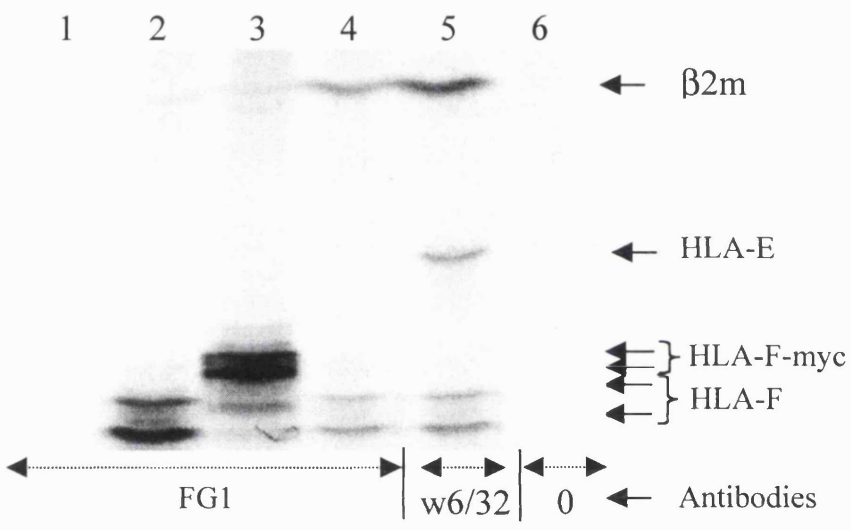

Figure 4. 7 FG1 can immunoprecipite HLA-F from metabolically labelled cell lines.

FG1 was used to immunoprecipitate HLA-F from 293 T cells transfected respectively with β2m alone (lane 1), or β2m and HLA-F (lane 2) or β2m F-Myc/His (lane 3). Untransfected LCL 721.221 cells were subjected to immunoprecipitation respectively with FG1 (lane 4), w6/32 (lane 5) or isotype control (lane 6). Isoelectric focusing was then used to analyse the proteins immunoprecipitated.

FE6 was used in an identical experiment. The two bands identified as HLA-F were also present when FE6 antibody was used to immunoprecipitate HLA-F protein from 293T cells transfected with HLA-F. However, FE6 did not efficiently immunoprecipitate HLA-F from 721.221 (Figure 4.8).

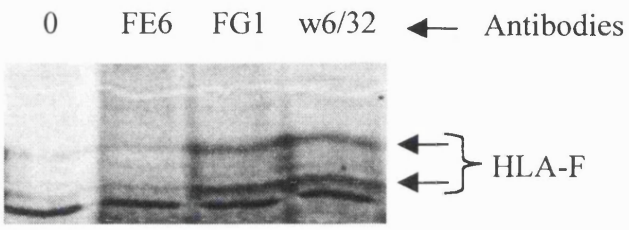

Figure 4. 8 FE6 can not immunoprecipitate HLA-F from metabolically labelled cell lines.

721.221 cells were metabolically labelled and protein immunoprecipitated with no antibodies (0), FE6, FG1 or w6/32 antibodies. Isoelectric focusing was then used to analyse the proteins immunoprecipitated.

FG1 monoclonal antibodies were tested for their specificity to HLA-F among other MHC class I molecules. Immunoprecipitation with FG1 from cell lines expressing different HLA haplotypes does not reveal any cross-reactivity with any other class Ia molecules expressed by the cell lines tested (Chapter 5). Finally, FG1 did not stain HLA-E and HLA-G transfected cell lines when analysed by flow cytometry. Thus no cross reactivity of FG1 antibody with other MHC class I molecules has been detected.

4.6 Discussion

The strategy of anti-HLA-F monoclonal antibody production was based on the generation of the HLA-F ectodomain from recombinant proteins expressed in *E.coli*, as discussed in Chapter 3. The removal of the trans-membrane and cytoplasmic domains was necessary to allow refolding, but also to allow the production of monoclonal antibodies. The trans-membrane region would not be accessible to antibodies and the cytoplasmic region is very short and of limited divergence compared with other class I molecules. Another important aspect of the immunisation strategy was the use of HLA-B27 and human β2m transgenic mice. This aimed to limit the number of cross-reactive antibodies to other HLA molecules. However, this strategy also potentially makes HLA-F recombinant protein less immunogenic. Indeed, a very low number of hybridomas were produced for each fusion and few antibodies recognised HLA-F. We attempted to improve the immunisation protocol by increasing the number of injections and the gap between injections, but without success. In addition, cross-reactive antibodies detected by ELISA and flow cytometry staining were still produced.

The production of specific anti-HLA-F monoclonal antibodies is a key determinant in the study of HLA-F function. Different modifications of the fusion protocol and the screening strategy were also tested to improve the monoclonal antibody production. The fusion protocol was improved using new partner cells and by modifying the ratio of partner cells/splenocytes. The best results were obtained using NS-1 partner cells at a ratio of 1 to 4 splenocytes. In these conditions 620 wells containing Hybridomas were obtained in fusion 4.

The screening method determined the characteristics of the antibodies generated. It would have been ideal to base the screening method on the technique designed to study HLA-F. We wanted to produce antibodies which were able to specifically recognise HLA-F expressed *in vivo*. However, little information was available on HLA-F protein expression *in vivo*. In addition, it was not possible to detect recombinant HLA-F proteins at the cell surface of F-Myc/His transfected 293T cells.

Two methods of screening were tested. Culture supernatants were tested on HLA-F recombinant proteins by ELISA and on HLA-F transfected 293T cells by intracellular immuostaining. Immunostaining of transfected cells proved to be impractical and unsuccessful. Finally, we decided to use the recombinant protein for immunisation as well as for the principal screening. Using this strategy, we ensured selection of antibodies produced by immunisation. ELISA was chosen as a rapid and reliable technique to screen antibodies against recombinant HLA-F. It was a concern that the direct absorption of HLA-F on plastic might alter the epitope availability however, specific monoclonal antibodies were produced from recombinant HLA-F.

Two specific monoclonal antibodies to HLA-F were eventually identified, FE6 and FG1, the properties of these antibodies are compared in Table 4.3. FE6 was able to detect HLA-F on western blotting but could not immunoprecipitate HLA-F from 721.221 cell lines, suggesting that it is a low affinity antibody. On the contrary, FG1 was able to immunoprecipitate HLA-F in a complex with β2m from 721.221 cells but was unable to detect HLA-F heavy chain in its denatured form by Western blot. FG1,

could also immunoprecipitate HLA-F heavy chain without β2m. FG1 cross reactivity with other class I molecules was excluded by screening against a panel of recombinant proteins, flow cytometry, and by immunoprecipitation associated with IEF.

	FE6	FG1
Isotype	Ig G1	Ig G2b
Western blot	+	–
Immunochemistry	+/–	+
Immunoprecipitation	+/–	+

Table 4. 3 Summary of FE6 and FG1 monoclonal antibody properties.

The use of peptide fragments typically leads to the generation of lower affinity antibodies. A peptide fragment of HLA-F was used by Wainwright et al. to produce a specific monoclonal antibody which only detected the denatured form of HLA-F (Wainwright, Biro and Holmes, 2000). In our study, the use of recombinant protein as an immunogen and for antibody screening allowed the production of a specific antibody (FG1) able to directly detect and purify HLA-F from human B cell lines. FG1 was able to immunoprecipitate HLA-F in association with β2m and could specifically detect HLA-F in cell lines and tissue sections.

Chapter 5 - HLA-F : Immunochemical Studies

5.1 Introduction

Chapter 4 described how HLA-F molecules were generated from recombinant proteins produced in bacteria, and how these proteins were subsequently used to produce two specific monoclonal antibodies, FE6 and FG1. This chapter presents the direct study of the HLA-F protein using these two monoclonal antibodies.

The function of MHC class Ia proteins is to present endogenous or viral peptides to cytotoxic T cells. HLA-E and HLA-G have evolved specialised immunological functions and also participate in immunological recognition. However, expression of HLA-F recombinant proteins in mammalian cell line experiments presented in Chapter 3 and previous studies (Shimizu et al., 1988) do not demonstrate HLA-F at the cell surface. A recognition role for HLA-F is difficult to conceive if HLA-F does not reach the cell surface.

The pattern of HLA-F protein expression was studied first. Cell lines and tissue sections were screened for HLA-F protein using different immunochemistry techniques. Cell lines showing expression were further investigated for HLA-F interactions with the classical class I pathway molecules, in particular TAP (transporter associated with antigen processing) molecules.

The antigen presenting function of class Ia molecules is highly dependent on their interaction with TAP molecules. Thus, an experiment using specific monoclonal antibodies to HLA-F and TAP molecules was designed to determine the potential interaction between these two molecules. Regulation of HLA-F expression and cellular localisation was also investigated.

Previous Analysis of the HLA-F promoter suggested that, like MHC class I molecules HLA-F expression levels could be up-regulated by IFNγ (Gobin et al., 1999). EBV (Epstein-Barr virus) transformed B cells with constitutive HLA-F expression were tested for increased expression and for cell surface expression after IFN-γ treatment. In addition, the effect of growth at low temperatures on HLA-F cell surface expression was investigated.

5.2 Expression of HLA-F proteins in cell lines

The first general study of the HLA-F transcript was performed using an RNase protection assay (Geraghty et al., 1990). HLA-F was detected at higher levels in B and T cells compared to non-lymphoid cells. In cell lines, expression was observed in HUT-78 (cutaneous T cell leukemia) but not in Molt-4 (T cell leukemia) (Geraghty et al., 1990). In tissue, HLA-F transcript was detected in the skin and not detected in adult liver. The first results indicated that HLA-F expression was tissue or cell specific. More recently, HLA-F transcript was discovered in both first and second trimester foetal liver, but undetected in fibroblasts, myelomonocytic leukaemia cells, placental trophoblast cells, and Jurkat (a T cell leukemia) (Houlihan et al., 1992; Lury, Epstein and Holmes,

1990). FE6 and FG1 were thus used to detect HLA-F in a range of cell lines and tissue sections including lymphoid cells and lymphoid tissues.

5.2.1 Western blotting with FE6

FE6 was the first monoclonal antibody specific for HLA-F to be produced. FE6 could not detect HLA-F in non-transfected 721.221 cells by immunoprecipitation, but FE6 could detect HLA-F protein from HLA-F transfected 293 T cell lines by Western blot. Thus Non-transfected cell lines derived from lymphoid tissues and non-lymphoid cell lines were grown, and FE6 was used to study HLA-F protein expression by Western blot (Figure 5.1). 10^5 cells were lysed and analysed for each cell line. In addition, cell membrane prepared by centrifugation of lysed EBV transformed B cell line (the TN B cell line) and Jurkat cell line were also analysed. 293T cells transfected with the untagged version of HLA-F were used as a positive control. Jurkat cells, for which no HLA-F transcript was detected, was used as negative control (Lury, Epstein and Holmes, 1990). A band of identical molecular size to the positive control was only detected from the B cell line (TN BCL) lysate and a membrane preparation. This experiment suggested that HLA-F proteins were expressed as a membrane protein in B cell lines. No expression was detected in Jurkat (T cell Leukemia), U937 (histiocytic lymphoma), astrocytoma, COS-1 (monkey kidney fibroblast) or THP-1 (monocytic leukemia) cell lines. Bands of lower molecular weight were observed in astrocytoma, Jurkat and the B cell lines. These bands were also present in the negative control and the Jurkat cell line corresponded to background.

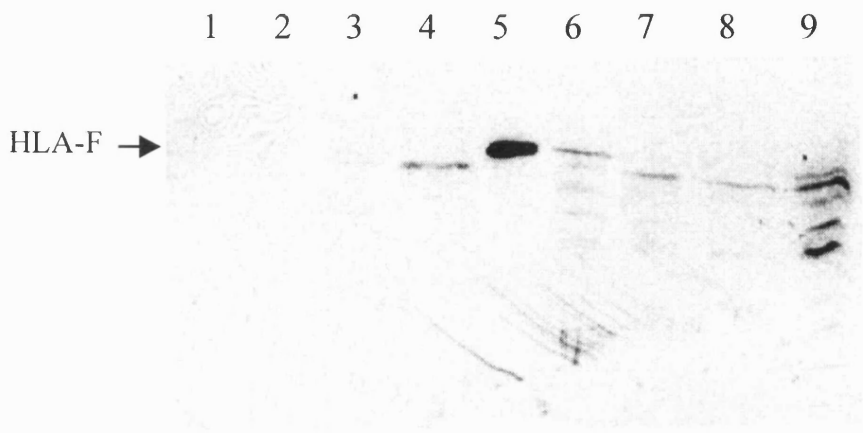

Figure 5. 1 HLA-F could be detected by FE6 antibody in a B cell line by Western blot.

10^5cells were lysed in NP-40 buffer and loaded in each lane. The cell lines tested were COS-1 (lane 1), THP-1 (lane 2), U937 (lane 3), astrocytoma (lane 4), Jurkat (lane 8), TN BCL (lane 9). Membrane preparations of two cell lines, TN BCL (lane 6) and Jurkat (lane 7) were also tested. In lane 5 the positive control was 293T cells transfected with the untagged version of HLA-F. FE6 detected HLA-F protein in the TN BCL membrane preparation lane 6.

5.2.2 Immunoprecipitation with FG1

FG1, another monoclonal antibody specific to HLA-F was produced after FE6. FG1 was able to directly detect HLA-F by immunoprecipitation or immunohistochemistry, as shown in Chapter 4. Immunoprecipitation of HLA-F with FG1, a specific monoclonal antibody, combined with IEF gel separation allows formal identification of the HLA-F heavy chain as a doublet.

Preliminary screening for HLA-F expression was performed by using FG1 to immunoprecipiate HLA-F from metabolically labelled cell lines. The two bands characteristic of HLA-F when analysed on iso-electic focusing gel, were previously detected for 722.221, (Figure 4.7). We also detected HLA-F expression in JY BCL, T2, and HUT-78 a T cell line (Figure 5.2). Further observations could be made from these gels. Firstly, bands corresponding to the class I molecule immunoprecipitated with w6/32 were not present when FG1 was used, except for the two bands corresponding to HLA-F and the band corresponding to β2m. These further demonstrate the specificity of FG1 to HLA-F among class Ia molecules. Secondly, FG1 immunoprecipitated HLA-F heavy chain without β2m from T2 cells. Finally, the level of expression of HLA-F is very low compared to the other class Ia molecules.

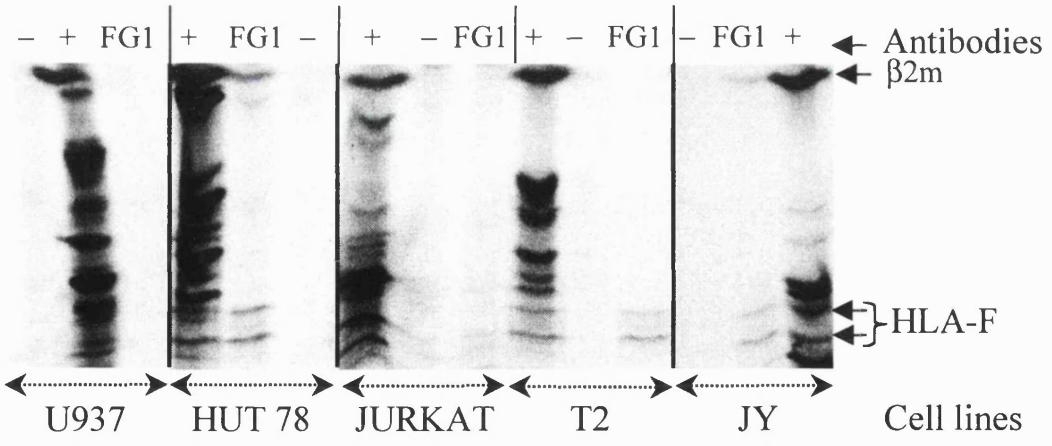

Figure 5. 2 HLA-F is expressed in B cell lines and a T cell line, HUT 78.

Cell lines were metabolically labelled, and proteins immunoprecipitated with FG1, w6/32 (+) or Isotype control (-). Samples were analysed by iso-electric focusing gel.

5.2.3 Immunocytochemistry

FG1 was able to specifically and directly detect HLA-F by immunohistochemistry. Fresh cells from different cell lines were immobilised on slides, then permeabilised and fixed with acetone. HLA-F was detected by immunoperoxidase staining using FG1 as a primary antibody. Using immunoperoxidase staining HLA-F was not detected in a range of human cell lines including K562 (chronic myelogenous leukemia), THIEL (plasma cell), Jurkat (T cell leukemia), HEL (erythroleukemia), RH-30 (rhadbomyosarcoma), KMH2 (Hodgkin's lymphoma), REH (pre B lymphoma), A431 (epidermoid carcinoma) and Nalm-1 (chronic myeloid leukemia). HUT 78 (cutaneous T lymphoma), which expressed the lowest level of HLA-F when detected by immunoprecipitation, and LCL 721.221 were both used as positive controls (Figure 5.3). Staining in positive cells was predominantly cytoplasmic. These experiments were carried out in collaboration with the Department of Cellular Science, Oxford University.

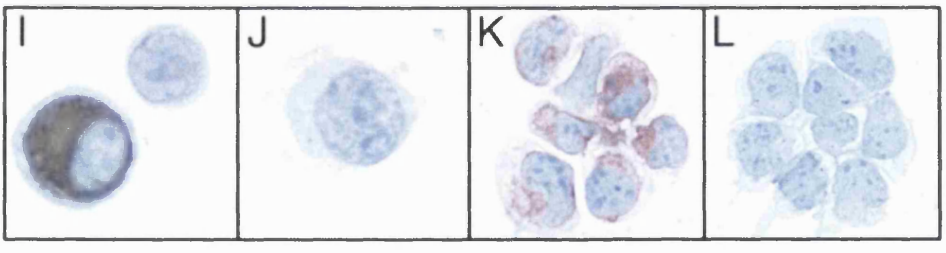

Figure 5. 3 Intracellular staining of LCL 721.221 and HUT-78 cell lines.

Peroxidase staining of LCL 721.221 cells (I, J) and HUT-78 (K, L) at high power. (J) and (L) are negative staining controls performed without specific antibody.

5.2.4 Summary of HLA-F distribution in cell lines

In summary, HLA-F was only detected in B cell lines and HUT 78 a human cutaneous T cell lymphoma. FG1 detected HLA-F by immunoprecipitation from all EBV transformed B cells tested including JY, TH, TN, NAN and 721.221 a LCL. HLA-F was also immunoprecipitated from T2, a B and T cell Hybrid and HUT 78 a human cutaneous T cell lymphoma.

HLA-F could not be detected in Jurkat cells (acute T cell leukemia) and histiocytic lymphoma cells (U937), K562 (chronic myelogenous leukemia), THIEL (plasma cell), HEL (erythroleukemia), RH-30 (rhadbomyosarcoma), KMH2 (Hodgkin's lymphoma), REH (pre B lymphoma), A431 (epidermoid carcinoma) and Nalm-1(chronic myeloid leukemia).

5.3 Tissue distribution of HLA-F

Immunoperoxidase techniques using FG1 as a first antibody were able to specifically stain cell lines which have been shown to express HLA-F. To extend this research, the same technique was used to detect HLA-F in tissue sections (Figure 5.4). Post-mortem and surgically removed tissues were obtained from the Histopathology Department of the John Radcliffe Hospital, Oxford, UK. Staining was found to be negative within the small bowel, kidney, adult and foetal liver, brain, oesophagus, prostate and skin tissue sections (not shown). Restricted expression patterns were observed in tonsil, spleen, and thymus. In the tonsil, positively stained cells were mainly concentrated within the mantle zone. Small cells were stained in the T cell area, but no staining was observed in

germinal centres. Positive staining was also observed in endothelial cells of the tonsil. Within the spleen, the principal feature was strong staining of endothelial cells (splenic sinusoids). Small cells in the lymphoid area were also positively stained, but there was no staining of the mantle zone or vessels. The thymus showed weak staining restricted to a heterogeneous population of cells within the medulla. There was no staining in the cortical area nor in endothelial cells. Staining was predominantly cytoplasmic in all stained cells.

5.4 TAP interaction

Class Ia molecules bind peptides resulting from protein degradation in the cytosol. The association of the class I heavy chain, β2-microglobulin, and peptide to form stable class I molecules occurs in the endoplasmic reticulum. A number of chaperone proteins are involved in the formation of stable class I molecules. The TAP (transporter associated with antigen processing) molecule has a central role in this process. Class I molecules associate with the TAP multicomplex and are retained in the endoplasmic reticulum until released by the binding of peptide. The resulting stable complex is then transported to the cell surface. Class I molecules which do not bind peptide will be retained in the endoplasmic reticulum until they are eventually degraded.

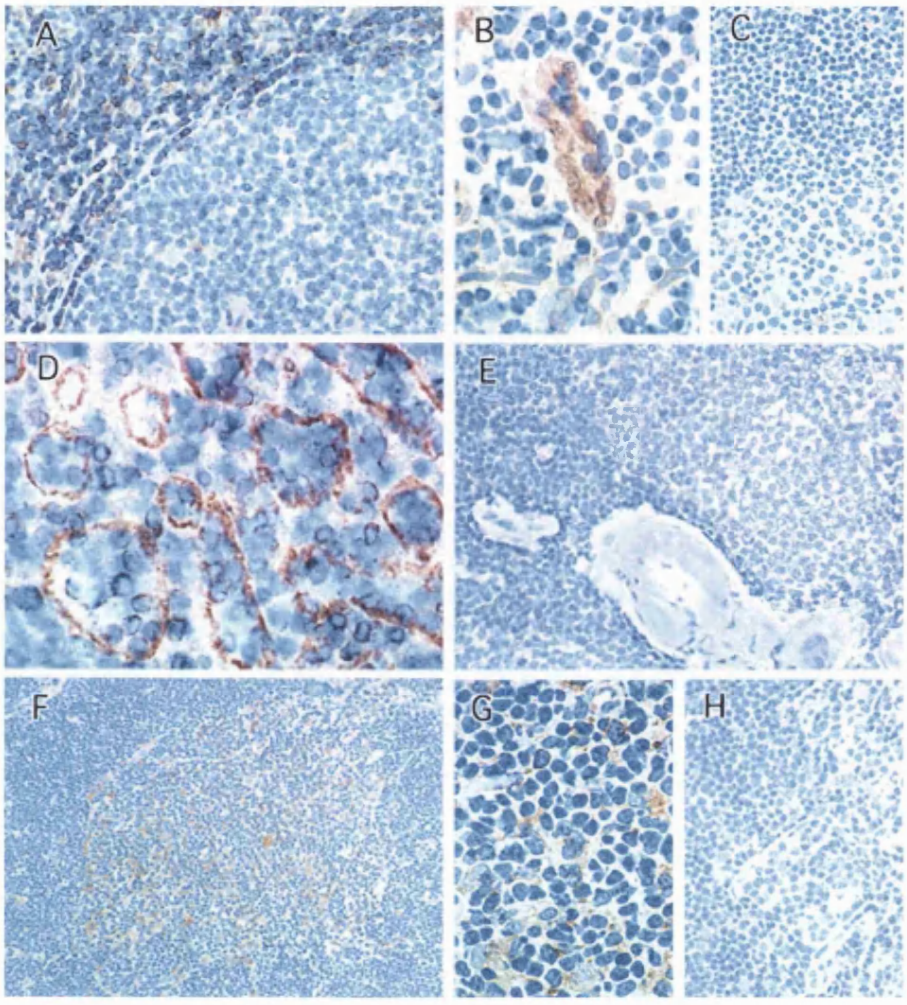

Figure 5. 4 Tissue distribution of HLA-F.

Peroxidase staining of tonsil tissue sections (ABC). (A) germinal centre and mantle zone, low power. (B) vessel, high power. (C) negative control, low power. Peroxidase staining of spleen tissue sections (D, E). (D) Lymphoid area, low power. (E) negative control, low power. Peroxidase staining of thymus tissue sections (F, G, H). (F) medulla, low power. (G) medulla high power. (H) negative control.

To investigate the possible association of HLA-F with TAP molecules, TAP-1 was immunoprecipitated from digitonin lysates of LCL 721.221 (B cell line) and T2 (TAP negative T cell line) cells. The immunoprecipitated proteins were then analysed by iso-electric focusing (Figure 5.5). We previously demonstrated that HLA-F was expressed in both cell lines. Two bands corresponding to HLA-F were co-precipitated from LCL 721.221 cells, but not from the TAP negative cell line T2. To verify that those two bands were indeed HLA-F, the immunoprecipitate was transferred to NP40 lysis buffer to destabilise any intermolecular associations. When a second immunoprecipitation was performed with FG1, HLA-F was recovered. These observations demonstrate that HLA-F interacts with the TAP peptide transporter in LCL 721.221. Other bands were detected after co-immunoprecipitation of TAP molecules. One band corresponded to HLA-E and an unidentified band between the band corresponding to HLA-F and HLA-E, was also detected. However both bands were undetected after immunoprecipitation by FG1.

5.5 Cellular Localisation of HLA-F molecule

It has been shown that in B cell lines HLA-F proteins are expressed and translocated in the endoplasmic reticulum where it associates with TAP molecules. Following on from this we investigated cellular trafficking and the cell surface expression of HLA-F. In Chapter 3, 9E10 (Myc) antibodies were used to detect HLA-F in 293T or Hela cells transfected with a Myc/His tagged HLA-F. Immunostaining of transfected cells showed predominant intracellular localisation of the tagged HLA-F. The production of a

specific antibody to HLA-F allowed the study of HLA-F maturation under physiological conditions.

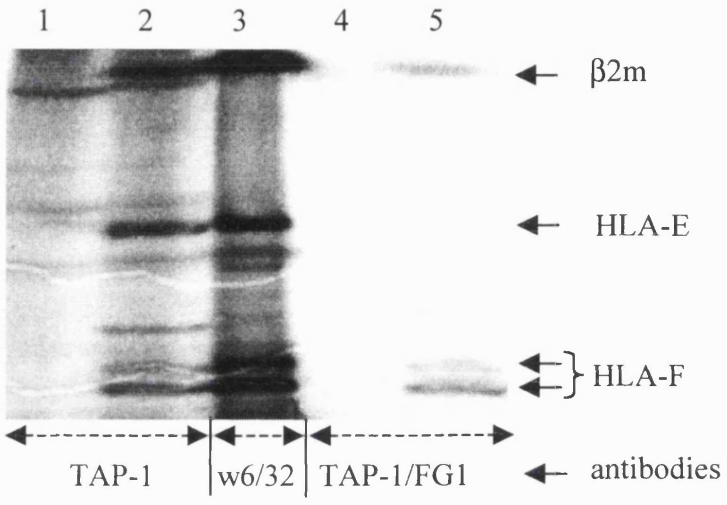

Figure 5. 5 HLA-F interacts with TAP-1.

Immunoprecipitates were analysed by iso-electric focusing

Lanes 1 and 2: TAP-1 co-immunoprecipitations were performed on T2 cells (lane 1) and LCL 721.221 cells (lane 2) in digitonin . The anti humanTAP-1 antibody used was 148.3 (Hughes and Cresswell, 1998). Lane 3: HLA-E and HLA-F were immunoprecipitated with w6/32 from LCL 721.221 cells to determine precisely their positions on the gel. Lane 4 and 5: immunoprecipitation with FG1 of HLA-F from the TAP-1 co-immunoprecipitated sample (as represented in lane 1 and 2) from T2 cells (lane 4) and LCL 721.221 cells (lane 5). This experiment demonstrates that HLA-F is associated with the TAP-1 molecule.

No cell surface staining was observed when FG1 was used to detect HLA-F on 721.221 cells or EBV transformed B cells, cells which have been shown to express HLA-F. Using the same antibody, immunostaining of HUT-78, 721.221 and tissue sections also showed predominantly cytoplasmic staining (Figure 5.3). All these experiments suggest that HLA-F does not reach the cell surface under the conditions studied.

The study of HLA-F trafficking after its synthesis in the cytosol is important in the determination of HLA-F function, as it might not follow the usual class I pathway or might diverge from it. HLA-F has a single N-linked glycosylation site, an asparagine in the α1 domain at position 86. The trafficking of a molecule can be studied by monitoring N-linked glycosylation which is initiated in the ER and completed in the Golgi. As soon as a protein is exposed in the lumen of the ER, an oligosaccharide is attached to the N-linked glycosylation site. Subsequent, trimming of this oligosccharide arises quickly in the ER generating a high mannose oligosaccharide. Subsequently the transit from the cis to the trans Golgi results in further modifications leading to complex oligosaccharides. Tests to determine whether a glycoprotein has left the ER are based on the two last modifications occuring in the Golgi. In the medial Golgi the oligosaccharide becomes resistant to degradation by endoglycosidase H (Endo H). Finally in the trans Golgy there is addition of further residues (Sialylation) which change the charge of the molecule.

We looked for siaylated forms of HLA-F in pulse chase experiments. HLA-F was immunoprecipitated from these cells with FG1 and analysed by iso-electric focusing

(Figure 5.6). The appearance of sialylated forms of class Ia molecules are usually observed after 15 to 30 minutes. However, no additional bands were observed for HLA-F even after a four hour pulse, suggesting that HLA-F could not be efficiently transported from the endoplasmic reticulum to the Golgi under the conditions employed.

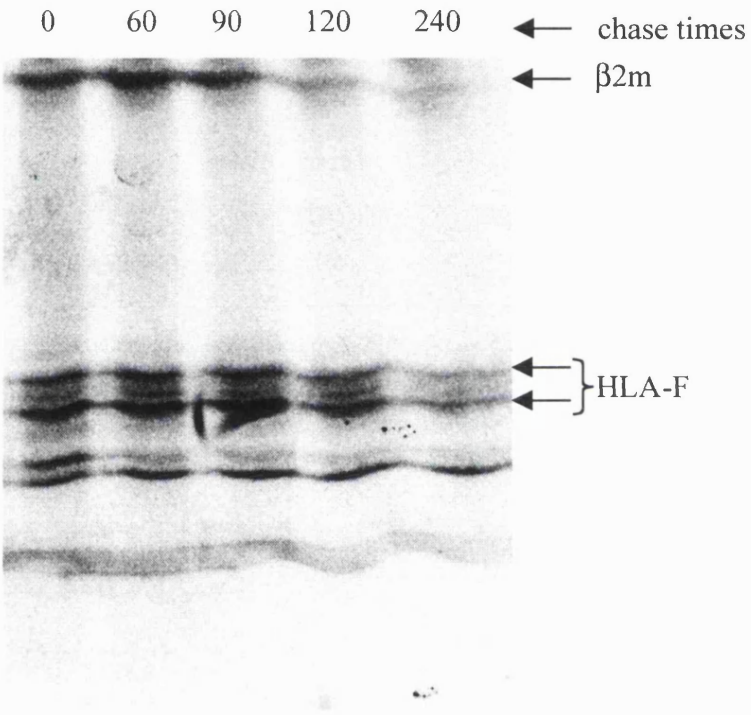

Figure 5. 6 Analysis of HLA-F maturation in B cells.

Following a 15 minute pulse with ^{35}S methionine, NAN-BCLs were harvested after the chase times shown at the top of the gel (in minutes) and HLA-F was immunoprecipitated with FG1 mAb and analysed by iso-electric focusing

5.6 Low temperature cell growth

The antigen processing pathway can be altered, and it has been shown that cell surface expression of class Ia molecules can be induced at low temperatures in the absence of peptides (Ljunggren et al., 1990). However, this observation is not universally true, as H2-M3, a mouse class Ib molecule is not expressed at the cell surface in the absence of peptide even when the cells are grown at 26°C (Chiu et al., 1999).

To determine if surface expression of HLA-F was promoted in the cold and could be detected by FG1, 721.221 cells were cultured overnight at a range of temperatures from 24°C to 37°C. FG1, w6/32 and the isotype control were used to stain the cells and cell surface expression was analysed by flow cytometry. A small but detectable increase in fluorescence could be seen when these cells were grown at 26 °C compared to 37°C (Figure 5.7). The increase in staining was strongest for w6/32, weaker for DT9, and even weaker with FG1. Flow cytometry results were further confirmed by immunoprecipitation of HLA-F from I^{125} radio labelled 721.221 cell surface, grown at 37°C or 26°C again using FG1 antibodies (Figure 5.8). A very small amount of class I was present at the cell surface of 721.221 cells grown at 37°C. At 26°C, more class I molecules could be immunoprecipitated, and FG1 immunoprecipitated a molecule of molecular weight corresponding to HLA-F heavy chain. However, the β2m band was not seen. These results demonstrate that a very small amount of HLA-F heavy chain reaches the cell surface in the cold. The effect was so small that terminal glycan modifications (sialylation) were not detected when pulse chase experiments were conducted at 26°C rather than 37°C (data not shown).

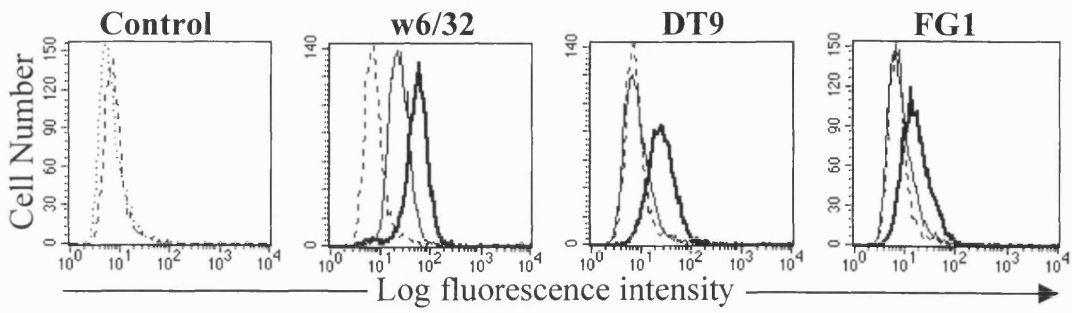

Figure 5. 7 Limited HLA-F cell surface staining of 721.221 cells grown at 26°C.

721.221 cells were grown for 24h at 37°C (thin line) or 26 °C (thick line). Class I molecules, HLA-E and HLA-F were respectively detected with w6/32, DT9 and FG1. The second antibody was an anti-mouse Ig (DAKO,) PE- conjugated. Staining with only the second antibody of cells grown at 37°C (dotted line) and 26°C (dashed line) were used as controls.

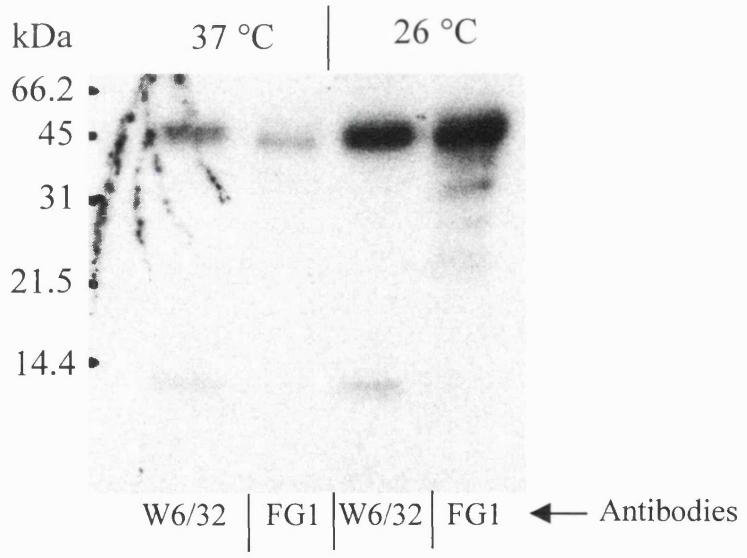

Figure 5. 8 Class I expression at the cell surface of 721.221 cell grown at 26 °C.

721.221 cells were grown overnight at 37 or 26 °C, surface-labelled by iodination and lysed. Labelled proteins were immunoprecipitated with w6/32 or FG1 and analysed on SDS-PAGE. Film was exposed for 11 days at –70°C.

5.7 IFN-γ does not enhance cell surface expression of HLA-F

Interferon (IFN)-γ is a key regulator of MHC class I expression and acts via intermediary transcription factors on upstream elements in the human MHC class Ia genes (Gobin et al., 1999). The upstream region of the HLA-F gene encodes a potential IFN-γ-stimulated response element (ISRE) (Gobin et al., 1999). We thought to establish whether IFN-γ could upregulate the surface expression of HLA-F. Cells were treated with IFN-γ and the surface expression of MHC molecules examined by flow cytometry of permeabilised and non-permeabilised cells stained with the antibodies w6/32, a pan class I antibody and FG1, a specific HLA-F antibody (Lepin et al., 2000). There was a strong increase in the overall level of MHC class I molecule expression as detected with the anti-class I antibody w6/32 in HeLa and U937 cells, confirming the biological activity of the IFN-γ exposure. Western blotting detected a minimal increase in total cellular HLA-F protein levels following IFN-γ treatment of B cell lines which express HLA-F (Figure 5.9). However, in multiple experiments we could not detect any increase in cell surface expression of HLA-F in response to IFN-γ in such B cell lines.

5.8 HLA-F associated proteins

HLA-A2 B cell line (TH-BCL) was used to compare protein associated with HLA-A2 and HLA-F (Figure 5.10). Like HLA-A2, HLA-F appears to interact with a number of proteins. Identification of these proteins is currently being undertaken in collaboration with the laboratory of Professor Peter Cresswell.

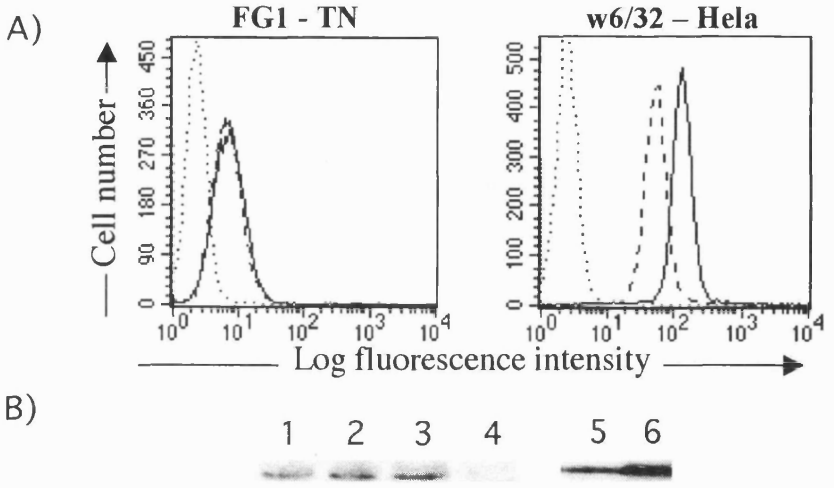

Figure 5. 9 Effects of IFN-γ on HLA-F expression in B cells

(A) Flow cytometry. TN BCL and HeLa cells were treated or mock treated for 24h with 500U/ml of IFN-γ. Cells were then permeabilised and stained with the antibodies FG1 or w6/32 for flow cytometry. The secondary antibody was phycoerythrin-labeled anti-mouse Ig (DAKO, CA). The dotted line represents cells stained with the secondary antibody alone. Cells treated with IFN-γ are shown with a continuous thin line and untreated cells are shown with a dashed line. There was no substantial increase in HLA-F expression as detected by FG1 staining when cells were treated with IFN-γ, although as expected there was an increase in w6/32 staining reflecting the induction of other class I molecules.

(B) Western blotting.The effect of IFN-γ treatment on HLA-F expression was analysed by Western blotting with anti-HLA-F. In (B) TN B cell lines were left untreated (lane 1) or treated for 24hours with 250U/ml of IFN-γ (lane 2) or with 500U/ml of IFN-γ (lane 3). Untreated Jurkat cells were loaded in lane 4. Minimal weak induction of HLA-F expression was detected with this sensitive approach. As a postive control for the biological activity of the IFN-γ in the assay conditions, U937 cells were treated for 24hours with 500U/ml of IFN-γ (lane 6) or untreated (lane 5) and stained with the monoclonal anti-HLA-A and HLA-B heavy chain antibody HC-10.

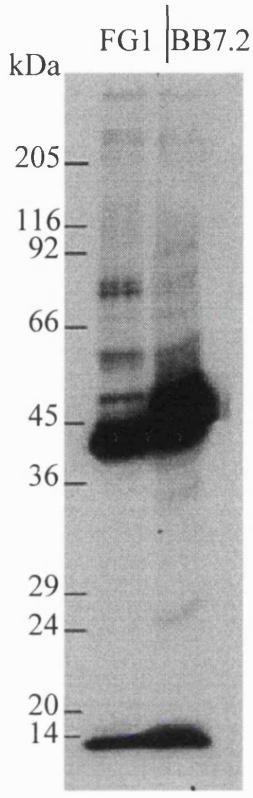

Figure 5. 10 Coimmunoprecipitation of protein associated with HLA-F.

Metabolically labelled HT B cell lines were lysed in digitonin and protein associated with HLA-A2 or HLA-F immunoprecipitated. HLA-F and HLA-A2 were respectively immunoprecipitated with FG1 and BB7.2 monoclonal antibodies.

5.9 Discussion

The investigation of the HLA-F protein was carried out using FE6 and FG1, two monoclonal antibodies described in Chapter 4. Initial investigation concerned the pattern of expression of HLA-F in different cell lines. Previous studies of HLA-F transcript have indicated a very low level of expression in general, with the highest

level of expression in B cell lines (Lury, Epstein and Holmes, 1990). This suggests that the detection of HLA-F would need a particularly good quality antibody and sensitive technique. Indeed, FE6 could specifically and clearly detect HLA-F from transfected cells. However, very low levels of HLA-F expression was detected in membrane preparation from a B cell line. Furthermore even after this first step, purification contaminant bands were detected by FE6.

Immunoprecipitation of HLA-F with FG1, a higher affinity monoclonal antibody, conjugated with iso-electric focusing analysis allowed unambiguous identification of HLA-F. HLA-F was detected in 3 different B cell lines, the T2 cell line and HUT-78 (a T cell line), but was undetected in U937 and Jurkat cell lines. The level of expression was very low compared to other class Ia molecules, which is in accord with the level of transcription. These results were obtained before the publication of similar results (Wainwright, Biro and Holmes, 2000). Wainwright et al. have produced a specific monoclonal antibody (Fpep1.1) using a synthetic peptide. This antibody was used to indirectly detect HLA-F by Western blot, which allowed the study of HLA-F expression. Fpep1 antibody was not able to detect HLA-F protein by immunoprecipitation or immunochemistry. It seems likely that our antibody, FG1 has higher affinity to HLA-F compared to Fpep1, which did not detect HLA-F in the HUT-78 cell line.

FG1 antibody was also highly effective for staining tissue sections and this enabled us to perform further analysis of HLA-F expression. Immunochemistry results show that

HLA-F is expressed at significant levels in a range of immunological tissues including tonsil, spleen and thymus. It is now necessary to determine the nature of the different cells involved to have a better idea of the functional consequence of that pattern of expression. However, even without clear identification of the cells stained, several observations can be made. Although HLA-F is mainly expressed in B cells and B cell lines, the germinal centres which are a site of B cell proliferation are unstained. Secondly, the mantle zone composed of B and T cells shows the strongest cell staining observed after the endothelial cells of the spleen. This might indicate that expression of HLA-F in B cells may fluctuate as a function of their stage of differentiation. Another characteristic is the staining of endothelial cells in tonsil and spleen. The most significant information is the specific pattern of expression in lymphoid tissues. Lymphoid organs have highly organised tissues where antigens are presented to cells of the immune system. This is consistent with a specialised function of HLA-F in the immune system.

It was shown that HLA-F heavy chain was associated with β2m in B cell lines and HUT-78. Assembly of MHC class I molecules with peptides is dependent on a number of chaperones. One of these chaperones, TAP has a central role in the assembly of MHC class I molecules. Therefore the interaction between HLA-F and TAP molecules was studied. Within a B cell line, we showed by co-immunoprecipitation that TAP-1 is associated with HLA-F. Experiments using different antibodies and a different technique confirmed the TAP interaction and demonstrated calreticulin interaction with HLA-F (Wainwright, Biro and Holmes, 2000). These data prove that HLA-F is able to

bind TAP molecules. However, pulse chase experiments, cell surface labelling and staining suggested that HLA-F is retained in the endoplasmic reticulum under the conditions prevalent in our initial experiments. Retention of HLA-F in the ER observed in this study is consistent with HLA-F endo-H resistance (Wainwright, Biro and Holmes, 2000).

Class Ia molecules could be driven to the cell surface in the absence of peptide when cells were grown at low temperature (Ljunggren et al., 1990). However this was not observed with H2-M3 molecules, which were retained in the endoplasmic reticulum when cultured at 26°C (Chiu et al., 1999). A very limited proportion of HLA-F molecules reached the cell surface when 721.221 were grown at 26°C, as detected by the FG1 monoclonal antibody. *In vivo*, HLA-F seems to be retained inside the cell suggesting that there is selective pressure to retain HLA-F molecules intra-cellularly. It is possible that HLA-F, like HLA-E cell surface expression is highly dependent on the binding of specific peptide(s). These peptide(s) could be derived from pathogens. The restricted pattern of expression of HLA-F in immune effector cells makes it unlikely that HLA-F has a purely intracellular function linked to the class Ia pathway. More widespread expression would be predicted if it played a global role in the class I antigen processing pathway.

A modest increase in HLA-F expression of IFN-γ treated B cell lines was suggested (Wainwright, Biro and Holmes, 2000). Similarly, the results presented here suggest a very small increase of expression of both class I molecules and HLA-F proteins were

detected in B cell lines incubated with IFNγ. Finally cell surface expression of HLA-F was not detected in B cells activated with IFN-γ an observation also made by Shane Wainwright (Wainwright, Biro and Holmes, 2000).

The work presented in this Chapter has shown that HLA-F is mainly expressed in lymphoid tissues, B cells and in a T cell line (HUT 78) suggesting an immunological function. The association of the HLA-F heavy chain β2m complex with TAP (transporter associated with antigen processing) suggest that HLA-F may have a similar maturation path to MHC class Ia molecules. Thus, it is likely that HLA-F can bind peptide(s) and, under specific conditions, may reach the cell surface.

Chapter 6 - HLA-F receptors

6.1 introduction

The research presented in the previous Chapters suggests that HLA-F may have a similar maturation path to MHC class Ia molecules. However, HLA-F cell surface expression was only detected in specific conditions and a single peptide species was not identified after acid elution. Nevertheless, it is possible that like HLA-E, under specific conditions HLA-F would bind to peptide(s) and be transported to the cell surface much more efficiently. At the cell surface HLA-F could be recognised by receptors expressed on immune cells. One approach that would support this hypothesis is to identify potential ligands expressed at the surface of immune cells interacting with the HLA-F molecule.

Design of MHC class I tetrameric complexes or tetramers (Figure 6.1), a recent technological advance, permitted us to search for HLA-F ligands. HLA-E and HLA-G tetramers have been successfully used in previous studies to identify their respective ligands (Allan et al., 1999; Braud et al., 1998a). HLA-F tetramers were generated and used to screen peripheral blood leukocytes for cells expressing potential ligands to HLA-F.

Direct molecular binding between HLA-F and the receptors identified could then be analysed by SPR (Surface Plasmon Resonance), which permits direct monitoring of protein-protein interactions.

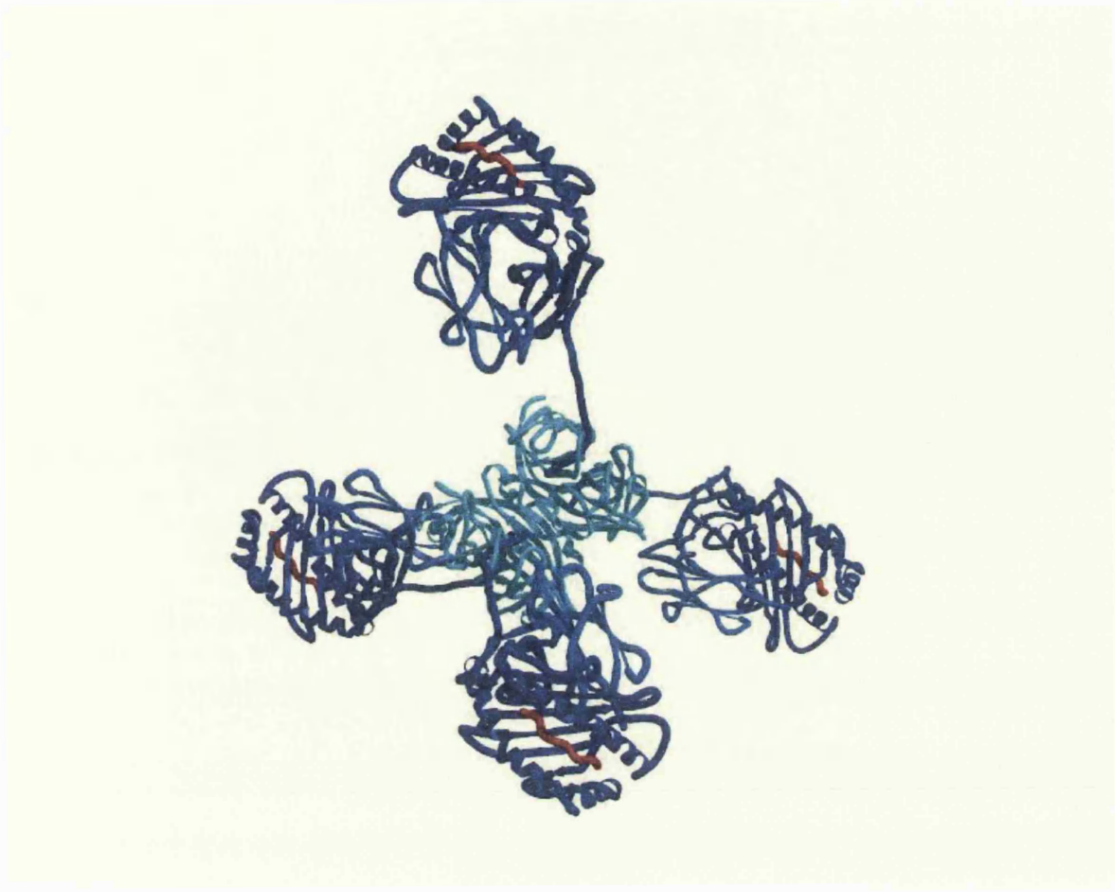

Figure 6. 1 Model of a MHC class I tetrameric complex.

Light blue, streptavidin; *dark blue*, B8 heavy chain; *intermediate blue*, beta-2 microglobulin; *red*, the peptide. This figure is adapted from McMichael and O'Callaghan, 1998.

6.2 Engineering of HLA-F recombinant protein to produce HLA-F tetramers

Soluble tetrameric complexes of class Ib recombinant proteins have been used as markers to identify ligand-receptor interaction (Allan et al., 1999; Braud et al., 1998a).

This application is based on the development of enzymatic biotinylation using recombinant BirA enzyme (Altman et al., 1996; O'Callaghan C et al., 1999). Addition of a BirA recognition site at the C-terminus of a recombinant protein allows specific biotinylation at the recognition site. Biotinylated proteins are able to bind to streptavidin derivatives. Streptavidin derivatives have four biotin binding sites, which means that up to four biotinylated molecules can be linked together in a specific orientation to form a tetramer. Streptavidin derivatives can themselves be conjugated to other chemicals like fluorochromes. This technology was first been applied for MHC class Ia molecules. The multimerisation increases the avidity of class Ia molecules because of a valency effect, and the fluorochrome allows flow cytometry analysis of specific T lympyocytes recognised by the class Ia molecule (Altman et al., 1996).

It was previously shown in Chapter 3, how recombinant HLA-F heavy chain could be expressed in *Escherichia coli* strain BL21 (DE3). To produce HLA-F tetramers, we engineered a recombinant HLA-F heavy chain with a BirA recognition site at the C terminus of the alpha 3 domain. The HLA-F sequence corresponding to the amino acids 1 to 280 was amplified by PCR from F0b2 clone described in Chapter 3 using TEF 5' and TEF 3' primers. PCR products were cloned in the pGMT7 expression vector (Reid et al., 1996) and TEF2 clone of the correct sequence was stocked. The N terminus $\alpha 1$ domain nucleotide sequence of this clone was altered synonymously as described in Chapter 3 to generate a high level of protein expression. The C terminus sequence of the $\alpha 3$ domain has in frame a linker (GS) and a BirA recognition site

(LNDIFEAQKIEWH). The BirA enzyme covalently links a Biotin molecule to the Lysine (K) in the recognition site.

6.3 Production of HLA-F tetramers

TEF2 was transfected into *E coli* strain BL21 (DE3) to express the HLA-F heavy chain tagged with the BirA recognition sequence. IPTG induction of the transfectant resulted in the appearance of a band of the correct molecular weight (34 kDa) when bacterial lysates were analysed by SDS-PAGE (data not shown). The yield was 120 mg of purified inclusion body by litre of culture. Inclusion bodies were solubilised in urea and checked on SDS-PAGE for purity. The refolding conditions used to produce the β2m/HLA-BirA tag complex were identical to that described in Chapter 3.

After concentration of the refolding reaction, a first step gel filtration purification of the complex HLA-F BirA /β2m was performed (Figure 6.2). The quantity of complex produced was similar to that obtained with the untagged HLA-F heavy chain described in Chapter 3. This pre-purified complex HLA-F BirA/β2m was then prepared for enzymatic biotinylation.

HLA-F/β2m protein was first exchanged to a BirA reaction buffer (100 mM Tris HCl pH 7.5, 5 mM MgCl2, 20 mM NaCl) using PD-10 column (Pharmacia, Sweden). Enzymatic reactions were then performed by adding d-Biotin, ATP and BirA enzyme to the protein sample in BirA reaction buffer. The BirA enzyme used was produced by Dr

Chris O'Callaghan (O'Callaghan C et al., 1999). Protease inhibitors were also added, and the enzymatic reaction incubated overnight at room temperature.

In the morning the complex was purified by gel purification (Figure 6.3), to remove the unbound biotin. The gel filtration histogram shows the appearance of an important aggregate peak. When checked on SDS-PAGE in reducing or non-reducing conditions, the complex produced appeared of good purity and none aggregated (Figure 6.4). The percentage of biotinylated protein was compared to previous biotinylated class I proteins by ELISA, and the stoichiometric quantity of Extravidine PE to form tetramer were added in several aliquots.

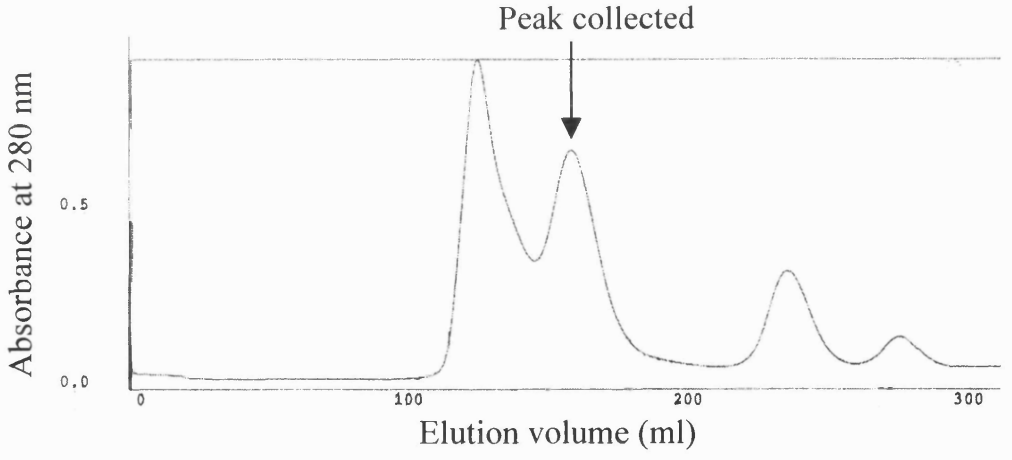

Figure 6. 2 Gel filtration purification of HLA-F BirA/β2m complex.

Gel filtration profile of concentrated refolding mix loaded on a Superdex G75 column. The second peak corresponding to the complex HLA-F BirA/β2m was collected, and proteins were enzymatically biotinylated.

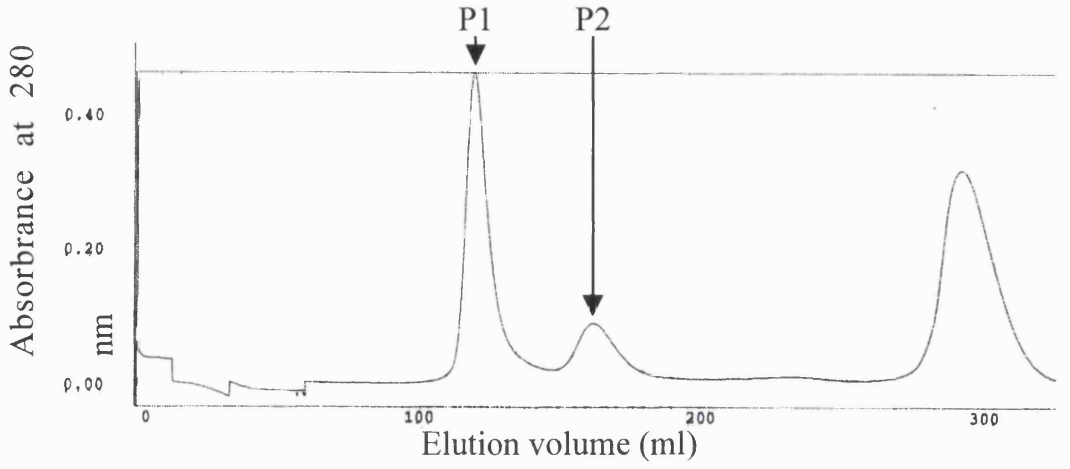

Figure 6. 3 Gel filtration purification of biotinylated HLA-F BirA/β2m sample.

Gel filtration profile. After overnight biotinylation the samples were loaded onto a Superdex G75. Samples from the first Peak (P1) and the second peak (P2) were analysed on SDS-PAGE.

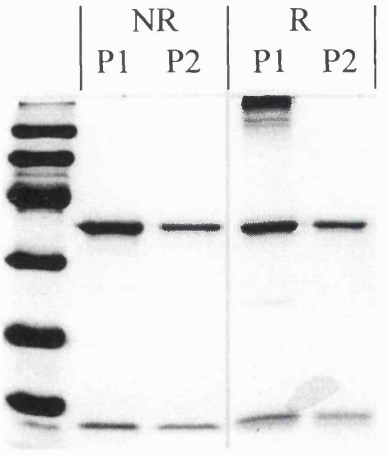

Figure 6. 4 SDS-PAGE analysis of the biotinylated complex.

A sample of the two peaks (P1 and P2) obtained after gel filtration of the Biotinylated complex shown in Figure 6.2 was run on SDS-PAGE. Both samples were analysed in non-reducing (NR), and reducing (R) conditions. (M) was the Molecular weight marker (97.4, 66.2, 45, 31, 21.5, 14.4 kDa).

To avoid degradation of the BirA tag the complex was tetramerised as soon as possible after purification of the refolded complex. Even though the quantity of complex purified diminished because of aggregation, it was still possible to produce sufficient amounts of biotinylated HLA-F/β2m complex to make tetramers. Around 300 to 400 μg of biotinylated HLA-F/β2m complex were produced from a 500ml of refolding reaction using 20 mg of β2m and 40 mg of HLA-F BirA recombinant protein.

6.4 HLA-F tetramers stain peripheral blood monocytes and B cells

PBMC (peripheral blood mononuclear cells) were extracted from healthy individuals using Ficoll-Hypaque™ separation. Fresh PBMC were stained at 4°C for one hour with tetramers, control, and marker antibodies to detect different cell populations. Cell surface staining was subsequently analysed by flow cytometry. HLA-F tetramers stained a restricted set of PBMC consisting of CD19+ and CD14+ cells (Figure 6.5). T cells (CD4+, CD8+), and natural killer cells (CD56+) were unstained. Within the myelo-monocytic gate, staining was observed on most CD14+ (monocytes) cells in both the CD14+ mid and CD14+ high populations. Within the CD19+ (B cells) cell population, there was a broad spread to the brightness of tetramer staining. On some occasions we observed some background with Extravidin PE on CD 19+ cells. Different batches of tetramer were tested on PBMC extracted from non related individuals. All experiments showed staining of B cells (CD19+), and monocytes (CD14+), but with different intensities.

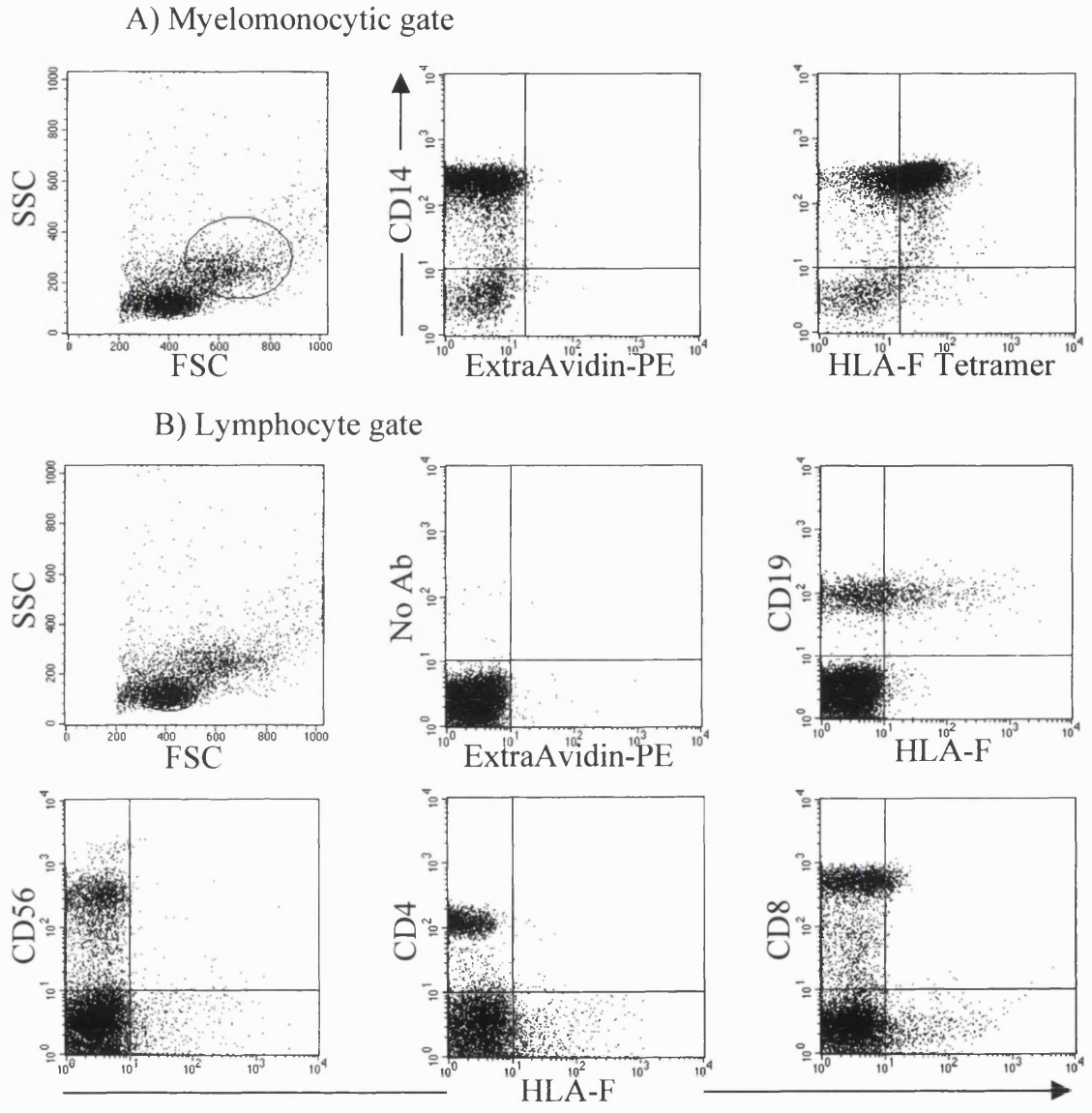

Figure 6. 5 HLA-F tetramers bind to B cell and myelo-monocytic cells.

PBMC from a healthy individual were stained with a phycoerythrin labelled HLA-F tetramer, or extravidin -phycoerythrin alone as a negative control, and CD4, CD8, CD14, CD19, or CD56 fluorescein-conjugated monoclonal antibodies, then analysed by flow cytometry. A) a myelo-monocyte gate was applied B) a lymphocyte gate was applied. HLA-F binds to a set of CD19+ and CD14+ cells.

6.5 HLA-F tetramers bind to ILT2 and ILT4

HLA-F tetramer staining of B cells and monocytic cells from PBMC might result from specific interactions between the HLA-F recombinant protein with receptors at the cell surface of these cells. Recently, many families of receptors expressed at the cell surface of leukocytes have been cloned. One of these families, ILT (immunoglobulin like transcript) / LIR (leukocyte immunoglobulin like transcript) has members like ILT2 which is mainly expressed on monocytes and B cells. ILT nomenclature will be used hereon for simplicity. Furthermore, it was suspected that ILT molecules, because of their homology with KIR receptors, would bind to class I molecules. However, most of ILT ligands are unknown. Only ILT2 and ILT4 are able to bind to a wide range of MHC class I molecules, including HLA-G (Allan et al., 1999). Recently it has been shown that ILT2 interacts with the conserved α3 domain of class Ia molecules (Chapman, Heikeman and Bjorkman, 1999).

It could be hypothesised that the pattern of staining observed on PBMC might be accounted for by binding to a subset of ILT receptors. Therefore, HLA-F tetramers were tested for their ability to bind to cells transfected with a range of ILT molecules (Figure 6.6). We also used HLA-F tetramer to stain cells transfected with CD94/NKG2A, a receptor for HLA-E, and KIR3DL2 a KIR receptor (Figure 6.7). Only ILT2 and ILT4 transfected cells were stained by HLA-F tetramer. Cells were a gift from M Colonna (Basel Institute for Immunology, Switzerland) and LL Lanier (University of California, San Francisco, USA).

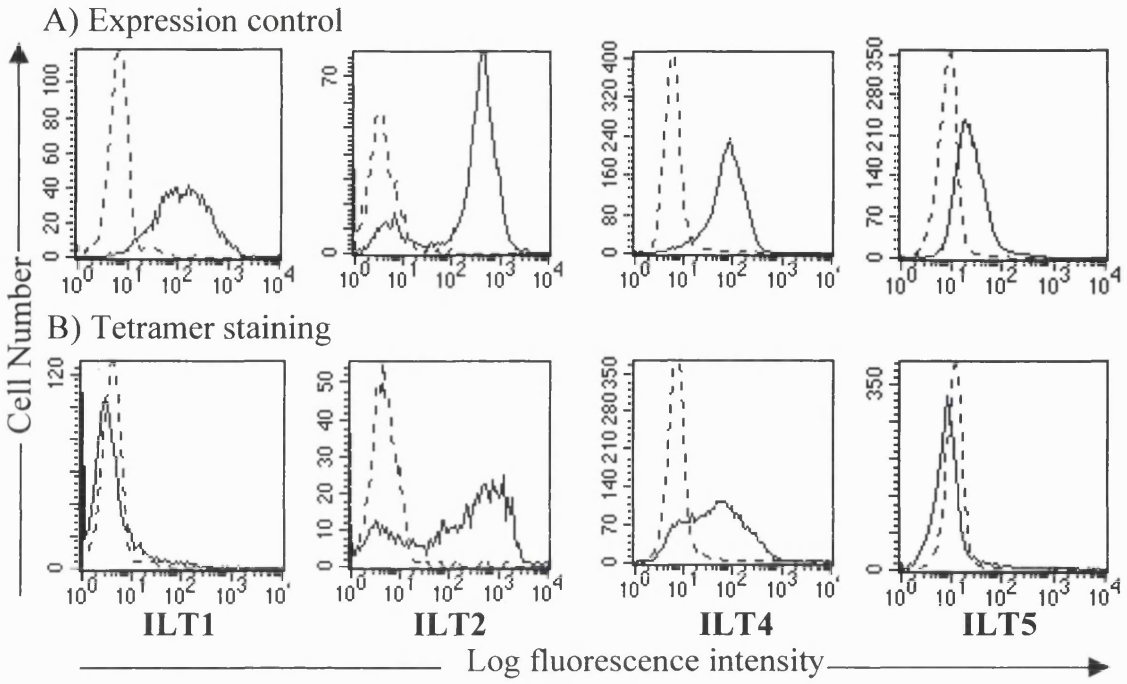

Figure 6.6 HLA-F tetramers bind to ILT2 and ILT4 receptors.

Staining of ILT transfected cells was analysed by flow cytometry. A) Full lines indicate levels of cell surface expression of ILT2, ILT4 and ILT5 (tested using 40H2 antibody) and ILT1 levels of expression (tested using an anti-FLAG antibody). Isotype control is represented by a dashed line. B) Transfected cells were stained with phycoerythrin labelled HLA-F tetramer (full line) or phycoerythrin labelled extravidin (dashed line).

HLA-F tetramers stain B cells and myelo-monocytic cells. To determine if this staining is the direct consequence of the HLA-F tetramer binding with ILT2 and ILT4 molecules, it was necessary to find a blocking antibody. Available anti-ILT2 and anti-ILT4 antibodies (Table 6.1) were tested for their ability to block the binding of HLA-F tetramers with ILT2 and ILT4 transfected cells (Figure 6.8 and 6.9). F1-HP, a specific anti-ILT2 antibody was the only one able to completely block the interaction between HLA-F tetramers and ILT2 transfected cells. 27D6, a specific ILT4 antibody blocked

the interaction between the HLA-F tetramer and ILT4 transfected cells. 28C8, an anti-ILT2 and anti-ILT4 antibody could block the binding of HLA-F tetramers with ILT4 transfected cells, but did not have the same effect on ILT2 transfected cells. These results suggest that HLA-F has different recognition sites to these two homologous receptors.

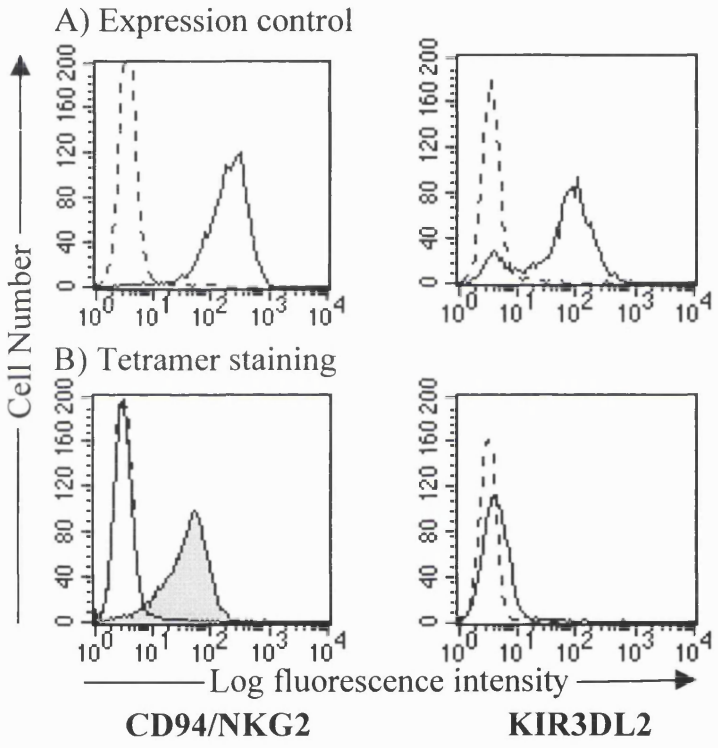

Figure 6.7 HLA-F tetramers do not bind to CD94/NKG2A and KIR3DL2 transfected cells

Cell staining of transfected cells was analysed by flow cytometry. A) Levels of cell surface expression CD94/NKG2a was tested using DX22 antibody, and KIR3DL2 level of expression was monitored using 5133 antibody (full line). Isotype control is represented by dashed lines. B) Transfected cells were stained with phycoerythrin labelled HLA-F tetramer (full line), phycoerythrin labelled HLA-E tetramer (filled in grey), or phycoerythrin labelled extravidin (dashed line).

mAb	HP-F1	GHI/75	VMP55	28C8	40H2	27D6
ILT detected	ILT2	ILT2	ILT2	ILT2 ILT4	ILT2 ILT4 ILT5 ILT6	ILT4

Table 6. 1 Reactivity of anti-ILT monoclonal antibodies (Colonna et al., 1999).

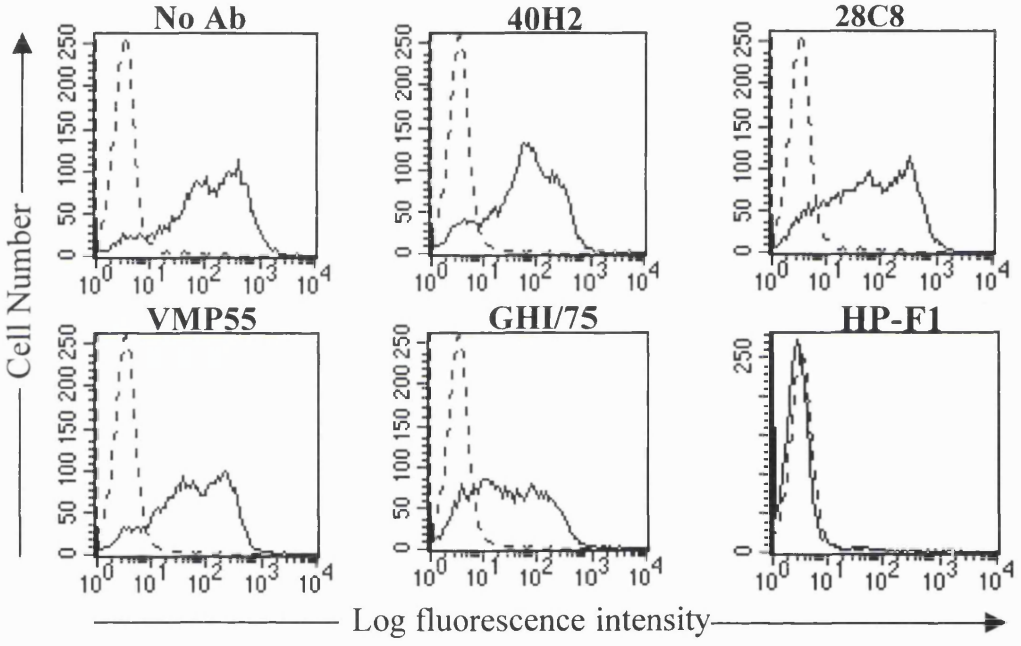

Figure 6.8 HP-F1 blocked the staining of ILT2 transfected cells by HLA-F tetramers.

Anti-ILT2 monoclonal antibodies 40H2, 28C8, VMP55, GHI/75, HP-F1 (Colonna et al., 1999) or no antibodies were incubated with ILT2 transfected cells for 10 minutes before staining with HLA-F tetramer. ILT2 transfected cells were stained with phycoerythrin labelled HLA-F tetramer (full line), or phycoerythrin labelled extravidin (dashed line). Staining was then analysed by flow cytometry.

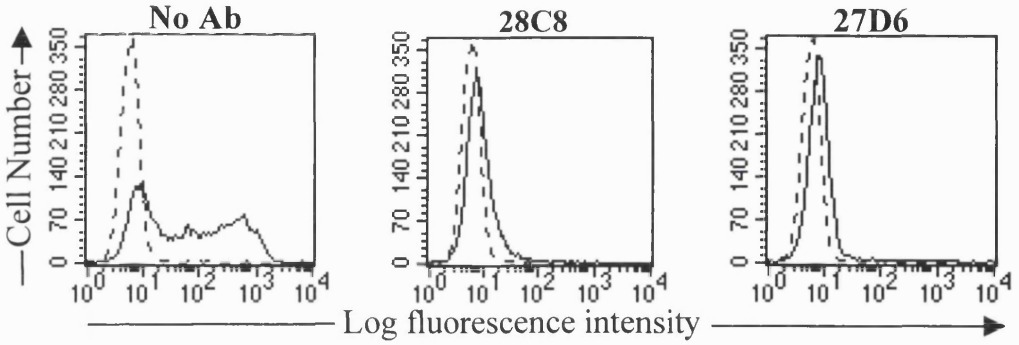

Figure 6. 9 28C8 and 27D6 blocked the staining of ILT4 transfected cells by HLA-F tetramers.

28C8, 27D6 antibodies or no antibodies were incubated with ILT4 transfected cells for 10 minutes before staining with HLA-F tetramer. ILT4 transfected cell were stained with phycoerythrin labelled HLA-F tetramer (full line), or phycoerythrin labelled extravidin (dashed line). Staining was then analysed by flow cytometry.

6.6 The interaction of HLA-F tetramers with blood monocytes and B cells is not only due to binding to ILT2 and ILT4

Different monoclonal antibodies, 28C8, HP-F1 and 27D6, were able to block the HLA-F tetramer interaction with ILT2 and ILT4. These antibodies were used to determine whether HLA-F tetramer staining on PBMC was the result of HLA-F interaction with ILT2 and ILT4 or if other receptor(s) were involved (Figure 6.10). Before the staining of PBMC with phycoerythrin labelled HLA-F tetramer, or phycoerythrin labelled extravidin, PBMC were incubated with different combinations of blocking antibodies. Complete inhibition of HLA-F tetramers staining on PBMC was not observed with any combination of blocking antibody even when HP-F1 (anti-ILT2) and 27D6 (anti-ILT4)

were used together (Figure 6.10). The binding of HLA-F tetramers was only partially inhibited by 28C8, and 27D6 predominantly in cells in the CD14+ mid population.

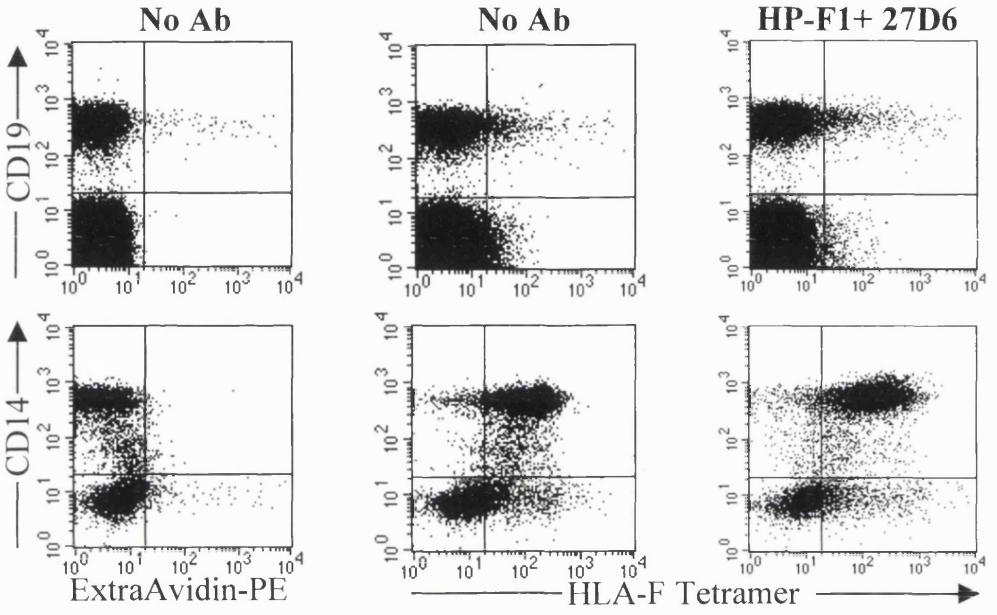

Figure 6. 10 HLA-F tetramers binding to B and monocytic cells are not blocked by HP-F1 + 27D6 anibodies.

PBMC from a healthy individual incubated with no antibody (no Ab) or HP-F1+27D6 were stained with a phycoerythrin labelled HLA-F tetramer, or extravidin-phycoerythrin alone as a negative control; CD14, CD19 fluorescein-conjugated monoclonal antibodies, then analysed by flow cytometry. Monocytic cells were identified as CD14 + cells in a myelomonocyte gate, and B cells were identified as CD19 + in a lymphocyte gate.

6.7 SPR analysis of HLA-F interaction with ILT2 and ILT4

Surface Plasmon Resonance (SPR) is a technique used to measure protein-protein interactions. This technique is based on an optical method to measure the refractive index near a sensor surface. Practically, a protein is immobilised on a sensor surface while another protein (the analyte) is injected over the sensor surface. Binding of the analyte to the immobilised protein will increase the local protein concentration at the sensor surface, which will change the refractive index. The measure of the binding is given in response units (RU). The concentration of protein is proportional to the signal detected and it is possible to study binding of the two molecules in real time. The analyte flows sequentially over four separate flow cells in a Biacore 2000 machine (Biacore AB UK). One of the proteins immobilised has to be an irrelevant protein. The response registered for this irrelevant protein, represents the background or negative control. The specific response for a ligand is the measure for this ligand subtracted by the non-specific background. Using SPR it is possible to study kinetics and to determine accurately the affinity of protein-protein interaction (Fivash, Towler and Fisher, 1998).

The HLA-F interaction with ILT2 and ILT4 was confirmed and analysed at the molecular level using SPR (Figure 6.11). ILT2 and ILT4 were immobilised on separate sensor surfaces. Chimeric ILT2 molecules, containing the extra-cellular domains of ILT2 fused with the Fc portion of human IgG1, were indirectly coupled to SPR sensor surface using an anti-human-Fc mAb (Colonna et al., 1997). A sensor surface coated with the mAb alone served as a control. Soluble extra-cellular domains of ILT4 tagged with a myc epitope and a poly-His tail was produced by M. Colonna (Basel, Institute for

Immunology, Switzerland). This ILT4 protein was indirectly coupled to the sensor surface using 40H2, an anti-ILT mAb. This antibody does not affect the binding of MHC class I tetramers. Again, a sensor surface coated with only 40H2 mAb served as a paired control. Recombinant HLA-F protein was injected over the four chips. Direct interaction of HLA-F protein with ILT2 and ILT4 proteins was demonstrated by an increase of response unit compared with the background controls. However, the dissociation curve obtained for ILT2 and ILT4 was biphasic, which was an indication of aggregation of HLA-F recombinant protein. Thus, measurement of the affinity between HLA-F and the two ILT molecules was not attempted in this orientation. SPR experiments were carried out in collaboration with Anton Van Der Merwe (Sir William Dunn School , Oxford) and David Allan (Human Immunology Unit, Oxford).

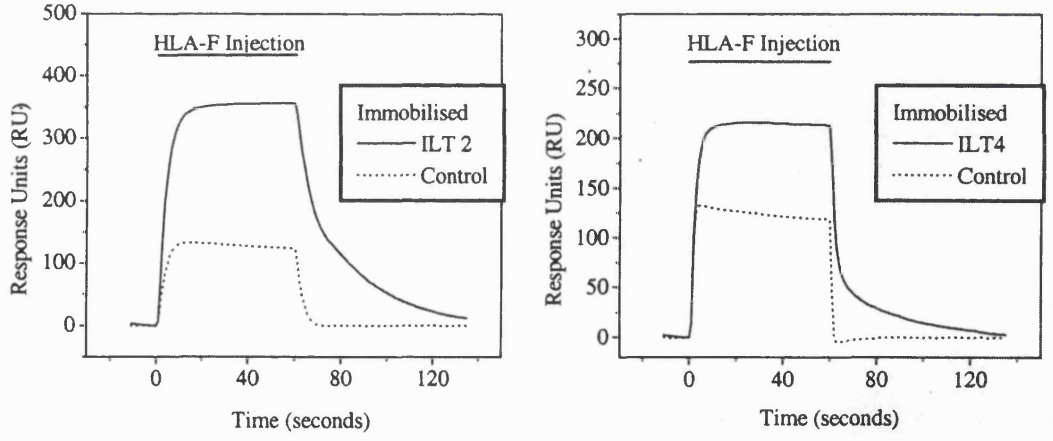

Figure 6. 11 Direct molecular binding interaction of HLA-F to ILT2 and ILT4.

Binding was analysed by surface plasmon resonance using a Biacore 2000 machine. ILT2 and ILT4 proteins were separately immobilised on chips, and HLA-F passed over the chips. Significant increases in resonance units indicate direct interactions of HLA-F with the ILT2 and ILT4 proteins.

ILT4 affinities to HLA-F, -G1 and B8 were calculated using the opposite orientation to the experiment described above. ILT4 recombinant protein was produced in a mammalian system of expression and purified on a nickel column. Class I molecules, and CD4 recombinant proteins were indirectly coupled to the chips to orient these molecules in a preferential direction. Biotinylated HLA-F, HLA-G1, HLA-B8, and CD4 were injected over Extravidin coated chip surfaces. CD4 was previously tested for the absence of any ILT4 interaction and used here as the negative control. Different concentrations of ILT4 were injected over the four chips, and the affinities to each class I molecule calculated from the data obtained (Figure 6.12). Results were confirmed in the other orientation for HLA-G1 and HLA-B8, which was not possible for HLA-F. The constant of dissociation (Kd) obtained for HLA-B8, HLA-F, and HLA-G1 were respectively 26.1μM, 14.7μM and 19μM.

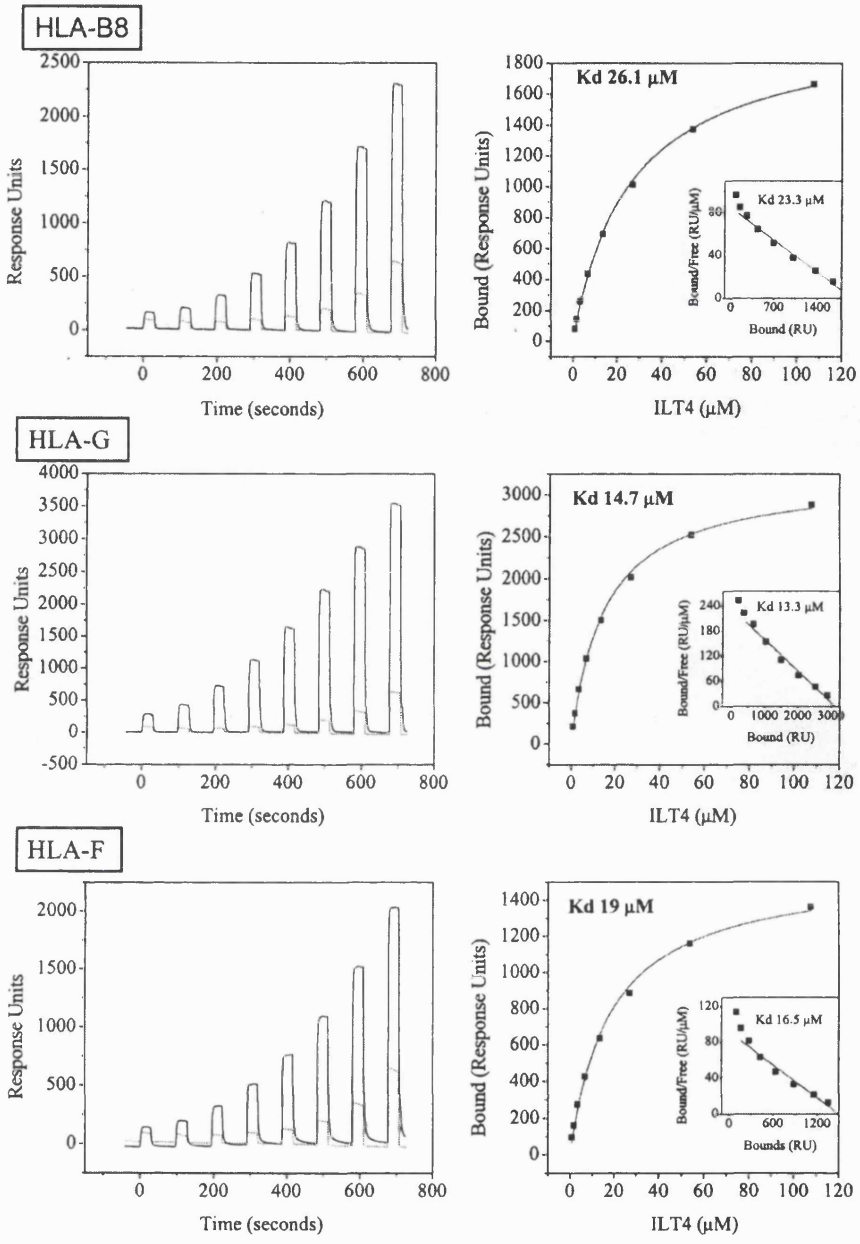

Figure 6. 12 ILT4 affinity to HLA-B8, HLA-G1 and HLA-F.

Affinity was calculated from SPR using a Biacore machine. Biotinylated HLA proteins and CD4 were separately immobilised on chips. Different concentrations of ILT4 were injected over the chips and each specific response unit calculated.

6.8 Discussion

Given the HLA-F association with TAP molecules, and the prediction of its binding to peptide(s), HLA-F may under specific conditions reach the cell surface. At the cell surface, this molecule could bind to ligands expressed on other cells. Recently HLA-E and HLA-G ligand expressing cells and the ligands themselves have been identified using tetramer technology (Allan et al., 1999;Braud et al., 1998a). We therefore applied the same strategy and engineered a BirA recognition tag on the C terminus of the HLA-F protein to produce HLA-F tetramers. Following the same protocol described in Chapter 3, a recombinant protein complex HLA-F-BirA tag/β2m was purified. It was possible to produce hundreds of micrograms of complex which, when analysed on SDS-PAGE was pure. This product was mixed with phycoerythrin-conjugated extravidin to form HLA-F tetramers.

HLA-F tetramers clearly stained a population of peripheral blood leukocytes identified as monocytes and B cells. The interactions of HLA-F with a subset of ILT receptors could account for the pattern of staining observed; this was tested. HLA-F tetramers were used to stain a range of ILT transfectants. The HLA-F tetramer staining was shown to be dependent on the presence of either ILT2 or ILT4.

The binding of HLA-F with ILT2 and ILT4 at the molecular level was confirmed using surface plasmon resonance. This technique can detect weak interactions, and allow quantitative measurement of affinities and kinetics. However, this method is very sensitive and valid quantitative results are difficult to obtain. The presence of an

aggregate in the analyte results in artefacts in quantitative studies. Using the HLA-F recombinant protein as the analyte, interaction of this protein with ILT2 and ILT4 was demonstrated. Quantitative measurements were not attempted in this orientation but, in the reverse orientation using purified ILT4 recombinant protein as the analyte which was injected over immobilised HLA-B8, HLA-G1 and HLA-F. It was possible to calculate the affinity between ILT4 and these class I molecules. The principal information, which can be extracted from these experiments, is that the ILT4 affinity to HLA-F seems very similar to HLA-B8, and HLA-G1. These results suggest that at equal concentrations, the three class I molecules have an equal role in their interaction with the ILT4 molecule.

ILT2 is mainly expressed on B cells and monocytes whereas ILT4 is expressed predominantly on monocytes. Thus, the staining of B cells and monocytes by HLA-F tetramers could be the direct result of HLA-F specifically binding to ILT2 and ILT4. To verify this hypothesis, monoclonal antibodies to ILT2 (HP-F1) and ILT4 (27D6 and 28C8) respectively capable at blocking the staining of HLA-tetramers with ILT2 or ILT4 transfected cells, were identified. However, combined use of these blocking antibodies on PBMC was not sufficient to completely inhibit the staining on B cells or monocytes by HLA-F tetramers, suggesting that HLF tetramers may also interact with other cell surface receptors on these cells.

HLA-G tetramer staining on PBMC could be completely blocked with 27D6, an anti-ILT4 showing that the staining of HLA-G tetramers with blood monocytes was largely

due to binding to ILT4 (Allan et al., 1999). However, a similar experiment showed that HLA-F tetramer interaction with ILT4 might only be involved in the staining of a limited population of monocytes, mainly mid CD14+.

The blocking of ILT2 receptors expressed on B cells and monocytes did not result in clear modifications of HLA-F tetramer staining on these cells. This implies that the interaction between HLA-F and ILT2 might not be responsible for any PBMC staining by HLA-F tetramers. However, it is possible that HP-F1 monoclonal antibodies are not able to block the interaction of ILT2 expressed on leukocytes with HLA-F tetramers. The non-recognition of ILT2 by class I tetramer might also be the result of steric conformation at the cell surface of leukocytes. It is also possible that the ILT2 molecules are constrained from binding in these cells, possibly by altered glycosylation. Similar results have been observed. HLA-G1 tetramers, able to bind to ILT2 transfected cells were unable to stain NK and B cells which express ILT2 (Allan et al., 1999). It has been shown that ILT2 (LIR1) interacts with the relative nonpolymorphic $\alpha 3$ domain of class I (Chapman, Heikeman and Bjorkman, 1999).

Binding of HLA-F tetramers to peripheral blood B cells is interesting as B cells have not been stained by any other class I tertramers. HLA-F may bind to a specific receptor on B cells different from known class I receptors.

We have certainly demonstrated that the HLA-F recombinant protein produced was able to bind to ILT2 and ILT4 receptors. These results confirm the biological activity of the

recombinant HLA-F molecule. However, any hypothetical binding of a specific peptide could influence the structure and biological activity of HLA-F.

Chapter 7 - Concluding Discussion

7.1 MHC class Ib molecules

HLA-E, -F and -G genes are located in the MHC and classified as non-classical class I or class Ib genes. Class Ib molecules have sequence and structural homology with class Ia molecules, consistent with a role for these molecules in immunological recognition. However, significant differences in Class Ib expression and peptide binding compared to class Ia molecules suggest that class Ib molecules have evolved different functions. HLA-E and HLA-G molecules have been intensively studied, and significant results have been obtained particularly for HLA-E.

The identification of MHC class I leader sequence peptides as specific ligands for the mouse molecule Qa-1 paved the way for studies of its human homologue HLA-E. HLA-E was shown to bind class I leader sequences that promote its cell surface expression, and enable HLA-E to interact with specific natural killer cell receptors, CD94/NKG2. Thus, a decrease of class Ia molecule expression, resulting from cell transformation or virus infection would be sensed by NK cells through a decrease of HLA-E cell surface expression. HLA-E appears as a key checkpoint for natural killer surveillance of intracellular activity (O'Callaghan and Bell, 1998).

HLA-G has been implicated in the protection of the foetus from the maternal immune system because of its expression in the placenta. HLA-G1 binds peptide and has been involved in inhibiting NK cell cytolysis. However the effect of HLA-G expression

might be indirect and linked to the up-regulation of HLA-E cell surface expression, which is an effect of HLA-G expression. HLA-G has been shown to interact with TCRs and CD8 as well as other leukocyte receptors such as ILT2, ILT4 and KIR2DL4. However, the function of HLA-G still remains unclear.

MHC class I molecules primarily interact with T cell receptors. It now appears that a number of other leukocyte receptors interact with class I molecules. Class Ib molecules, HLA-E and HLA-G seem to have specialised in the immune system regulation through their interactions with newly discovered receptors. Initial analysis of the HLA-F gene demonstrated MHC gene localisation, homology to class Ib genes and a low level of expression of the transcript in most cells (Geraghty et al., 1990). This suggested that HLA-F might also have evolved for a specific immunological function.

7.2 Characterisation of HLA-F: *in vitro* biochemistry

Reagents were produced to characterised the HLA-F molecule *in vitro* and *in vivo*. The HLA-F heavy chain ectodomain was expressed in bacteria after adequate modification of the N-terminal codon use. *In vitro*, HLA-F heavy chain and β2m proteins could combine in a 1:1 molar ratio to form a stable complex. This complex was of homogenous molecular size when purified by size exclusion chromatography. Furthermore, this complex reacted with w6/32, and was able to bind to ILT2 and ILT4 molecules in the same way as most class Ia molecules (Colonna et al., 1999). All these features are consistent with the correct conformation for the recombinant HLA-F-β2m complex. In the absence of information concerning the putative ligand for the peptide

binding groove, HLA-F was refolded without added peptide. Typically, class Ia and Ib molecules refold most efficiently when synthetic peptide is added (O'Callaghan et al., 1998; Reid et al., 1996).

In vitro the HLA-F/β2m complex was formed in the absence of synthetic peptide. Additionally, we were unable to demonstrate the presence of any peptides associated with the complex when the recombinant protein complexes were subjected to acid elution and high-pressure liquid chromatography. The ability of HLA-F to refold *in vitro* in the absence of synthetic peptide cannot be taken as evidence that the HLA-F-β2m complex does not require a third element to refold *in vivo. In vitro,* undetected bacterial peptides or other molecules from the production of HLA-F and β2m in *E.coli* are present in the refolding buffer. These molecules could stabilise the HLA-F-β2m complex. It is also possible that *in vitro* there may be a sufficient degree of stability in the absence of a third element to allow the formation of a stable complex.

7.3 HLA-F expression distribution

A specific high affinity antibody (FG1) was produced using the HLA-F-β2m complex as an immunogen and as a screening reagent. Cross reactivity of this antibody to other class I molecules was excluded by screening against a panel of recombinant proteins, immunoprecipitation and cell staining. Whole protein immunogens, rather than peptide fragments, typically lead to the generation of higher-affinity antibodies. Our antibody appeared to be of a higher affinity and work in a wider range of methods than that recently reported by Wainwright et al. (Wainwright, Biro and Holmes, 2000). FG1, as

w6/32 can immunoprecipitate a HLA-F heavy chain - β2m complex from B cell lines. However it is possible that FG1 might not detect one or more specific conformations or the maturation state of HLA-F. It is also possible that association with other molecules could mask the epitope recognised by FG1. One particular example is the immunoprecipitation of the HLA-F protein by an anti-β2m antibody from foetal liver. However, w6/32 (Wainwright, Biro and Holmes, 2000) and FG1 have been unable to detect HLA-F protein in the same tissues. In some cases it is also possible that the method used to study the pattern of expression of HLA-F is not sensitive enough to detect very low levels of expression.

We used the FG1 antibody to directly examine the pattern of expression of HLA-F by direct immunoprecipitation and cell staining. Immunoprecipitation of HLA-F by FG1 coupled with analysis on one dimentional isoelectric focusing gels allowed the demonstration of a specific pattern of expression among leukocyte cell lines. HLA-F was expressed in different B cell lines and HUT78, a T cell line. Expression was not detected in Jurkat, a T cell line, and U937 a histocytic lymphoma cell line. These results are consistent with previous studies on HLA-F mRNA expression (Geraghty et al., 1990; Lury, Epstein and Holmes, 1990).

In situ analysis of tissue sections with the same antibody demonstrate a restricted pattern of expression of HLA-F in a range of immunological tissues including tonsil, spleen and thymus. Expression was not detected within the small bowel, kidney, adult and foetal liver, brain, oesophagus, prostate and skin tissue sections. All these results

are consistent with a specialised function of HLA-F in the immune system. In tonsil, positively stained cells were mainly concentrated within the mantle zone which is a area of T and B cell interaction. However, cells were not stained in the germinal centre, a location of B cell proliferation. These results suggest that not all B cells are stained and it would be interesting to look at HLA-F expression of individual B and T cells as a function of differentiation markers. In the spleen, strong staining corresponding to the splenic sinusoid architecture was observed and staining of endothelial cells was also observed in the tonsil.

FG1 did not detect HLA-F protein in the foetal liver, but transcripts have been detected in this tissue (Houlihan et al., 1992), and HLA-F protein has been immunoprecipitated using an anti-β2m antibody in the same tissue (Wainwright, Biro and Holmes, 2000). The presence of HLA-F/β2m complex in foetal liver suggests that HLA-F might have a function in this tissue.

In conclusion, the FG1 monoclonal antibody detected HLA-F protein in B cells, one T cell line and lymphoid tissues containing B and T cells. Expression of HLA-F molecules in B cells and in tissues containing B cells was confirmed by another group using a different antibody, Fpep1.1 (Wainwright, Biro and Holmes, 2000). Thus expression of the HLA-F protein seems restricted to lymphoid tissues, and not as widely expressed as was suggested from the studies of HLA-F transcripts.

7.4 Expression control of HLA-F

It has been suggested that the IFN-γ treatment could increase the level of expression of HLA-F 35 times (Gobin et al., 1999). However, we have only been able to detect a small increase of HLA-F expression in B cell lines treated with INF-γ. Similarly, a weak increase in expression was observed by another group (Wainwright, Biro and Holmes, 2000). *In vivo*, the increase in expression might be lower than suggested. Alternatively, the increase in transcription may not be directly translated into an increase in protein, or low stability may lead to rapid protein degradation.

A role for IRF2 (Interferon regulated factor 2), which is the major ISRE binding protein in B cells, has been proposed for the expression of HLA-F protein in B cell lines (Gobin and van den Elsen, 2000). The constitutive level of expression of HLA-F in B cell and HUT 78 is low and as with other class I molecules, it is probable that the expression is modulated. Indeed, the HLA-F promoter seems to be more related to MHC class Ia genes than that of HLA-E or HLA-G. It is possible that cytokine treatment may cause expression of HLA-F in cells which otherwise do not express it.

7.5 Characterisation of HLA-F *in vivo* biochemistry

The first study of the HLA-F protein used an HLA-A, B, and C-null mutant 721.221 LCL (Lymphoblastoid cell line) transfected with the HLA-F gene (Shimizu et al., 1988). In these cells, BBM.1 (anti-β2m) and w6/32 antibodies were able to immunoprecipitate a class I molecule, but were unable to detect this molecule at the cell surface. We extended the study to other non-mutant cell lines using a construct

expressing HLA-F coupled with a myc tag. Anti-myc antibodies detected an intracellular localisation of HLA-F in all the transfected cell lines stained. In addition, limited maturation of HLA-F was confirmed in B cell lines for which endo-H resistance of HLA-F has been shown (Wainwright, Biro and Holmes, 2000). FG1 was not able to detect HLA-F at the surface of cells in which constitutive expression of HLA-F had previously been demonstrated, except when B cells were grown at 26 °C. This phenomenon was previously reported for class Ia molecules which were unable to bind peptides but could still reach the cell surface when the cells were cultured at 26°C (Ljunggren et al., 1990). It is possible that HLA-F could bind specific peptides and then efficiently reach the cell surface in cells grown at 37°C as observed for class Ia.

Inside B cells, it has been shown that HLA-F interacted with TAP molecules that reside in the ER. This was recently confirmed by another study which had also demonstrated HLA-F association with calreticulin (Wainwright, Biro and Holmes, 2000). Interaction of HLA-F with TAP and calreticulin molecules involved in the class Ia pathway suggests that HLA-F shares at least part of the class Ia pathway. However HLA-F molecules are trapped in the ER and fail to reach the cell surface except when cells were grown at 27°C.

HLA-F could have an intracellular function and never reach the cell surface. A global intracellular function for HLA-F linked to the class I pathway is nevertheless improbable, as HLA-F has a restricted pattern of expression.

7.5 Peptide binding

Class Ia molecules show a high level of polymorphism and have evolved to present a wide range of endogenous peptide at the cell surface. HLA-G and HLA-E also bind peptides. Comparison between peptides eluted from HLA-G1 and HLA-A2 has shown that the peptide diversity of HLA-G is lower than in class Ia molecules. HLA-E binds a very restricted set of peptides derived from class I leader sequences. Other class I-like molecules bind modified peptides or non-peptide ligands. For example, CD1 in humans and H2-M3 in the mouse have evolved to present specific molecules derived from intracellular pathogens (Chiu et al., 1999; Park and Bendelac, 2000). No ligands to HLA-F have yet been characterised.

Structural modelling of HLA-F has been performed by Dr Christopher O' Callaghan to identify any feature which might suggest the putative ligand preference of HLA-F (Figure 7.1). The crystal structure of HLA-E, the only human class Ib molecule whose structure is known, was used as the starting point from which to build a structural model of HLA-F using energy minimising computational algorithms. Overall, results suggest that the basic architectural framework structure of HLA-F is essentially similar to that of the other class Ia and class Ib MHC molecules and the two disulphide bonds are preserved between residue pairs Cys101-Cys164 and Cys203-Cys259. However, key similarities and differences are notable in the $\alpha 1$ and $\alpha 2$ domains around the peptide binding groove. In particular, at the region corresponding to the N-terminus of the bound peptide in the known class Ia and HLA-E structures, there is strict preservation of four tyrosine residues at positions 7, 59, 159 and 171, which typically interact with

the first N-terminal residue of the bound peptide. This strongly argues for the binding of a peptide or similar hydrogen bond-forming organic molecule within the groove. In most class Ia complexes a conserved hydrogen bond links the amide nitrogen of the second peptide residue to the terminal carboxyl oxygen of Glu-63 on the α1 helix. However, in HLA-F residue 63 is threonine which will have different hydrogen binding activity.

The floor of the groove of HLA-F is illustrated in Figure 7.1 and is significantly different to that of HLA-E and also to that of the class Ia molecules. In HLA-E, the floor is dominated by a hydrophobic stack which is absent in HLA-F. The mid portion of the groove is dominated by a centrally placed tryptophan at position 97 in HLA-E or a large charged molecule in the class Ia molecules. However, the presence of a small glycine residue at this position in HLA-F allows much greater space for a ligand in this portion of the groove. Overall, the groove would appear more spacious and more highly charged than that of HLA-E. Two histidines are spaced closely enough at positions 114 and 116 to form a hydrogen bond between their imidazole nitrogen atoms, so stabilising this portion of the floor of the groove. As the pKa of histidine is close to neutrality, a hydrogen bond would be highly sensitive to subtle changes in its local environment, such as the presence of charged groups on any potential incoming peptide or other ligand. A similar histidine pair has been seen at a different position in the peptide binding groove of HLA-E. Key residues in the first or 'A' pocket are all generally hydrophobic, but a Glu at postion 163 offers the potential for a charged interaction in this region of a putative peptide ligand. In the second or 'B' pocket region, most of the

residues are relatively small and this pocket is likely to be large and hydrophobic. The 'C' pocket which typically accommodates peptide residue 6, is likely to be large and polar, incorporating Asn-70 and Asp-74 and sharing some similarity with this region of HLA-B8. The small glycine at position 97 is likely to make the D pocket relatively large. In addition, the presence of an asparagine at position 99 is atypical and is consistent with a relatively charged environment in this region. There is a significant hydrophobicity to the 'E' pocket region which contains Trp-133, a residue that is conserved across all class Ia and Ib molecules. The 'F' pocket bears striking resemblance to that of HLA-E and is likely to be a deep hydrophobic pocket.

We have already discussed the apparent absence of peptides eluted from recombinant HLA-F molecules refolded *in vitro*. *In vivo*, it was shown that HLA-F interacts with TAP-1 in a B cell line. Interaction with the TAP multicomplex correlates with the loading of peptides on class Ia molecules. Class Ia molecules are stabilised by the binding of an appropriate peptide that allows cell surface expression of mature class Ia molecules. HLA-F molecules purified from B cells are unstable when incubated at 37 °C, suggesting that HLA-F is not associated with a peptide inside these cells (Wainwright, Biro and Holmes, 2000).

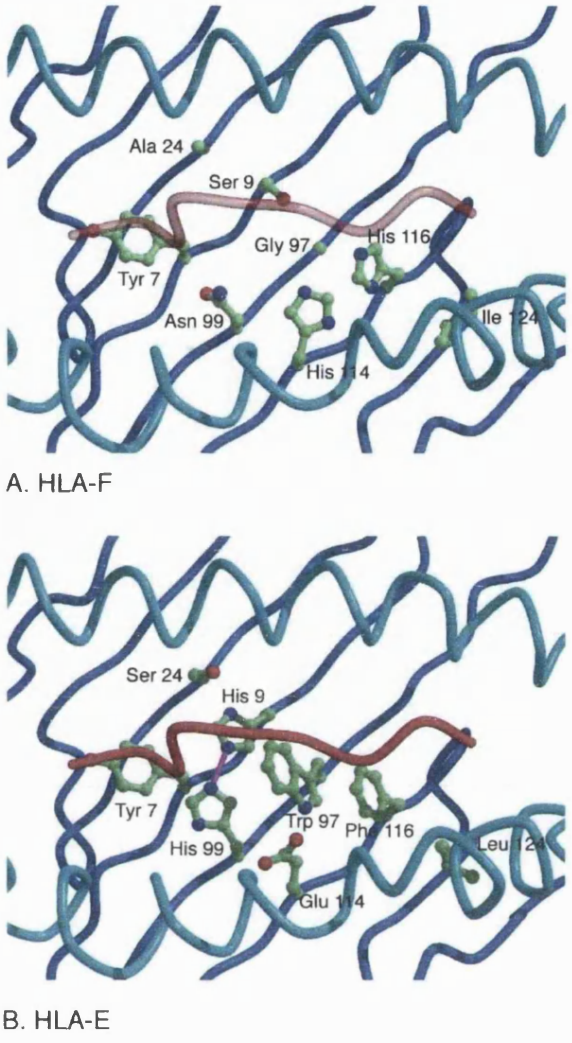

Figure 7. 1 Comparison of the putative peptide binding groove of HLA-F with the known structure of HLA-E (Dr C. O'Callaghan).

The alpha helices are coloured in light blue and the beta sheet strands are coloured in darker blue. The position of the peptide main chain in HLA-E is shown in red and for orientation in HLA-F, the equivalent position of this peptide is shown in translucent red. Key residues pointing into the central region of the floor of the grooves of both molecules are shown in green with sidechain nitrogen atoms coloured blue and sidechain oxygen atoms coloured red. Hydrogen bonds are shown as pink lines. The floor of the HLA-E groove contains bulky stacked hydrophobic residues, whereas the model of HLA-F indicates that there is more space in this central portion of the groove.

All the results presented suggest that HLA-F does not bind a peptide in the conditions studied. HLA-F could, as is the case for HLA-E, bind specific peptide only present in certain conditions. As with H2-M3, HLA-F may present specific conserved structures from a group of pathogens. However, HLA-F association with the transporter associated with antigen processing does not exclude HLA-F binding to non-peptide ligands.

7.6 HLA-F receptors

Until now, HLA-F has never been detected at the cell surface. Nevertheless we hypothesised that under certain conditions HLA-F molecules could reach the cell surface and be able to interact with receptors expressed at the cell surface of other immunological cells. To identify cells expressing potential receptors for HLA-F, we produced and used HLA-F tetramers. Monocytes and B cells were stained by HLA-F tetramers, and we identified two receptors ILT2 and ILT4. HLA-F at the cell surface could provide increased ligand density for the ILT 2 and ILT 4 molecules that could influence the activation threshold of cells displaying these receptors. Alternatively, the interaction of HLA-F with the ILT receptors could have an intracellular role in regulating the expression of such inhibitory receptors. It is also possible that other receptors could bind to the HLA-F tetramer. In particular, it is known that ILT2 binds to class I molecules through their α3 domain (Chapman, Heikeman and Bjorkman, 1999) and it remains possible that another receptor could interact with α1 and α2 domains and the putative peptide binding groove of HLA-F. Clearly the presence of a bound peptide species could influence such an interaction.

Binding of HLA-F recombinant protein to ILT2 and ILT4 recombinant proteins suggests that HLA-F might reach the cell surface. In addition, it was shown that HLA-F tetramers might bind other molecules expressed at the cell surface of monocytes and B cells in blood.

7.7 Future research

Few papers have been published on HLA-F and important issues remain to be addressed. No mouse homologue of HLA-F has been identified. Ideas concerning HLA-F function are mainly built around two hypotheses based on recent results. HLA-F might bind specific ligands and be transported to the cell surface where it would interact with receptors at the cell surface of immune cells, or HLA-F might have a restricted intracellular function which could be linked to class I antigen processing. One approach would be to try to identify potential ligands to HLA-F. Another approach would be to gather more information concerning HLA-F *in vivo* biochemistry and to study its possible function in the regulation of other class I molecule expression.

7.7.1 Identification of potential ligands to HLA-F recombinant proteins

In vitro, it has been shown that HLA-F and β2m recombinant proteins can associate without any synthetic peptides. However, HLA-F may not bind small and undetected bacterial molecules. Acid elution of potential ligands and analysis of the eluate by HPLC could detect a specific peptide. However, mixtures of small ligands would be difficult to detect using this technique. Similarly, the use of other analytical techniques such as mass spectrophotometery might be inefficient to detect a mixture of ligands.

More information could be gathered by crystallisation and solving the structure of HLA-F, the potential binding groove in particular. In preliminary work with the Division of Structural Biology at the University of Oxford, light scattering instruments were used to show that a structural study of HLA-F could be considered.

Another way to identify potential peptide ligands to HLA-F *in vitro* would be to screen a phage library. Phage can express random peptides of specific length on their surface. Ideally a phage whose peptide binds a class I molecule could be isolated and the sequence of the peptide expressed determined. This strategy has been successful used to identified CD1-d peptide ligands (Castano et al., 1995). The major constraint of this experimental procedure is that the peptides are attached to the phage and are not free. Class I molecules have closed peptide binding grooves which only bind free peptide of a correct length. CD1, a more distant class I homologue has an open binding groove. A structural model of the HLA-F binding groove demonstrates that it likely to be similar to the class I binding groove. However, it cannot be excluded that HLA-F could bind peptides expressed by phage.

7.7.2 *In vivo* identification of potential ligands to HLA-F

In vivo, in an effort to identify potential antigen presented by HLA-F, expression studies could be extended and studies of HLA-F expression in disease initiated. Cell surface expression is an indication that class I molecules bind with their ligands, thus FG1 could be used to monitor HLA-F cell surface expression.

HLA-F as well as HLA-E, and HLA-G transcripts has been detected in foetal liver (Houlihan et al., 1992). In this tissue HLA-F molecules could not be immunoprecipitated with w6/32 (Wainwright, Biro and Holmes, 2000) and we were unable to detect them with FG1. However, HLA-F was immunoprecipited using an anti-β2m antibody. These data differ from results obtained from B cell lines and further investigations are needed. General screening of HLA-F expression on other foetal tissues using FG1 antibody should be considered. Staining of placental cells which expressed the two other MHC class Ib molecules could also be undertaken.

Another interesting result of the tissue section expression screening is the strong staining of endothelial cells. We plan to perform HLA-F expression studies on kidneys, which are rich in endothelial cells. Thus the staining of healthy kidney and rejected transplanted kidney is being carried out in a collaborative study with Dr B. Hartley (St James Hospital, Leeds).

Although no disease has been associated with the HLA-F gene, we hypothesised that HLA-F could have a role in the control of the immune system. HLA-F expression and function might vary in specific disease. For example HLA-F might bind specific peptides from pathogens. Different groups of pathogens and different diseases could be screened for HLA-F cell surface expression. FG1 could be used to look at HLA-F cell surface expression on PBMC.

7.7.3 Study of HLA-F *in vivo* biochemistry

HLA-F, as class Ia molecules, interacts with TAP molecules in the ER. However, HLA-F does not reach the cell surface. Further studies or HLA-F intracellular trafficking and interaction with other proteins may reveal interesting results, especially if HLA-F has an intracellular function.

w6/32 does not bind to HLA molecules when they are associated with TAP (Sadasivan et al., 1996). Thus the amount of free HLA-F can be immunoprecipitated with w6/32 and compared with the amount associated with TAP, immunoprecipitated from a digitonin B cell lysate. Pulse chase experiments could be conducted and HLA-F immunoprecipitated with FG1 from digitonin cell lysate. Association of HLA-F with different intracellular proteins could be used to gather more data on the HLA-F maturation pathway. Differences between HLA-F and class Ia associated molecules could lead to further information on HLA-F function.

HLA-F could regulate class Ia antigen presentation function in specific cell lines. To study this hypothesis a class I molecule, such as HLA-A2 could be transfected on its own or in association with another class I gene in 721.221 cell lines which only express HLA-E and HLA-F. Effects on HLA-A2 cell surface expression and the range of peptides presented could be studied when high levels of HLA-F are expressed in these cell lines.

7.7.4 HLA-F receptors

We were not able to completely block B cell and monocytic cell staining by the HLA-F tetramer using anit-ILT2 and anti-ILT4 blocking antibodies. This suggests that other ligands expressed on these cells might be able to specifically bind HLA-F. Expression libraries of these cell types could be used as a comprehensive approach to identify potential receptors. The library could be screened using HLA-F tetramers.

Physiological studies of the effect of HLA-F tetramer on B cell lines such as calcium release, would be interesting and might show activation of these cells by HLA-F tetramers.

7.8 Conclusion

It is possible that HLA-F does not bind a ligand and has a strictly intracellular function. However, MHC Class I protein features are conserved for HLA-F and the structural model suggests that HLA-F would be able to bind peptides. The results presented here, demonstrating a restricted pattern of expression in lymphoid tissues, the association with TAP-1 and the identification of ILT2 and ILT4 as receptors to HLA-F, support the hypothesis that HLA-F could have a specialised immunological function comparable to other class Ib molecules. However, HLA-F cell surface expression could not be demonstrated except when cells are grown at 26°C, and peptide could not be identified. It is possible that, like HLA-E or H2-M3, HLA-F cell expression might be regulated by the presence of a very restricted peptide. The identification of the peptide would give a valuable indication of HLA-F functions, and this is now the main focus in HLA-F

studies. However, as for HLA-E, HLA-F ligand might ultimately be revealed by serendipity.

Appendix 1: Primers and Plasmids

PCR-Script constructs (Stratagene)

Primers

F L5' 5'CTC CTG CTG CTC TCA GGG GCC CTG3'

F U3' 5'GAG GCA CAA GTG CAA TTC TGC TAC3'

Clone name: **F0b2**

1F5' and 2F5' PCR oligos were used to clone HLA-F into PCR-Script (cloning vector). HLA-F sequence cloned start at 18 nucleotides after the A of the ATG (start codon), the last nucleotide being in the untranslated region 22 nucleotides before the polyadenylation signal. Blunt ended products of this PCR were cloned into PCR-Script digested with *Srf* I.

pET 30b constructs (Novagen)

Primers

1F5' 5'GTC ATA TGG GCT CCC ACT CCT TGA GGT ATT TCA GCA CCG CT 3'

2F5' 5'GTC ATA TGG Gaa gtC ACa gtT TGA GGT ATT TCA GCA CCG CT 3'

(Lower case letters represent nucleotides altered synoymously).

1F5' and 2F5' are two forward PCR oligos used to clone HLA-F (α1-α3 domains(1-275)) in pET 30b (bacterial system of expression). They have a Nde I restriction site.

1F3' 5'GTC TCG AGC CAT CTC AGG ATG AGG GGC TGG

Reverse PCR oligo to clone HLA-F (α1-α3 domains) in pET 30b (bacterial system of expression). It has a Xho I restriction site.

Clone name: **F1cl1**

A pET 30b construct for expression of HLA-F (α1-α3 domains(1-275) in bacteria.

PCR product from cDNA amplified with 1F5' and 1F3' primers and inserted between Nde I and Xoh I sites of pET 30b plasmid (Novagen). Kanamycin resistant plasmid.

Clone name: **F1cl2**

A pET 30b construct for expression of HLA-F (α1-α3 domains(1-275) in bacteria.

PCR product from cDNA amplified with 2F5' and 1F3' primers and inserted between Nde I and Xoh I sites of pET 30b plasmid (Novagen). Kanamycin resistant, plasmid.

pGMT7 constructs

Primers

FBL5' 5'TGA CAC CAT ATG Gga agt CAC agt TTG AGG

Forward PCR oligo to clone HLA-F (α1-α3) in pGMT7 (T7 bacterial expression vector). Nde I restriction site.

FBL13' 5'TCG CTA GGA TCC TCA GGG CTG GGG AGA CTG CTC CCA TCT CAG GAT GAG GGG

Reverse PCR oligo to clone HLA-F (α1-α3 280) in pGMT7 (T7 bacterial expression vector). BamH I restriction site.

FBL2 3' 5'TCG CTA GGA TCC TCA GGG AGA CTG CTC CCA TCT CAG GAT GAG GGG

Reverse PCR oligo to clone HLA-F (α1-α3 278) in pGMT7 (T7 bacterial expression vector). BamH I restriction site.

FBL3 3' 5'TTG GCT AGG ATC CTC ACT GCT CCC ATC TCA GGA TGA GGG G

Reverse PCR oligo to clone HLA-F (α1-α3 276) in pGMT7 (T7 bacterial expression vector). BamH I restriction site.

TEF 5' 5'TGA CAC CAT ATG GGA AGT CAC AGT TTG AGG3'

Contains NdeI site to clone in pGMT7 and the modified N terminus alpha 1 domain sequence necessary for high protein expression level. (cf 2F5' primer)

TEF 3' 5'TGT GAT AAG CTT AAT GCC ATT CAA TTT TCT GTG CTT CAA AAA TAT CAT TCA GGG ATC CGG GCT GGG GAG ACT GCT C3'

Contains in frame the C terminus sequence of α3 domain, a linker (GS), a BirA recognition site, and a Hind III site (for cloning in pGMT7).The recognition site is (LNDIFEAQKIEWH).

Clone name: **F1**

A pGMT7 construct for expression of HLA-F (α1-α3 domains (1-280 aa)) in bacteria. PCR product from F0b2 amplified with FBL 5' and FBL1 3' primers and inserted between Nde I and BamH I sites of pGMT7 plasmid (ampicillin resistant).

Clone name: **F2**

A pGMT7 construct for expression of HLA-F (α1-α3 domains (1-278 aa)) in bacteria. PCR product from F0b2 amplified with FBL 5' and FBL1 3' primers and inserted between Nde I and BamH I sites of pGMT7 plasmid (ampicillin resistant).

Clone name: **F3**

A pGMT7 construct for expression in bacteria of HLA-F (α1-α3 domains (1-276 aa).

PCR product from F0b2 amplified with FBL 5' and FBL1 3' primers and inserted between Nde I and BamH I sites of pGMT7 plasmid (ampicillin resistant).

Clone name: **TEF2**

A pGMT7 construct for expression in bacteria of HLA-F (α1-α3 domains (1-280 aa) with a BirA recognition tag. PCR product from F0b2 amplified with TEF 3' and TEF 5' primers and inserted between NdeI and BamH I sites of pGMT7. Ampicillin resistant plasmid.

Eukaryotic expression pcDNA plasmids (invitrogen)

Primers

CO48 5'CGT GCG GGA TCC GCC ACC ATG GCG CCC CGA AGC CTC CTC CTG CTG CTC TCA3'

Forward PCR oligo to clone HLA-F (full length) in pcDNA3.1a (eukaryotic system of expression). It has a BamH I restriction site. A Kozak sequence preceding the AUG initiator was added to enhance expression (Kozak, 1987).

CO49 5'GTA GTT GGT ACC CAC TGC AGC CTG AGA GTA GCT CCC TCT GTT

Reverse PCR oligo to clone HLA-F (full length) in pcDNA3.1a (eukaryotic system of expression). It has a Kpn I restriction site.

CO50 5'CGT GCG GGT ACC GCC ACC ATG GCG CCC CGA AGC CTC CTC CTG CTG CTC TCA

Forward PCR oligo to clone HLA-F (full length) in p cDNA3.0 (eukaryotic system of expression). It has a Kpn I restriction site. A Kozak sequence preceding the AUG initiator was added to enhance expression (Kozak, 1987).

CO51 5'GTA GTT GGA TCC TCA CAC TGC AGC CTG AGA GTA GCT CCC TCT GTT

Reverse PCR oligo to clone HLA-F (full length) in pcDNA3.0 (eukaryotic system of expression). It has a BamH I restriction site.

Clone name: **F/Myc-His**

A pcDNA 3.1a construct for expression in mammalian cell of HLA-F complete open reading frame with the adjunction of a myc and a his tag. PCR product from F0b2 amplified with CO48 and CO49 primers and inserted between BamH I and Kpn I sites of p cDNA 3.1a. The result is the expression in mammalian cells of the complete HLA-F protein. The protein expressed is HLA-F tagged with a myc and a his tag at the C-terminus of the protein.

Clone name: **F**

pcDNA 3.0 construct for expression in mammalian cell of HLA-F (complete open reading frame). PCR product from F0b2 amplified with CO48 and CO49 primers and inserted between BamH I and Kpn I sites of p cDNA 3.0. The result is the expression in mammalian cells of the complete HLA-F protein.

Clone name: **β2m**

β2m cloned in pcDNA 3.0 was a gift from Dr O'Callaghan.

Appendix 2: Surface Plasmon Resonance

The affinity of a protein/protein interaction can be measured at equilibrium using SPR.

Considering the binding event:

$$A+B \underset{Kd}{\overset{Ka}{\rightleftharpoons}} AB$$

The dissociation constant (Kd) has Molar units and is preferred to the association constant (Ka).

$$Ka = \frac{[AB]}{[A][B]} \quad \text{and} \quad Kd = \frac{[A][B]}{[AB]}$$

At equilibrium

the net rate of complex association = the net rate of complex dissociation

$$Ka\,[A][B] = Kd\,[AB]$$

This equation can be modified to express the constant of dissociation (Kd) only in function of:

-Concentration of injected analyte (A)

-Bound analyte measured in response units (Bound)

-Maximun of response units (Max)

$$Bound = \frac{A * Max}{A + Kd}$$

Practically the binding response at equilibrium (Bound) was analysed in function of different concentrations of analyte (A). Kd and Max were then calculated by non-linear cure fitting to the data obtained using the computer software Origin.

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