HLA-B27 subtypes differentially associated to an autoimmune disease: Analysis of peptide display and attempt to define their recognition pattern by the Killer cell Ig-like receptor KIR3DL1

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The important thing is not to stop questioning
Curiosity has its own reason for existing

Albert Einstei

Contents

ABBREVIATIONS

Amp	Ampicillin
APC	Antigen Presenting Cell
APS	Ammonium persulphate
AS	Ankylosing Spondylitis
Å	Angstrom (0.1 nm)
bp	base pair
BSA	Bovine Serum Albumin
CD	Circular Dichroism
CTL	Cytotoxic T Lymphocyte
Da	Dalton
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DSC	Differential Scanning Calorimetry
dsDNA	double-stranded DNA
DTT	Dithiotreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
FPLC	Fast Performance Liquid Chromatography
g	g-force
g	gram
IPTG	Isopropyl-β-D-thiogalactopyranoside
K	Kelvin
kDa	kilo Dalton
kb	kilobase
KIR	Killer Cell Immunoglobulin-like Receptor
l	liter
LB	Luria-Bertani
LIR-1	Leukocyte immunoglobulin-like receptor-1
LPS	Lipopolysaccharide
M	molar (mole/liter)
mA	milliampere

VI Abbreviations

MALDI-TOF/TOF	Matrix-assisted laser desorption/ionization time-of-flight/ time-of-flight
min	minutes
MHC	Major Histocompatibility Complex
mM	millimolar
MW	molecular weight
NCBI	National Center for Biotechnology Information
NK	Natural Killer cells
nm	nanometer
OD	optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBT	Phosphate buffered saline + Tween
PCR	Polymerase chain reaction
PDB	Protein Data Bank
pMHC	MHC antigen complexed with a peptide
PMSF	Phenylmethylsulfonylfluoride
rpm	revolutions per minute
RT	room temperature
SEC	size exclusion chromatography
SDS	Sodium dodecyl sulfate
s	second
TCR	T-Cell Receptor
TE	Tris-EDTA buffer
TEMED	Tetramethylethylenediamine
Tris	Trihydroxymethylaminomethane
Triton X-100	Polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
Tween-20	Polyoxyethylene (20) sorbitan monolaurate
U	unit
UV	ultraviolet light
w/v	weight/volume
WBC	White Blood Cell
X-Gal	5-bromo-4-chloro-3-indolyl-β-galactosidase

Symbols of amino acids

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

1. Introduction

1.1 The human immune system

The mammalian immune system has evolved to protect from pathogens (a great variety of infectious viruses, bacteria, parasites and malignant cells) which are constantly threatening the integrity of the organism. It is composed of a complex of cells, organs and tissues, arranged in an elaborate and dynamic communication network. The immune system is, in its simplest form, a cascade of detection and adaptation, which is remarkably effective. Since it must manage with many different types of malignant cells, a wide variety of immune responses are required. The immune system includes effector mechanisms that destroy a broad range of microbial cells, and therefore it has to differentiate between pathogen-derived, dangerous antigens ("non-self") and antigens which come from the organism itself ("self") and are needed for a proper functioning of the body. Avoidance of destruction of the mammalian host's own tissues is referred to as self-tolerance whereas failure of self-tolerance leads to autoimmune diseases (Chaplin, 2003).

There are different ways in which the immune system can fail and cause immunopathological reactions leading to autoimmune diseases. However, the primary role of the immune system is to protect the body from infection by creating and maintaining barriers that prevent bacteria and viruses from invading the host. One component of immunity, the phylogenetically ancient innate immune response, fights infections from the moment of first contact and is the fundamental defensive weapon of multicellular organisms (Kimbrell and Beutler, 2001). If a pathogen enters the body, the innate immune system which is equipped with specialized cells (macrophages, polymorphonuclear leukocytes and mast cells) for detection and often elimination of the invader before it is able to reproduce and cause serious injury to the host, will often be sufficient to prevent harm to the host. A pathogen that successfully evades the innate immune cells faces a second barrier, the adaptive immune system. The important difference between these two is that an adaptive immune response is highly specific for the pathogen whereas innate immune defense is non-specific, and recognizes and responds to a pathogen in a generic way (Janeway, 1989). Moreover, the adaptive system gains the ability to recognize a pathogen, and to mount stronger attacks each time that the same pathogen is encountered later in life.

1.1.1 Innate immunity

Innate immunity developed before the emergence of vertebrates, and most multicellular organisms exclusively depend on it. The innate immune system is comprised of cells and mechanisms that defend the host from infection by other organisms, in a non-specific manner. It is a system that acts effectively without previous exposure to a pathogen. Therefore, it is the first line of defense against infection (Kimbrell and Beutler, 2001). Responses here have broad-spectrum, pathogen exposure results in no immunologic memory and there is a limited repertoire of recognition molecules (Janeway, 1989). The same molecular modules are found in plants and animals, indicating that it arose before these two kingdoms split (Hoffmann et al., 1999).

There are a number of barriers which prevent pathogens from entering an organism. From the anatomic point of view these are mainly skin layers which have low pH (caused by lactic and fatty acids), tightly packed epidermal cells and keratin. There are also internal, impermeable mucosal epithelial surfaces that overlay the epithelium in the respiratory, gastrointestinal and genitourinary tracts (Isberg 1991). The flushing action of tears and saliva helps to prevent infection of the eyes and mouth whereas the trapping effect of mucus helps protecting the lungs and digestive systems from infection (Roit et al., 1985). The normal temperature of the body of vertebrates also inhibits growth of many microorganisms. Except for these factors there are additionally chemical ones, soluble proteins (lysozyme, anti-microbial substances, interferon) which are either released from the cells when activated or constitutively present in biological fluid (i.e. defensins, low molecular weight proteins with antimicrobial activity that are found in the lung and gastrointestinal tract), (Frank and Fries, 1991; Yang et al., 2002).

The innate immune system uses a variety of pattern recognition receptors, which can be expressed in intracellular compartments, on the cell surface or can be secreted into the bloodstream (Medzhitov and Janeway, 1997). The major functions of the innate response include recruitment of immune cells to sites of inflammation, through the production of chemical factors like chemical mediators (cytokines). Once a pathogen penetrates the skin or mucosal epithelium, it usually establishes a local infection (Janeway and Medzhitov, 2002). Tissue damage and pathogen-derived antigens are signals for tissue macrophages to secrete chemotactic cytokines.

The roles of innate immunity are also identification and removal of foreign substances (present in organs, tissues, the blood and lymph), by specialized white blood cells (WBC) and activation of the adaptive immune system (by a process called antigen presentation; Figure 1.8), (www.vetmed.wsu.edu). All white blood cells are known as leukocytes. They act like independent, single-celled organisms since they are not exclusively associated with any organ or

tissue (Alberts et al., 1994). To the innate leukocytes we subsume mast cells, eosinophils, basophils, natural killer cells, and the phagocytes (macrophages, neutrophils and dendritic cells). All of them function in identifying pathogens that might cause infection and eliminating them by direct killing or by engulfment and destruction (Chaplin, 2003). NK cells develop in the bone marrow under the influence of IL-2, IL-15 and stromal cells. They occur in peripheral blood, in the spleen and other secondary lymphoid tissues. The cytotoxic activity of NK cells is inhibited by the receptors on their surface (see also section 1.5) thus they kill self cells with down regulated expression of HLA class I molecules. NK cells express also activating receptors on their surface and can kill other cells by antibody-dependent cell-mediated cytotoxicity (Brown et al., 2001).

1.1.2 Adaptive immunity

The adaptive immune system is a relative newcomer by evolution. It is composed of highly specialized, systemically acting cells and carries out processes that eliminate pathogens that have entered the body. Mechanisms of receptors that generate adaptive responses involve variability and rearrangement of receptor gene segments, and consequently the recognition of the antigens is specific and provides immunological memory of infection (Janeway and Medzhitov, 2002). The adaptive immune system augments the responses of the innate system. While the second one is our first line of defense, the adaptive immune system acts as a second line of defense and affords protection against re-exposure to the same pathogen. Highly specialized cells of the adaptive immune system proliferate when a pathogen threatens the organism to mount an effective response (Chaplin, 2003).

The adaptive immune response provides the immune system with the ability to recognize and remember specific pathogens. This will in future result in better immunity each time when the pathogen is encountered and it is called adaptive immunity because the body's immune system prepares itself for future challenges. Adaptive immunity is triggered when a pathogen escapes the innate immune system and generates a threshold level of antigen (Medzhitov and Janeway 1999). The major function of the adaptive immune system is to distinguish specific “non-self” antigens from the “self” components of the body. This takes place during the process of antigen presentation described in more details in section 1.2.2. Adaptive immunity generates the responses that are tailored to maximally eliminate specific pathogens or pathogen-infected cells. Moreover, the specific immune system develops the immunological memory in which each pathogen is “remembered” by the cells of immune system. The antigen-specific receptors of the

adaptive immune system are assembled by rearrangement of germ line gene elements to form T cell receptors (TCR) and immunoglobulins (Ig) (B cell antigen receptor). From the few hundred gene elements encoded by the germ line, astronomical numbers of distinct antigen receptors can be formed, each with unique specificity (Chaplin, 2003).

The cells of the adaptive immune system are called lymphocytes. They are specialized leukocytes termed B cells and T cells, and are derived from hematopoetic stem cells from the bone marrow (Schwarz and Bhandoola, 2006). B cells play a role in humoral immune responses, whereas T cells are involved in cell-mediated immune responses. Both B cells and T cells are equipped with receptor molecules that allow them to recognize and respond to their specific targets. A critical difference between B cells and T cells is how each lymphocyte recognizes its antigen. B cells recognize it in its native form, either as a free antigen in the blood or in lymph as cell-bound form (e.g. on a bacterial surface), whereas T cells recognize their antigen in a processed form, as a peptide fragment presented by a major histocompatibility complex (MHC) molecule to the TCR on T-cells.

T cells recognize a “non-self” target, such as a pathogen, only after it has been processed and presented by a receptor molecule specified by the MHC. During their migration through the thymus T cells differentiate into two subsets, each with specific functions: $CD8^+$ T cells kill cells harboring intracellular parasites like viruses and bacteria or cells that are otherwise damaged (Mescher, 1995), while $CD4^+$ helper T cells act to regulate cellular and humoral immune responses (Williams et al., 1991). The major difference between them is that the killer T cells recognize antigens complexed with class I MHC antigens, while helper T cells recognize antigens presented by class II MHC antigens. Helper T cells are immune response mediators and have no cytotoxic activity. Therefore, they cannot kill infected cells but their role in the immune response is directing other cells to perform these tasks (Janeway, 2001). They provide extra signals to activate both killer cells and antibody production by B cells (Janeway, 2001).

The recognition of a specific pMHC complex (an MHC molecule carrying an antigenic peptide) is the task of T-cell receptors (TCR). The TCR has no measurable affinity for antigenic peptide alone and low affinity for MHC containing other peptides (Chaplin, 2003). This fact forms the basis for the phenomenon of MHC restriction, originally described by Zinkernagel and Doherty (Zinkernagel and Doherty, 1997). When the T cell receptor interacts with an antigen bound to MHC Class I molecules, it forms a complex with a series of polypeptides constituting the CD3 antigen (Peterson et al., 1998; Kuhns and Davis, 2007), which in turn initiates a cascade of phosphorylation events (Christensen and Geisler, 2000) and provides a partial signal for cell activation. The whole process involves several steps, one of which is the activation of ITAM

(immunoreceptor tyrosine-based activation motif) of the TCR complex (Weis, 1993; Gruta et al., 2004). There are three ITAMs in the ζ chain of CD3, one in the cytoplasmic domain of CD3 γ, one in δ, ε (Haks et al., 2002), Igα, and Igβ (Gold et al., 1991; Flaswinkel and Reth, 1994). Such motifs are also found in activating receptors on NK cells (Christensen and Geisler, 2000). Phosphorylation of the tyrosine residues within the ITAMs of CD3 recruits a Protein Tyrosine Kinase (PTK ZAP-70), which activates Src homology 2 (SH2) domains (Haks et al., 2002). Subsequential phosphorylation of signaling proteins leads to the activation of protein kinase C (PKC), constituting an early membrane signaling event in T cell activation. This leads to the formation of a complex composed of many adapters and to an increased intracellular level of Ca^{2+}. This cascade activates calcineurin, which regulates transcription factors (Christensen and Geisler, 2000).

The full activation of killer T cells requires costimulatory molecules like CD28 on the T cell surface and CD80 or CD86 on the antigen presenting cell (APC) (Lenschow et al., 1996; Hayakawa et al., 2001). When exposed to infected cells, killer cells release cytotoxins, which cause apoptosis of the infected cell. Killer T cell activation is tightly controlled and generally requires a very strong activation signal, or additional activation signals provided by helper T-cells (Janeway, 2001).

1.2 The Major Histocompatibility Complex (MHC)

MHC is the immunologically most important genome region in vertebrates (Horton et al. 2004). Antigens encoded by the MHC were discovered on the surface of leukocytes. In human, the MHC is also called Human Leukocyte Antigen (HLA) complex. The development of the MHC concept and its nomenclature were formulated in the late 50s and at the beginning of the 60s of the 20th century. In 1958 Jean Dausset reported the first HLA antigen but the idea about the polymorphic nature of the HLA system appeared five years later, in 1963 (reviewed by Terasaki, 1990). Finally, intense investigations of this genomic region resulted in a crude map of the human MHC in the year 1993 (Campbell and Trowsdale, 1993). At the beginning of 21st century it was proposed that there was an extended MHC (xMHC) in humans. Sequencing of entire chromosome 6 in the year 2003 resulted in revealing the xMHC sequence which covers 7.6 Mb of the short arm of this chromosome (Horton et al., 2004, and Figure 1.1).

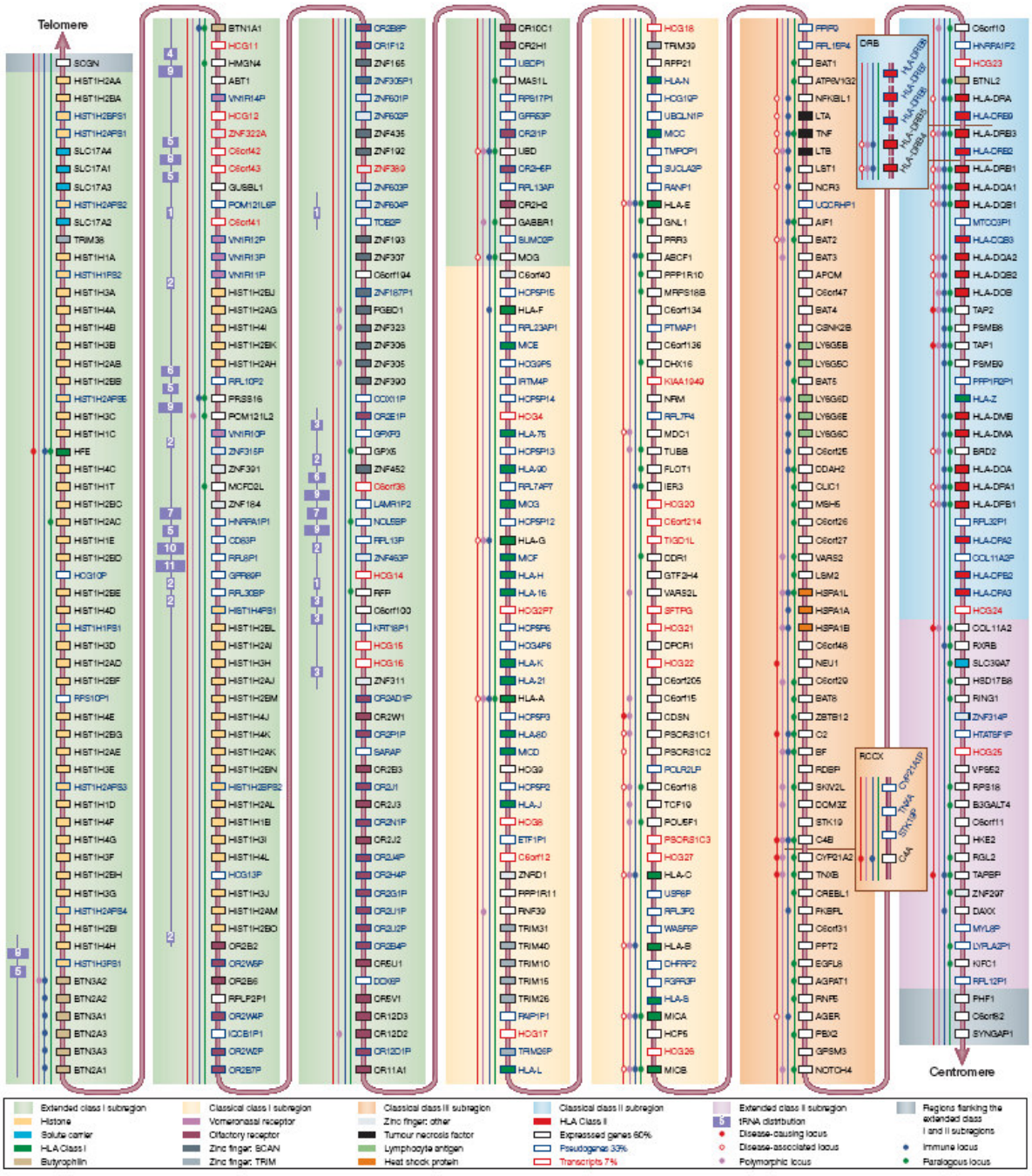

Figure 1.1. Gene map of the extended MHC on chromosome 6. All genes encoding MHC molecules are localized on the short arm of chromosome 6. The gene map is shown from telomere (left) to centromere (right). The extended class I subregion - green block, 3.9 Mb, the classical class I subregion - yellow block, 1.9 Mb, the classical class III subregion - orange block, 0.7 Mb, the classical class II subregion - blue block, 0.9 Mb and the extended class II subregion - pink block, 0.2 Mb. From Horton et al., 2004.

The HLA nomenclature has been changed many times. Now it reflects the way in which these molecules were characterized, i.e. as antigens that can be recognized by lymphocytes or antibodies from another strain (Roitt et al., 1985). The different definitions and nomenclature of HLA genes were recognized by Jan van Rood, Rose Payne, Ruggiero Ceppellini, and Walter

Bodmer and others in the 60s, indicating the need for International Standardization. This was assured by a series of International Workshops, starting in 1964 (Glen 1991). The current name has been assigned by the HUGO Gene Nomenclature Committee (HGNC) and the IMGT/HLA Sequence Databases (Robinson et al., 2003; Wain et al., 2004).

Many years of research gave a wide knowledge about the MHC. It is the most gene-dense region of the mammalian genome and plays a crucial role in the innate and adaptive immune systems as well as in autoimmunity. It is known that several genes of MHC are the result of duplication, many are highly polymorphic and a number of them are associated with diseases (Horton et al., 2004). The MHC contains 421 loci, 139 (33%) of which were classified as pseudogenes on the basis of similarity to already known proteins (Horton et al., 2004). Duplication of MHC genes resulted in the formation of six clusters and six superclusters. The HLA class I supercluster comprises genes encoding the class I proteins HLA-A, -B, -C, -E, -F, and -G and several class I-like genes (Radosavljevic and Bahram, 2003). The HLA class II cluster contains genes encoding the HLA-DR, -DQ and -DP proteins (Campbell and Trowsdale, 1997) and non-classical class II genes as well (Alfonso and Karlsson, 2000). A region between these two gene groups on chromosome 6 encodes class III molecules. A schematic representation of HLA genes on chromosome 6 is presented in figure 1.1.

It has been established that more than 5% of the expressed genes in human are involved in immune defense (Trowsdale and Parham, 2004). Many MHC genes, including HLA class I and II are engaged in responding to very variable antigens as part of the adaptive immune system. Class I molecules are not only involved in antigen presentation to T cells, but are also recognized by natural killer cells, which are part of the innate immune system. The MHC may therefore be considered as a linker between innate and adaptive immunity (Horton et al., 2004). Regions within the MHC that are associated with immunity can either be involved in antigen processing/presentation, inflammation, leukocyte maturation, the complement cascade, immune regulation or stress responses. A variety of functions as well as mutations in MHC genes influence the association with hundreds of diseases (Feder et al., 1996) among them most of the autoimmune disorders (Lechler and Warrens, 2000; Lazarus et al., 2002).

1.2.1 Structure and function of HLA class I molecules

MHC class I molecules (MHC) consist of a heavy chain (HC; about 43 kDa) that is non-covalently bound to β_2-microglobulin (β_2m or light chain; 12 kDa), encoded on chromosome 15. β_2m is a polypeptide which is non-polymorphic in humans and can also be found in free form in

the serum. The class I heavy chain consists of three extracellular domains (α1, α2 and α3, each about 90 amino acids long, which can be cleaved from the cell surface by proteolytic enzymes), a transmembrane domain and a cytoplasmic tail. The α2 and α3 domains both have internal disulphide bonds enclosing loops of 63 and 86 amino acid residues, respectively. The extracellular region of the HC is glycosylated, and the degree of glycosylation depends on the species and haplotype. The association with β_2-microglobulin is essential for expression of all typical class I molecules at the cell surface. When properly folded, both molecules are composed of β strands and are stabilized by intrachain disulphide bonds (Madden, 1995). However, both the α1- and α2 domains are also characterized by presence of a long α-helical region.

A schematic representation of an MHC class I molecule is shown in the figure 1.2 a, and the overall structure of the extracellular part of an MHC class I molecule in ribbon representation is presented in figure 1.2 b.

a) b)

Figure 1.2. Schematic representation of the MHC class I (a) and the extracellular region of the HLA class I in complex with β_2m and a peptide (b). a) The α1, α2 and α3 domains of heavy chain are colored in violet, α1 and α2 create a binding groove at the top of the molecule where the peptides bind and are presented to specific T cells receptors. The β_2-microglobulin (pink) is non-covalently associated with α1 of the heavy chain. The whole complex is docked in the outer membrane by the transmembrane domain. b) The three domains of the heavy chain, α1, α2, and α3, in ribbon representation are shown in blue, and are complexed with β_2m (red). The peptide binding groove is formed by the α1, and α2 domains, the peptide sitting in the groove is colored with yellow.

It is known from X-ray crystal structures that the α1- and α2 domains constitute a platform of eight anti-parallel β strands which support the two α helices that are oriented in anti-parallel fashion as well (Bjorkman and Parham, 1990, Parham, 1989) (Figure 1.3).

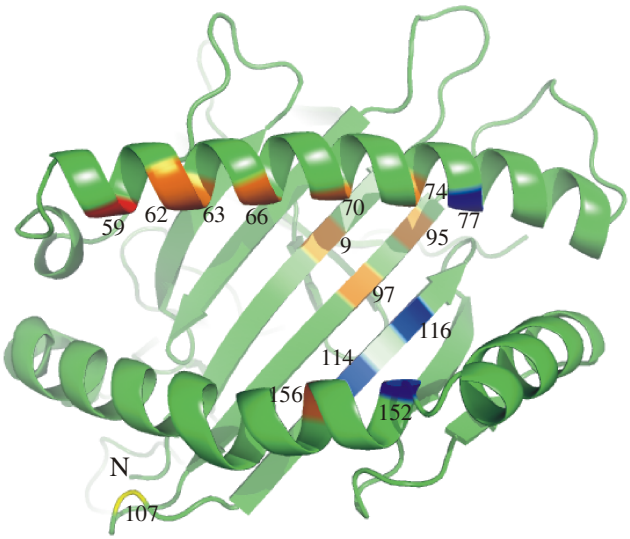

Figure 1.3. The HLA-B27 peptide binding groove seen from the top. The α1 and α2 domains consist each of four anti-parallel β strands and of two long α-helices. Both domains together form the so called peptide binding groove with eight-stranded β sheet and two α helices at the top. The locations of the most polymorphic residues are highlighted. The ones which occur in the peptide binding groove are orange, with exception of 59 (red – polymorphic residue in B*2703), 77, 114, 116, 152 (blue - polymorphic residues in B*2704 and B*2706).

The structure of HLA-A2 showed initially that the region between the two helices contains extra electron density representing the processed antigen peptide (Bjorkman et al., 1987). The binding groove of MHC class II is more open than in class I molecules, and longer peptides can be accommodated. In order to be bound, the peptide must be complementary to the MHC binding groove (Stern et al., 1994; Madden, 1995; Diegel et al., 2003).

Up to six pockets along the groove interact with peptide residues in case of MHC class I antigens. They are designated A through F. The A- and F pocket accommodate free N^- and C^- terminal side chains, respectively, thereby anchoring a peptide within the MHC class I molecule (Heemels and Ploegh, 1995). The mid-section pockets (B through E) show allele-dependent sizes and properties. Self-peptides eluted from MHC class I molecules have been shown to share the main-chain structure and be about nine amino acids long (Madden et al., 1991; Madden et al., 1992). For peptides bound by particular MHC molecules, several characteristic residues were identified; most often at N- and C-termini of the peptide (Madden et al., 1992; Bouvier and Wiley, 1994). These motifs distinguish the peptide repertoire for different MHC class I molecules. Thanks to analyses of three-dimensional structures of class I molecules it was shown that the peptide termini are buried at both ends of the binding groove whereas the centre of the peptide bulges out. Numerous hydrogen bonds stabilizing peptide binding are formed between residues of the class I HC and those of the peptide (Stern and Wiley, 1994). The main difference noticed in the groove of class II HLAs is that the ends of the peptide binding groove are not

closed and this is the reason why the peptides bound to class II molecules are longer (15 or more residues) and may extend beyond the ends of the groove (Rammensee, 1995). Similarly to MHC class I, in MHC class II anchor residues were recognized, however they are usually more difficult to identify. Class II binding pockets are generally more permissive with respect to the side chain which they will accommodate. A residue may act as an anchor, an inhibitor or be entirely neutral. Predicting whether a given peptide will bind is especially difficult as there is no upper limit to the length of the bound peptide (Davies and Flower, 2001; http://pps00.cryst.bbk.ac.uk/course/section11/Matt/PPS2.htm).

Variations in amino acid sequence between different HLA class I subtypes can give rise to dramatic changes in antigen presentation. The polymorphism of amino acids within the peptide binding groove can change the position of the pockets which is the structural basis for a different peptide binding affinity and antigen presentation to T cells (Bjorkman et al., 1987; Garratt et al., 1989).

The MHC class I molecules are expressed on the surface of most nucleated cells (Garcia-Fernández et al., 2001). They present proteolytic fragments of proteins that are synthesized inside the cell to a variety of ligands, in particular T cell receptors, but also to killer inhibitory receptors (KIR) residing on the surface of natural killer (NK) cells (Peruzzi et al., 1996; Pamer and Cresswell 1998). The purpose of this surveillance system is to identify abnormal body cells, such as those infected with viruses or those that have turned malignant. Cells displaying unfamiliar peptide antigens, e.g. fragments of viral proteins, are attacked and destroyed. A detailed description of antigen presentation is given in section 1.2.2.

1.2.2 Antigen presentation by HLA class I molecules

As already mentioned, the antigens that elicit the immune response in the body are displayed to T cells by molecules of the MHC. Antigens that are derived from proteins synthesized within the cell are called endogenous antigens and associate with class I molecules. Although most of the events that lead to assembly of class I antigens occur in the endoplasmic reticulum (ER) (Bijlmakers and Ploegh, 1993), proteins are processed already in the cytosol (Parham, 1996). Cytosolic processing renders antigens suitable for transfer to the ER lumen and class I restricted presentation (Heemels and Ploegh, 1995). The molecular machinery that generates peptide fragments for presentation by HLA class I molecules (Figure 1.8), is the proteasome, a proteolytic factory (Huston, 1997; Niedermann, 2002). This multisubunit complex displays minimally three distinct proteolytic activities *in vitro*. It cleaves at the C-terminus of basic,

hydrophobic, and acidic amino acids. Two subunits of proteasome, LMP2 and LMP7 (low molecular mass polypetides), have been extensively investigated and they might be involved in antigen presentation. Mutant cell lines with a deletion in the MHC region encoding both LMPs and peptide transporter genes are not able to present endogenously processed antigens (Cerundolo et al., 1990; Hosken and Bevan, 1990). Also proteases localized in the ER lumen are involved in peptide processing (e.g. cleavage of ER signal sequence during or after translocation to the ER). It was shown that proteases responsible for cutting peptides to a favored size are present within ER (Eisenlohr et al., 1992).

A specialized transporter complex, TAP (Transporter associated with Antigen Processing), that is encoded by the MHC, transports the peptide into the endoplasmic reticulum (Momburg and Tan, 2002). TAP is a heterodimeric complex consisting of TAP1 and TAP2, two transmembrane proteins, and is a member of the ATP-binding cassette family of transporters. The transmembrane segments contain polar residues which might form a hydrophilic pore through which the peptides are transported (Heemels and Ploegh, 1995). It was shown that hydrolysis is required for the translocation process, and both TAP1 and TAP2 seem to contribute to peptide binding site formation (Androlewicz and Cresswell, 1994; Androlewicz et al., 1994). TAP interacts with preassembled MHC:β_2-microglobulin complexes (Ortmann et al., 1994; Suh et al., 1994) and its dissociation occurs when the MHC complex accommodates a peptide and leaves the ER lumen. The process of heavy and light chain folding, assembly and peptide binding is mediated by molecular chaperones (e.g. calnexin, calreticulin) which are present in the ER lumen (Antoniou et al., 2003). Only properly folded protein complexes can be transported via the Golgi apparatus to the cell surface where they will be displayed for recognition by T cell receptors or receptors on NK cells. The antigen presentation by the endogenous pathway is shown on figure 1.4.

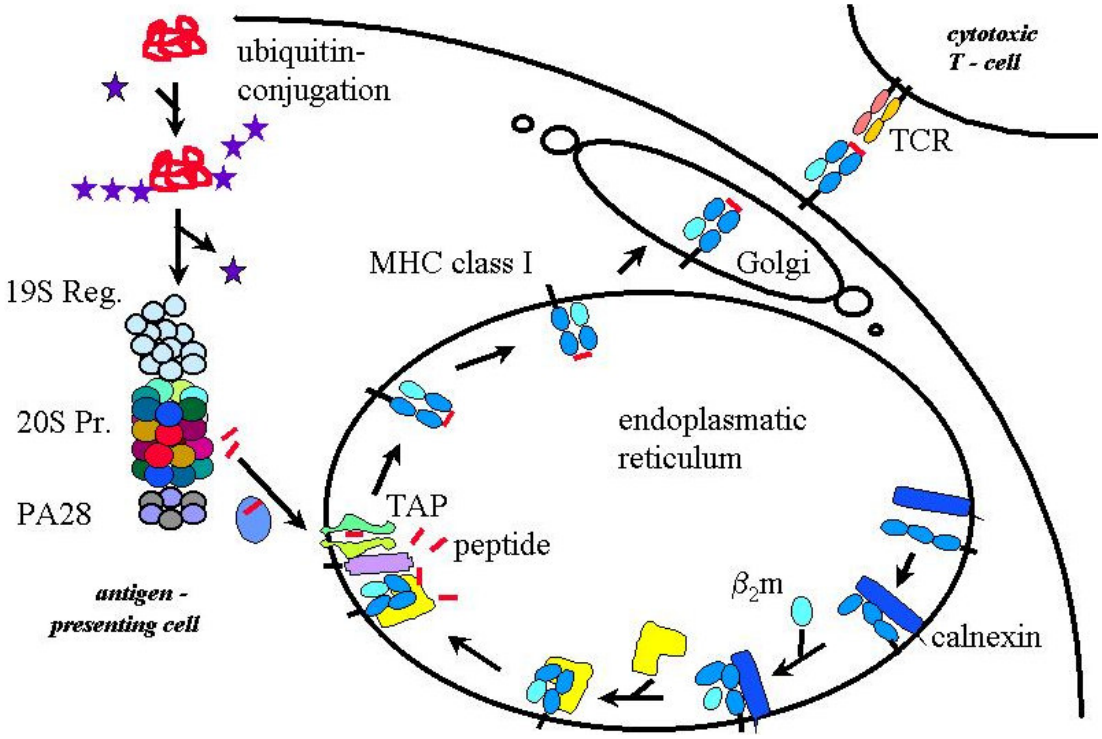

Figure 1.4. Model of MHC class I pathway of antigen presentation. Peptides are generated by proteasome complex that simultaneously bind 19S regulator and PA28. Antigen fragments are transported to ER lumen by TAP. Here they bind the peptide-binding groove of class I molecules, which facilitates final folding, release from ER retention and migration to the cell surface. If the antigen receptor of cytotoxic T cells recognizes the appropriate MHC carrying peptide (possibly derived from a virus protein), the T cell becomes activated to kill the infected target cell. The molecules in the ER are color-coded: dark blue – calnexin, light blue – HLA heavy chain, cyan - β_2, yellow – calreticulin, red – peptide, violet – tapasin, green – TAP1 and TAP2. www.uni-konstanz.de.

1.3 Autoimmunity and antigen presentation

Antigens presented by the MHC are displayed to T cells and NK cells. Both of these cells possess specialized receptors which can recognize the peptide localized in the binding groove of the HLA molecule together with a part of the HLA molecule. This stimulates the cells to react to the antigen presentation. As already mentioned, MHC molecules can display both "self" molecules which originate from the body's proteins and "non-self", foreign peptides derived from pathogens which infected the organism. Therefore it is of great importance that the receptors on T cells differentiate between self and pathogenic peptides. This capability is acquired in the process called positive and negative selection which occurs in thymus.

Maturation of T cells is initiated in bone marrow and requires migration of T cell precursors (pre-T cells) from the marrow to the thymus (Solof et al., 1992). The stages of T cell development are identified by the expression of specific cell surface markers, such as TCR (T Cell Receptor), CD3 (which serves as signal transduction component of TCR), and CD4/CD8

(Sebzda et al., 1999). Direct cell to cell interaction between these cells and thymic cells induces their proliferation and also differentiation (Goldrath and Bevan, 1999). Pre-T cells are CD4/CD8- double negative and need to undergo gene rearrangement to reach their mature form. Double negative cells rearrange either gamma and delta or alpha and betha chains of TCR genes. CD3+ CD4- CD8- γ/δ T cells are exported to the periphery in small numbers, whereas the majority of double negative cells will express α and β chain (Roit et al., 2001). After the α and β chain of the TCR undergo rearrangement, CD4 and CD8 are also expressed, and the cells on this stage (called double positive) are ready to go through positive and negative selection (von Boehmer et al., 2003; Starr at al., 2003). T cells recognize the peptide only when it is presented by the MHC molecules. During positive selection (thymic education) T cells that recognize the MHC with moderate affinity develop further and those which display very high or very low affinity to MHC molecules undergo apoptosis (Sebzda et al., 1999). Positive selection also assures that the proper TCR will select the adequate CD4 or CD8. CD4+ T cells recognize MHC II, and CD8+ T cells recognize MHC I (von Boehmer et al., 2003; Starr at al., 2003). Most of the positively selected T cells are believed to have receptors that recognize peptides derived from self-proteins. Many of these are eliminated during negative selection. Only T cells that fail to recognize self-antigens with high affinity can enter the periphery.

The whole immune system is organized in such a way that it reacts only to “non-self” peptides; however it may also happen that cells of the immune system will generate immune responses against peptides derived from proteins of the organism. There are some lymphocytes which are sensitized against "self" tissue cells, but they are usually controlled (suppressed) by other lymphocytes (Janeway et al, 2001). Autoimmune disorders are characterized by cellular or antibody responses against self-antigens and occur when the normal control process is disrupted or if normal body tissue is altered so that it is no longer recognized as "self."

Autoimmune disorders result in destruction of one or more types of body tissues, abnormal growth of an organ, or changes in organ function. The disorder may affect only one organ or tissue type or may affect multiple organs and tissues. Organs and tissues commonly affected by autoimmune disorders include blood components such as red blood cells, blood vessels, connective tissues, muscles, joints, and skin (Ramos and López de Castro, 2002). Examples of autoimmune and autoimmune-related disorders include inter alias: type I diabetes, rheumatoid arthritis, multiple sclerosis, and ankylosing spondylitis.

Ankylosing spondylitis (AS) is a member of the spondylarthropaties family, which in general affects joints and often the spine (Reveille and Arnett, 2005). AS causes arthritis of sacroiliac joints and since it is also a systemic rheumatic disease, it can affect other tissues

throughout the body. Accordingly, it can cause injury to joints other than the spine and further organs, such as the eyes, lungs, kidneys, and heart valves. However, these other manifestations are less frequent (Lutermann and Braun, 2002). This rheumatic disease can have a variable course, from episodes of back pain to a severe chronic inflammation of the spine, peripheral joints and back stiffness, loss of motion and deformity as life progresses (Wanders et al., 2005). A dramatic loss of flexibility in the lumbar spine is an early sign of AS. Arthritis may also occur in the shoulder, hips and feet. AS is 2-3 times more common in males than in females. In women, it most often attacks joints other than the spine. AS affects all age groups, including children, however, the most common age of onset of symptoms is in the second and third decades of life (Feldtkeller et al., 2003).

Other members of the spondyloarthropathies are reactive arthritis (ReA) and Reiter's disease. ReA is a chronic form of arthritis characterized by inflammation of joints, eyes, and genital, urinary or gastrointestinal systems. As ReA is an autoimmune response to inflammation in another part of the body, it is called "reactive arthritis" (Leirisalo-Repo, 2005). Patient's immune systems react aberrantly when the genital, urinary, and gastrointestinal systems or the upper respiratory tract are exposed to certain bacteria (Ramos and López de Castro, 2002). The aberrant reaction of the immune system leads to spontaneous inflammation in the joints and eyes (Gaston and Lilicrap, 2003). Reiter's syndrome is a disorder that causes three seemingly unrelated symptoms: arthritis, redness of the eyes, and urinary tract signs. Less common symptoms are mouth ulcers, skin rashes, and heart-valve problems (Uystepuyst et al., 1998). The signs may be so mild that patients do not notice them. They usually come and go over a period of several weeks to several months. Reiter's disease is most common in young men between age 20 and 40. Women can also develop the disorder, although less often than men and with features which are often milder and more subtle (Gaston and Lilicrap, 2003).

The role of antigens specified by the major histocompatibility complex (MHC) in these diseases is crucial. In case of the human MHC, the class I gene *HLA-B27* is associated with the rheumatoid disease Ankylosing Spondylitis (AS), and it is the strongest predisposing factor for pathogenesis (Ramos and López de Castro, 2002). Developing spondylarthropaties is most likely inherited, and most patients with AS and related diseases are carrying the *HLA-B27* gene (Reveille, 1998; Braun and Sieper, 2007). However, the *HLA-B27* gene appears only to increase the tendency of developing ankylosing spondylitis, while further genetic and environmental factors are necessary for the disease to manifest itself (Sampaio-Barros et al., 2007).

1.4 Association of HLA-B27 with Ankylosing Spondylitis

The association of the *HLA-B27* gene with AS and related spondyloarthropathies is among the strongest observed for any HLA gene (Brewerton et al., 1973; Lechler and Warrens, 2000, Ramos and López de Castro, 2002).

There are more than 40 *HLA-B27* subtypes described to date. Their structural patterns and ethnic distribution are consistent with *B*2705* being the ancestral allele (Ramos and López de Castro, 2002), which is also most frequent and widely distributed (Khan, 1995). Other subtypes are related to B*2705 by one to few amino acid exchanges (Table 1.1; only the first 25 subtypes are shown). Some of them, like B*2702, B*2704 and B*2707 are linked to the disease (Breur-Vriesendorp et al., 1987; López-Larrea et al., 1995). There are however studies which showed that B*2706 (López-Larrea et al., 1995) and B*2709 (D'Amato et al., 1995) are not associated to the disease. In particular, it is interesting to investigate the possibility of differential, subtype-dependent molecular mimicry (Lang et al., 2002) between pLMP2 and pVIPR.

Table 1.1 Amino acid changes among the HLA-B27 subtypes (from Ramos and López de Castro, 2002).

	Residue number†																									
	L	α1												α2												α3
Subtype	−20	59	63	67	69	70	71	74	77	80	81	82	83	94	95	97	103	113	114	116	131	143	152	156	163	211
B*2705	A	Y	E	C	A	K	A	D	D	T	L	L	R	T	L	N	V	Y	H	D	S	T	V	L	E	A
B*2701	ND	–	–	–	–	–	–	Y	N	–	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B*2702	–	–	–	–	–	–	–	–	N	I	A	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B*2703	–	H	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B*2704	–	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–	E	–	–	G
B*2706	–	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–	D	Y	–	–	E	–	–	G
B*2707	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	S	–	H	N	Y	R	–	–	–	–	–
B*2708	–	–	–	–	–	–	–	–	S	N	–	R	G	–	–	–	–	–	–	–	–	–	–	–	–	–
B*2709	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	H	–	–	–	–	–	–
B*2710	ND	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	E	–	–	ND
B*2711	–	–	–	–	–	–	–	–	S	–	–	–	–	–	–	S	–	H	N	Y	R	–	–	–	–	–
B*2712	–	–	–	–	T	N	T	–	S	N	–	R	G	–	–	–	–	–	–	–	–	–	–	–	–	–
B*2713	E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
B*2714	–	–	–	–	–	–	–	–	–	–	–	–	–	–	W	T	L	–	–	–	–	–	–	–	–	ND
B*2715	ND	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–	E	–	T	ND
B*2716	ND	–	–	–	T	N	T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND
B*2717	ND	F	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND
B*2718	–	–	–	S	T	N	T	Y	S	N	–	R	G	–	–	–	–	–	–	–	–	–	E	–	–	ND
B*2719	ND	–	–	–	–	–	–	–	–	–	–	–	–	I	I	R	–	–	–	–	–	–	–	–	–	ND
B*2720	ND	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	H	N	Y	R	–	E	–	–	ND
B*2721	ND	–	–	–	–	–	–	–	S	–	–	–	–	–	–	R	–	–	D	Y	–	–	E	–	–	ND
B*2723	ND	–	N	F	T	N	T	Y	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	ND
B*2724	ND	–	–	–	–	–	–	–	S	–	–	–	–	–	–	S	–	H	N	Y	R	S	E	–	–	ND
B*2725	ND	–	–	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–	–	–	–	–	E	W	L	ND

*B*2722 was erroneously reported as a novel allele, as its sequence was identical to B*2706. Thus, it was removed from the HLA database.
†Dashes (–) indicate identity with B*2705.
ND = not determined.

The polymorphisms can influence the peptide binding specificity and other biochemical and functional features of the HLA-B27 molecule (Ramos and López de Castro, 2002). An issue of highest interest is the differential association of certain *HLA-B27* subtypes to AS. *B*2705* and *B*2709* occur in Sardinian, and are known to be differentially AS-associated (D'Amato et al., 1995): *B*2705* is AS-associated whereas *B*2709* is not. They differ only in one amino acid residue in the binding groove (Asp116His). Another pair of subtypes with different AS-association is *B*2704* and *B*2706*. *B*2706* has been reported to be only weakly or not associated with AS in South-East Asian populations in which the subtype *B*2704* is very frequently observed in AS patients (López-Larrea et al., 1995; Garcia-Fernández et al., 2001). The products of these subtypes differ in two residues of their heavy chains located at the floor of the peptide binding groove (Vega et al., 1986). In contrast, the association of *B*2703* to AS is questionable (Ramos and López de Castro, 2002). This subtype is nearly exclusively restricted to black individuals (Rojo et al., 1987; Choo et al., 1988; Gonzalez et al., 2002) and differs from B*2705 only by a single amino acid exchange at position 59 which may affect the A-pocket of the binding groove and thus anchoring of the N-terminal peptide residue (Table 1.1).

To explain the pathogenic role of *HLA-B27*, several theories have been proposed (Benjamin and Parham, 1990; Colbert, 2000; Edwards et al., 2000; Uchanska-Ziegler and Ziegler, 2003). The arthritogenic peptide theory (Benjamin and Parham, 1990; Ramos and López de Castro, 2002) and the β_2m deposition hypothesis (Uchanska-Ziegler and Ziegler, 2003) assume that peptide-dependent properties of the molecules are central to AS pathogenesis. According to the former theory, particular features of its peptide binding groove enable the HLA-B27 to present specific self-peptides with high sequence homology to peptides of bacterial or viral origin, so called arthritogenic peptides, allowing their recognition by crossreactive cytotoxic T cells (CTL). The β_2m deposition hypothesis assumes that in cells with AS-associated *HLA-B27* subtypes, β_2m dissociates from HLA:peptide complex more often than from cells with non-AS-associated subtypes (Uchanska-Ziegler and Ziegler, 2003). β_2m might accumulate within synovia and cause inflammation, culminating in destructive spondyloarthropathy. However, no amyloid β_2m deposits were found in tissues showing axial lesions of rats with AS (Tran et al., 2006).

It has been also shown that HC misfolding might be relevant in the pathogenesis of spondyloarthropathies. HLA-B27 misfolding occurs even in the presence of an intact antigen processing and assembly conditions (Colbert, 2000), what distinguish it from other MHC class I molecules. It has been suggested that aberrant folding (dimers formation) is dependent on the B-pocket which is important for peptide binding specificity thus linked to arthritogenicity.

HC misfolding could also trigger an innate immune response and cause inflammation or be a tendency of HLA-B27 for creating immunogenic structures such as dimers (Colbert, 2000). It has been previously described that the HLA-B27 molecules form disulphide-bonded HC homodimers (HC-HLA; Allen et al., 1999) which might present new peptides and induce inflammation. Signalling through the altered molecule might also lead to modified immune response (Edwards et al., 2000).

More recent observations of HLA-B27 heavy chain misfolding in rats with high transgenic copies of HLA-B27 (Tran et al., 2004), suggest triggering of the unfolded protein response (UPR) as well as downstream inflammation by HLA-B27 heavy chain misfolding (Turner et al., 2005) as putative mechanisms explaining role of HLA-B27 in inflammatory disease. However, additional human β_2m reduces HLA-B27 misfolding and UPR triggering, while it also increases the severity of arthritis and spondylitis (Tran et al., 2006). This indicates that HLA-B27 misfolding is not crucial in the development of the associated arthropathy and supports an involvement of β_2m in AS pathogenesis, as suggested by Uchanska-Ziegler and Ziegler (2003).

An involvement of HLA-B27 molecules in AS pathogenesis is supported by the correlation of AS with reactivity of cytotoxic T cells to an HLA-B27-restricted self-peptide (Fiorillo et al., 2000). This self-peptide is derived from vasoactive intestinal peptide type 1 receptor (pVIPR, RRKWRRWHL) and displays potential arthritogenic properties (Fiorillo et al., 2000). pVIPR exhibits sequence similarity to a peptide derived from the latent membrane protein 2 of Epstein Barr-virus (pLMP2; RRRWRRLTV) (Brooks et. al., 1993). The numbers of CTLs against pVIPR increase during progression of AS in patients typing as *B*2705*, but CTL against the same peptide are only rarely present in individuals with the AS-non-associated subtype *B**2709 (Fiorillo et al., 2000). However, while TCR are thought to play a role in the context of the arthritogenic peptide hypothesis, KIR (KIR3DL1 in case of HLA-B27) have been implicated in interactions with HLA-B27 HC dimers, which could also contribute to AS pathogenesis (Edwards et al., 2000; Allen et al., 2001). It must be pointed out, that it has not been possible so far to reconcile any of the various theories on *HLA-B27* and AS with all biochemical, genetic, and clinical data.

1.5 Structures of HLA-B27 subtypes

HLA-B27 was among the first HLA molecules whose structures were elucidated (Madden et al., 1992), but high-resolution comparative crystal structures of HLA-B27 subtypes bound to individual peptides, including pVIPR and pLMP2 (Figure 1.5), have only recently been

determined (Hülsmeyer et al., 2002; Hülsmeyer et al., 2004; Hillig et al., 2004; Fiorillo et al., 2005; Hülsmeyer et al., 2005; Rückert et al., 2006; Uchanska-Ziegler et al., 2006). Drastically different binding modes for both peptides in B*2705 and B*2709 have been observed (Figure 1.5). This has led to an explanation for subtype-specific differences, linking the generation of dissimilar T-cell repertoires against pVIPR/pLMP2 to the Asp116His polymorphism. These structural studies support the concept of molecular mimicry between the two peptides (Figure 1.5 and 1.6), (Fiorillo et al., 2005) and a further self-peptide (Rückert et al., 2006).

a) b)

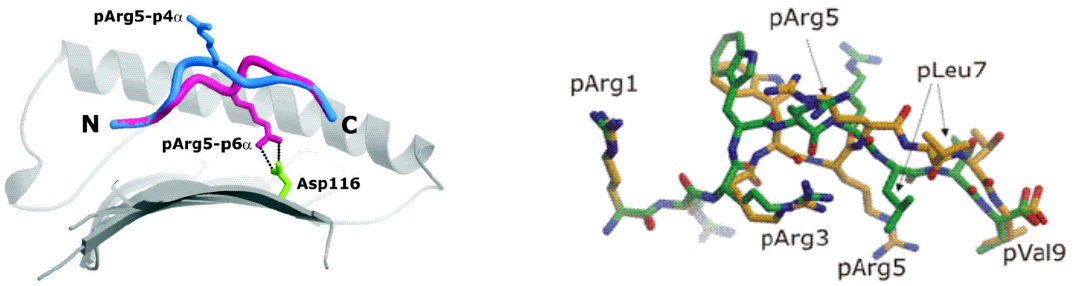

Figure 1.5. pVIPR peptide conformations in the binding sites of HLA*B2709 and B*2705 (Hülsmeyer et al., 2004) (a); Superimposed conformations of pLMP2 in B*2705 (orange) and B*2709 (green) (Fiorillo et al., 2005) (b). a) The α1-helix of the heavy chain and the floor of the peptide-binding groove are shown (grey), whereas the α2-helix in the front has been cut away to provide better visibility of the peptide. b) The peptide conformations in the two subtypes differ drastically in their middle sections as pArg5 forms a salt bridge with Asp116 only in the B*2705 subtype (compare figure a). Functionally important atoms are indicated in red (oxygen) and blue (nitrogen). Both peptides exhibit the common p4α conformation in B*2709 (α-helical φ/ψ torsion angle only at position 4), while the p6α binding mode is only observed in B*2705 with α-helical φ/ψ torsion angle at position 6.

The classical conformation observed also previously (Stern et al., 1994) is here called p4α because the α-helical φ/ψ torsion angle occurs only at position 4 of the peptide backbone. This binding mode is seen for the pVIPR peptide when bound to B*2709 (Hülsmeyer et al., 2004; Fiorillo et al., 2005), while the pLMP2 peptide bound to B*2705 occurs in p6α conformation, where an α-helical φ/ψ torsion angle is at position 6 (Fiorillo et al., 2005). Interestingly, the self peptide pVIPR adopts in B*2705 both modes in the groove (Hülsmeyer et al., 2004) and molecular mimicry hypothesis has been proposed to explain this phenomenon (Fiorillo et al., 2005).

Additional surface representation analyses (Figure 1.6) support this hypothesis (Fiorillo et al., 2005). The structure of B*2705:β_2m:pLMP2 (p6α conformation) is extremely similar to the structure of B*2705:β_2m:pVIPR in p6α conformation. This means that the T cell receptor may not differentiate between self and viral peptide, and this could lead to an autoimmune response. A widespread reactivity in patients with AS against a self-epitope, that exhibits some features of a putative “arthritogenic” peptide, was found in cytotoxicity assays

using pLMP2- and pVIPR-specific cytotoxic T lymphocytes. Individuals carrying both B*2705 and B*2709 possess pLMP2-specific T cells, whereas nearly exclusively B*2705 subjects respond to pVIPR (Fiorillo et al., 2000). It was shown that the number of CTLs against the pVIPR peptide increases during AS progression in patients bearing the *B*2705* allele (Fiorillo et al., 2000). These data indicate that pVIPR-specific reactivity is a feature of patients suffering from AS.

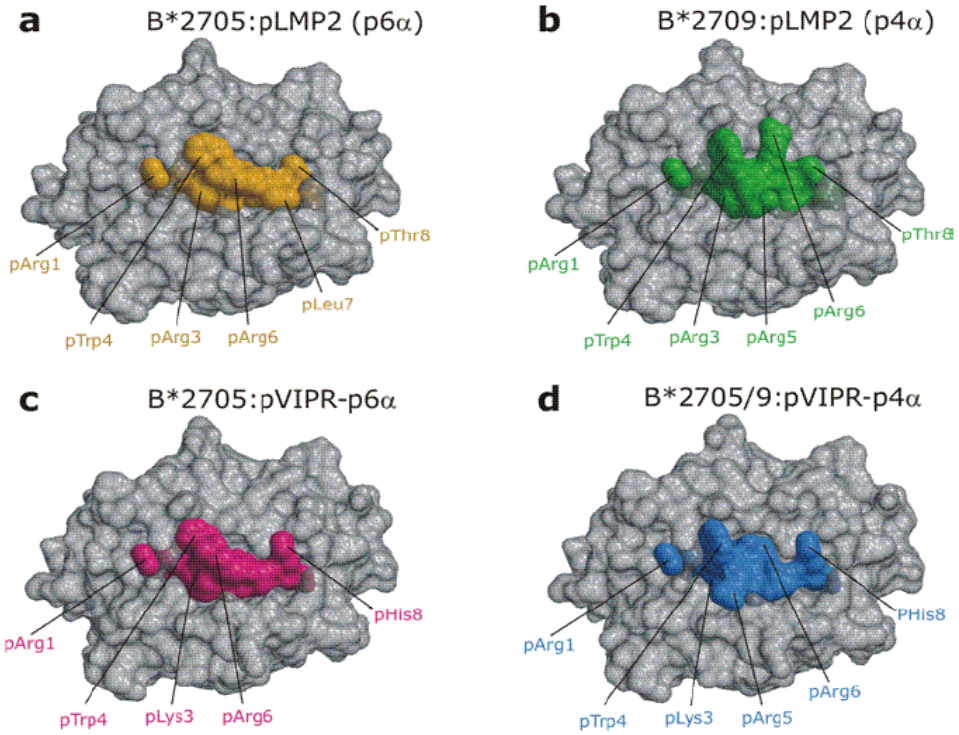

Figure 1.6. Molecular surface representations of B*2705 (a, c) and B*2709 (b, d) complexed with pLMP2 or pVIPR (Fiorillo et al., 2005). The similar structures of the peptides being presented in a and c imply the existence of molecular mimicry between pLMP2 and pVIPR in the context of B*2705 (only when the two peptides are presented in the p6α conformations) but not in B*2709 (where both peptides can only be presented in dissimilar p4α binding modes).

1.6 HLA-B27 subtypes with different AS-association

All three HLA subtypes, B*2703, B*2704 and B*2706 are very closely related to the ancestral allele B*2705 (Table 1.1). They differ in one to several amino acids residues in the heavy chain, in their AS association and in ethnic distribution. Based on the previously performed analyses of solved structures of HLA-B27 molecules (B*2705 and B*2709 with self- and viral peptides), we assumed that the crystal structures of the three additional HLA-B27 subtypes (B*2703, B*2704 and B*2706) would shed light on the differential involvement of HLA-B27 molecules in AS pathogenesis.

1.6.1. HLA-B*2703

Whereas HLA-B*2705 is the most common HLA-B27 subtype in Euro-Caucasoid populations (Khan, 1995; Gonzalez et al., 2002) and is AS associated, HLA-B*2703 is nearly exclusively restricted to black individuals (Rojo et al., 1987; Choo et al., 1988; Gonzalez et al., 2002) and shows questionable AS association. The B*2703 subtype differs from the ancestral subtype B*2705 by a single amino acid exchange in the binding groove (His59 instead of Tyr59 in the A pocket) (Choo et al., 1988). The exchange of the extremely conserved residue Tyr59 occurs only in B*2703 (His59) and B*2717 (Phe59) (Rojo et al., 1987). Since this residue is known to stabilize peptide binding to class I molecules, B*2703 might be expected to exhibit biochemical and functional properties distinguishing it from other HLA-B27 subtypes (Madden, 1995).

It was previously shown that substitution of Phe for Tyr at either position 7 or 171 in HLA-A2 (Latron et al., 1992) or position 59 in B*2705 dramatically influences peptide presentation (Colbert et al., 1994), demonstrating a functional importance of these tyrosines. The exchange at position 59 in B*2703 is expected to affect the A-pocket in the binding groove, as the anchoring the N-terminus of the peptide occurs through a highly conserved, characteristic pentagonal network of hydrogen bonds (Madden et al., 1992; Madden, 1995). The influence of another naturally occurring A-pocket amino acid exchanges on the binding of the peptide N-terminus has already been structurally investigated for B*5101, one of the few subtypes where the common Tyr171 is replaced by His171 (Maenaka et al., 2000). The crystal structures of B*5101 in complex with the immunodominant peptide epitopes from HIV-1 (8-mer KM2 - TAFTTPSI and 9-mer KM1 - LPPVVAKEI) revealed a nonstandard interaction within the A-pocket where the N-terminal amino acid is lifted up with regard to B*2703. The data which were available at the beginning of this work are based on computer modeling of B*2703 in complex with several different peptides. They suggested a weakening of hydrogen bonds and movement of the α1-helix (Griffin et al., 1997).

1.6.2. HLA-B*2704 and HLA-B*2706

The human leukocyte antigen alleles *HLA-B*2704 and HLA-B*2706,* although closely related, show an ethnically restricted distribution. The HLA-B*2704 subtype is restricted to Orientals, in particular individuals from China and Southeast Asia, (López-Larrea et al., 1995; Nasution et al., 1997; Ren et al., 1997; Chen et al., 2002; Ramos and López de Castro, 2002; Dhaliwal et al., 2003) whereas B*2706 occurs in Southeast Asia and Pacific. The products of the two alleles

differ by only two amino acids, at heavy chain residues 114 (His in B*2704; Asp in B*2706) and 116 (Asp in B*2704; Tyr in B*2706) which are located at the floor of the peptide binding groove (Vega et al., 1986; Vilches et al., 1994). Both residues could be involved in contacting amino acids of a bound peptide, suggesting that self-peptides presented by these subtypes may play a role in disease pathogenesis. Both subtypes share about 90 % of their peptide repertoires (Sesma et al., 2002). The main features that distinguish B*2704 and B*2706 subtypes from B*2705 are: failure to present peptides with C-terminal basic residues, and allowance for polar and nonpolar residues at the p3 peptide position (Garcia et al., 1997). Peptides which can bind to B*2704 have Tyr or Arg at the C-terminus and a strong preference for an aliphatic/aromatic p3 residue (Sesma et al., 2002) which was not detected among peptides from B*2706. The use of Tyr or basic residues at the C-terminus is a feature shared by the disease associated B*2705, B*2702 and B*2704 subtypes (Garcia et al., 1997), suggesting that this property might be critical for the association with the disease.

Differences in the sequence in comparison to the B*2705 subtype are shown in Table 1.1. The change of Asp to Ser77 involves loss of an acidic residue in the C/F pocket. The second exchange of Val to Glu152 in the binding groove (Vega et al., 1985; Rudwaleit et al., 1996) involves substitution of an acidic residue for an aliphatic one near the place where the HLA molecule binds the C-terminus of the peptide (Madden et al., 1991; Madden et al., 1992). These two changes might therefore have an influence on the peptide binding mode.

Structural comparisons of B*2706 and B*2704 with other HLA-B27 subtypes complexed with self and viral peptides are likely to contribute to a better understanding of the effect of amino acid exchanges at the floor of the peptide binding groove on the repertoire of bound peptides. Using X-ray crystallography, the two B*2704 and B*2706 subtypes complexed with the peptides pVIPR and pLMP2 were analyzed in this study, and a comparison of these structures with previously solved B*2705 and B*2709 structures (Hülsmeyer et al., 2004; Fiorillo et al., 2005) became possible.

1.7 Killer cell Immunoglobulin-like Receptors

Killer cell Immunoglobulin-like Receptors (KIR) belongs to a multigene family of Ig-like extracellular domain receptors which contains 15 genes and 2 pseudogenes (Kiessling et al., 1975; Kimber and Moore, 1985; Valiante et al., 1997; Moretta et al., 2001; Volz and Radeloff, 2006). They are members of a gene cluster of a highly variable leukocyte receptor complex on human chromosome 19q13.4 (Uhrberg et al., 1997; Wende et al., 2000; Wilson et al., 2000;

Hsu et al., 2002; Yawata et al., 2002; Volz and Radeloff, 2006). They reside on the surface of Natural Killer (NK) cells (Peruzzi et al., 1996; Vilches and Parham, 2002), and are the main receptors for both classical HLA-A, -B and -C (only with a subset of alleles; Cella et al., 1994; Pende et al., 1996, Vitale et al., 1996) and also non-classical HLA-G molecules. The physiological functions of the classical HLA molecules are to present peptides to T cells, to serve as interaction partner for T cells, but also to inhibit or permit the activity of natural killer cells.

NK cells were described in the early 1970s as a third group of lymphocytes (Cudkowicz and Bennett, 1971; Rosenberg et al., 1972; Oldham and Herberman, 1973; Thornthwaite and Leif, 1974) which comprises about 5–15% of lymphocytes in spleen, liver and peripheral blood. At lower frequencies they are also present in thymus and lymph nodes (Lian and Kumar, 2002) and are characterized by cytotoxic reactivity immediately after the first contact. NK cells constitute a major component of the innate immune system, and they attack cells that have been infected by microbes, but not microbes themselves. NK cells are cytotoxic due to small granules containing special proteins in their cytoplasm such as perforin and granulozymes (Smyth and Trapani, 1995), and their lytic response can be triggered within minutes. After release of perforin, pores are formed in the cell membrane of the target cell through which proteases and associated molecules can enter, inducing apoptosis.

To defend the body against viruses and other pathogens, NK cells require mechanisms which enable the determination whether a cell was infected or not. They distinguish between healthy and abnormal cells by a repertoire of cell surface receptors with the ability to control activation, proliferation and function of NK cells (Spits et al., 1998). The majority of NK receptors is not exclusively expressed on NK cells and can be present on a subset of T lymphocytes as well (i.e. γδTCR and activated CD8+), (Phillips et al., 1995; Lanier, 2005). To control their lytic activity, NK cells possess two types of receptors residing on their surface: activating and inhibitory. The name "natural killer" comes from the initial idea that they do not require a target-specific educational process to render the effector cells cytotoxic (Kiessling et al., 1975 a; Kiessling et al., 1975 b). NK cells were defined as non-MHC-restricted. It was described that their reactivity against MHC-negative cells was strong and cytotoxicity was not reduced even when antibodies masking MHC antigens were used (Koren and Williams, 1978). Confirmation of the hypothesis about NK cells ability to kill cells lacking MHC (Stern et al., 1980) was surprising in an age of discovery that T cell recognition by MHC is required for cell lysis (Zinkernagel and Doherty, 1974).

The missing-self model (Kärre et al., 1986; Ljunggren and Karre, 1990; Kärre, 2002) was proposed to explain the function of NK cells. According to this hypothesis one function of NK cells is detecting and eliminating cells which fail to express normal self markers. They either lack MHC class I or have lowered expression of this antigen (Ljunggren and Kärre, 1985; Moretta et al., 2002; Kärre, 2002; Volz and Radeloff, 2006) and/or stress-induced proteins (Bauer, 1999). The activation of NK cells is strongly controlled by inhibitory receptors, however, in some cases signals from activating receptors are sufficient to stimulate NK cells despite of the presence of inhibitory ones caused by the impaired expression of MHC class I molecules (Chambers et al., 1996; Cerwenka et al., 2001; Moretta et al., 2001). Current modifications of the "missing-self" hypothesis state that NK cells look for cells lacking MHC class I or cells which overexpress ligands for activating NK cell receptors. The inhibitory receptors on NK cells regulate signals from activating receptors but can not terminate completely NK cell effector function (Lanier, 2005). Therefore, the reduced level of MHC expression is not a prerequisite for the activation of natural killer cells. Also the interaction of low affinity cell surface receptors on the NK cells with the Fc fragment of antibodies can induce cytotoxicity (Lanier et al., 1988; Ravetch and Bolland, 2001; Lanier, 2005).

The KIR on NK cells fall into two subsets having either two or three Ig domains which are thought to share a characteristic protein fold. The domains have been designated D0, D1 and D2, with D0 forming the N-terminal domain of three-domain KIRs. The structural characteristics correlate with function. The key difference between inhibitory and activating receptors is a long or short cytoplasmic tail and the presence of immunoreceptor tyrosine-based inhibitory motifs called ITIMs in inhibitory receptors. KIRs with short intracytoplasmic tails activate NK cells; these with long cytoplasmic domain inhibit NK cells. Activating KIRs have fewer cytoplasmic domains than inhibitory ones, and consequently the number of inhibitory motives is also lower (Volz and Radeloff, 2006). Their specificity seems to be weaker since they have lower affinity for putative HLA class I ligands (Williams et al., 2005).

Signaling by both inhibitory and activating NK receptors is mediated by a conserved sequence motif which in inhibitory receptors is defined as Ile/Val/Leu/Ser/x/Tyr/x/x/Leu/Val (x denotes any amino acid) called ITIM. Inhibitory KIRs interact with α1 and α2 domains of HLA and with positions 7 and 8 of bound peptide. ITIMs of cytopasmic tail are phosphorylated upon binding, and recruit tyrosine-specific phosphatases SHP-1 and SHP-2 which leads to the loss of effector function of NK cell (Lanier, 1998; Long, 1999).

After binding to pMHC on a potential target cell, inhibitory KIRs deliver signals into the interior of NK cells. The expression of inhibitory and activating KIRs on a single NK cell

controls its lytic potential. Inadequate expression of MHC class I molecules by a target cell will prevent the pMHC-KIR interaction. As a consequence, the target cell will be lysed by the NK cell. Reduced expression of MHC molecules on cells can be caused e.g. by a viral infection, malignant transformation, or an autoimmune disease (Vilches and Parham, 2002).

In contrast to pMHC, only very few structures for KIRs have been determined (Fan et al., 1999; Maenaka et al., 1999; Snyder et al., 1999) and no structure is available for a 3-domain KIR such as KIR3DL1. Two structures have been determined in which an HLA molecule is bound to a 2-domain KIR: HLA-Cw3:KIR2DL2 (Boyington et al., 2000; Figure 1.7) and HLA-Cw4:KIR2DL1 (Fan et al., 2001). In both cases, the KIR molecule contacts the C-terminal amino acids of the peptide and adjacent parts of the α1- and α2-helices of the HLA-C antigen.

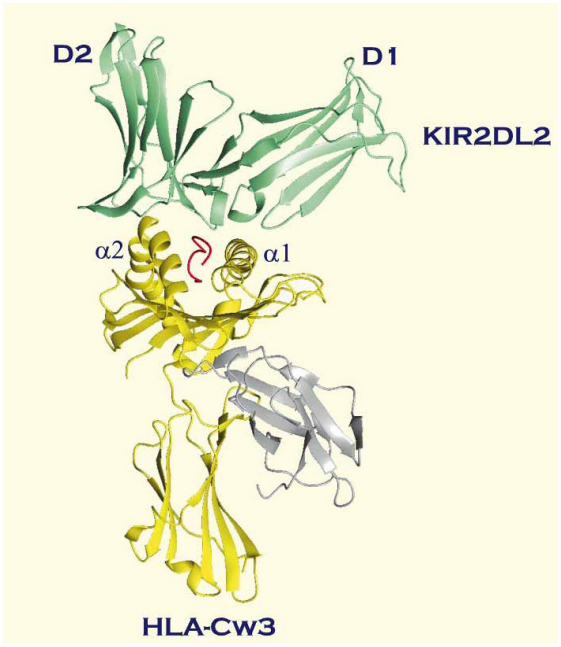

Figure 1.7.

The HLA-Cw3:GAVpeptide-KIR2DL2 complex (from Sawicki et al., 2001). The HLA-C HC is yellow, β_2m grey, peptide red, and two domains of KIR2DL2 are green.

Since KIR3DL1 binds only to HLA-B molecules with the Bw4 serological determinant (epitope within certain HLA-B molecules at position 77-83; DLRTLLR in B*2705; Gumperz et al., 1995) at the end of the α1-helix (Peruzzi et al., 1996), it is likely that the two KIR molecules bound to HLA-C (Boyington et al., 2000; Fan et al., 2001) exhibit binding modes similar to those of KIR3DL1. Interestingly, the D0 domain of KIR3DL1 which is absent in 2-domain KIRs is also needed for the recognition of HLA-Bw4 molecules with high affinity (Rojo et al., 1997; Khakoo et al., 2002). The two HLA-C-KIR structures show also that binding of KIR3DL1 to HLA-B27 may be influenced by distinct peptide binding modes.

It has been shown that expression of activating KIR, in the absence of inhibitory receptor for self MHC class I, may be a factor causing autoimmune disease like rheumatoid arthritis or psoriatic arthritis (Martin et al., 2002; Snyder et al., 2003), but it was also reported that these receptors may be beneficial in immune responses to viral pathogens (i.e. HIV) and tumors (Bishara et al., 2004).

1.8 Scopes and objectives of the project

While several X-ray structures of B*2705 and B*2709 are already known, including those with the self-peptide pVIPR and the viral-peptide pLMP2, the binding of these peptides to B*2703, B*2704 and B*2706 and formation of a complex with a killer Ig-like receptor (KIR3DL1) have not been studied so far. Comparison of another pair of differentially AS-associated subtypes of HLA-B27 might aid in explaining, whether molecular mimicry can be generalized, and provide a key structural contribution to the potential role of the pVIPR and pLMP2 peptides in an autoimmune disease.

Solving the crystal structures of B*2703 in complex with pLMP2 and pVIPR would enable comparison to the recently determined crystal structures of B*2705 and B*2709 in complex with the same peptides (Hülsmeyer et al., 2004, Fiorillo et al., 2005). This would permit to discuss the influence of different polymorphic residues on the peptide binding and relation to AS association. Additional thermodynamic studies, circular dichroism (CD) and differential scanning calorimetry (DSC) are expected to provide insights into the thermodynamic properties of B*2703:β_2m:pLMP2 and B*2703:β_2m:pVIPR.

Furthermore, knowledge of the structure of a 3-domains KIR would permit an insight into how this crucial receptor recognizes HLA-B molecules bearing the Bw4 determinant, a key event in controlling NK cell cytotoxicity. Therefore I also tried to crystallize for the first time the 3-domain KIR in complex with B*2705 bound to a peptide.
This project was aimed at addressing the following questions:

1. How are peptides such as pVIPR and pLMP2, whose binding modes have already been determined in the B*2705 and B*2709 subtypes, bound to B*2703?
2. How does the His59Tyr replacement affect the binding mode of the N-terminal amino acid of the peptide within the A-pocket? Can the distinct conformations which characterize the binding of pVIPR and pLMP2 in the B*2705 subtype also be observed in B*2703?

3. Do polymorphisms between B*2704 and B*2706 lead to distinct binding modes of the pVIPR and pLMP2 peptides as in the case of B*2705 and B*2709?
4. Do the conformations of pVIPR and pLMP2 bound to B*2704/B*2706 resemble those found in B*2705/B*2709? Is molecular mimicry observed in B*2704 and B*2706 as well?
5. Can expression and reconstitution systems for KIR3DL1 be developed?
6. Do the results support any of the currently discussed hypotheses seeking to explain AS pathogenesis?