HLA-B27-specific Immune Response to Influenza is Critically Dependent on ERAP-1 and on MHC-I Allelic Co-dominant Expression

by

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy in Medical Sciences
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University of Toronto

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Abstract

Genetic studies have demonstrated that both the HLA-B27 and endoplasmic reticulum aminopeptidase (ERAP) are contributing factors to the pathogenesis of ankylosing spondylitis (AS). In my studies I have used HLA transgenic (Tg) mice to examine the contribution of HLA-B7 and ERAP1 to the HLA-B27-mediated immune response to influenza (flu) infection, results of which could be used as proof of principle for AS.

Influenza infection affects millions of individuals worldwide annually. Because of the well-established HLA allele-specific epitope recognition, I used flu infection in HLA Tg mice deficient of endogenous MHC-I expression; i.e., H2-D^d, H2-K^k or double knock out (DKO) in a proof-of-concept strategy to define the details of first, the interaction of HLA-B7 and HLA-B27 and secondly, the contribution of ERAP to allele-specific immunodominance.

Immunodominance during a viral infection is influenced by many factors. To assess whether co-expression of HLA-B7 and HLA-B27 would influence the immune response in double HLA Tg mice, I created double Tg B7/B27 mice with intact ERAP expression on a DKO
background. Following flu infection, the dominant anti-viral CTL responses was surprisingly directed predominantly at the B7-restricted NP418-426 epitope while the CTL response to the B27-restricted NP383-391 epitope was significantly reduced. Using chimeras and flu-specific tetramer staining I showed that the reduced B27/NP383-391 CTL response in B7/B27 Tg mice was due to negative selection of T cells specific to NP383-391 recognition. Secondly, recent genome-wide association studies (GWAS) with AS patients have shown that loss-of-function genetic variants of ERAP1 are protective, and that this relationship is seen exclusively in B27+ patients. To investigate these interactions in an in vivo model, I created single Tg B7 and B27 mice in the absence of ERAP. Following flu infection the B27/NP383-391 CTL response was reduced in B27/ERAP−/− Tg mice compared to B27/ERAP+/+ mice. No difference in the CTL response to the B7/NP418-426 epitope was observed between the B7/ERAP−/− and B7/ERAP+/+ mice. This is the first in vivo validation of the gene-gene interaction between B27 and ERAP1 which had been suggested by prior genetic studies.
Acknowledgments

This has been a long journey and I would not have been able to go through this voyage without the support of my wife, my son and my family.

My beautiful wife encouraged me to achieve my goals when it seemed almost impossible. Her continuous support and love enabled me to fulfill my dream.

I love you very much Ghizal Jan!

Qand Pader, Yusuf Aryan Jan, you are the best gift God could have given us.

Having you and playing with you on a daily basis changed me forever.

I love you so much Babeh Babah!
Contributions

Chapter 1

A part of this chapter describing the factors contributing to immunodominance was published in the journal of Clinical Immunology. The citation for this article is: Akram A and Inman RD. Immunodominance: a pivotal principle in host response to viral infections. Clin Immunol. 2012 May; 143(2):99-115. Some minor parts of this chapter was also published as part of my MSc thesis.

Chapter 3

This chapter describing the effects of dual HLA allele co-expression influencing the flu response was published in the European Journal of Immunology. I did all the experiments and analysis. I prepared the draft for publication. The citation for this article is: Akram A and Inman RD. Co-expression of HLA-B7 and HLA-B27 alleles is associated with B7-restricted immunodominant responses following influenza infection. Eur J of Immunol 2013 Dec; 43(12): 3254-67.

Chapter 4

This chapter describing the role of ERAP in flu response of HLA Tg mice has been published in the Journal of Immunology. The citation for this article is: Ali Akram, Aifeng Lin, Eric Gracey, Cathy Streutker, Robert D. Inman. HLA-B27, but not HLA-B7, Immunodominance to Influenza is Uniquely ERAP Dependent. J Immunol. 2014 Jun 15; 192(12):5520-8
Aifeng Lin and Eric Gracey helped with mouse genotyping. Cathy Streutker helped with the scoring of the H and E slides. All authors approved the draft. I did all the described experiments and analysis. I prepared the draft for publication.
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<tbody>
<tr>
<td>A2</td>
<td>HLA-A2 (Transgenic HLA-A2/H2 DKO)</td>
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<td>A2/B7</td>
<td>HLA-A2/B7 (Transgenic HLA-A2/B7/H2 DKO)</td>
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<td>A2/B27</td>
<td>HLA-A2/B27 (Transgenic HLA-A2/B27/H2 DKO)</td>
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<td>Ab</td>
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<td>ABC</td>
<td>ATP Binding Cassette</td>
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<td>ACE</td>
<td>Angiotensin-Converting Enzyme</td>
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<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<td>A-LAP</td>
<td>Leucine Aminopeptidase</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<td>Ankylosing Spondylitis</td>
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<td>HLA-B7 (Transgenic HLA-B7/H2 DKO)</td>
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<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;m</td>
<td>MHC β&lt;sub&gt;2&lt;/sub&gt;-Microglobulin</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>CLIP</td>
<td>Class II-associated Invariant Chain Peptide</td>
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<td>Abbreviation</td>
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<tr>
<td>CMV</td>
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<td>DKO</td>
<td>H2-K/H2-D MHC Class I Deficient Mice</td>
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<tr>
<td>DOX</td>
<td>Doxycycline</td>
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<td>DRIPS</td>
<td>Defective Ribosomal Products</td>
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<td>EBV</td>
<td>Epstein Barr Virus</td>
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<tr>
<td>ER</td>
<td>Endoplastic Reticulum</td>
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<td>ERAAP</td>
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</tr>
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</tr>
<tr>
<td>HA</td>
<td>Haemagglutinin</td>
</tr>
<tr>
<td>HAUs</td>
<td>Hemagglutinating Units</td>
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</table>
HBV  Hepatitis B Virus (HBV)
HC   MHC Class I Heavy Chain
HCV  Hepatitis C Virus
HIV  Human Immunodeficiency Virus
HLA  Human Leucocyte Antigen
HPV  Human Papillomavirus
HSP60 Heat Shock Protein 60kDa
HUGO Human Genome Organization
IBD  Inflammatory Bowel Disease
IBP  Inflammatory Back Pain
ICAM-1 Intracellular Adhesion Molecule 1
IFN-γ Interferon Gamma
Ig   Immunoglobulin
IL   Interleukin
IL-6 Interleukin 6
IL-1α Interleukin 1 Alpha
ImDc Immunodominance
ImD  Immunodominant
i.n.  Intranasal Infection
i.p.  Intraperitoneal Infection
kDa  Kilo Dalton
KIR  Killer Cell Immunoglobulin-like Receptor
KO   Knock-Out
<table>
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<tr>
<td>L-Amc</td>
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<td>MHC Class I Light Chain</td>
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<tr>
<td>LIR</td>
<td>Leukocyte Immunoglobulin-like Receptor</td>
</tr>
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<td>LMP2</td>
<td>Low Molecular Protein 2 (Proteasomal Component)</td>
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<td>mAb</td>
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<td>MPER</td>
<td>Membrane Proximal External Region</td>
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<td>Abbreviation</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>PA</td>
<td>Polymerase A Protein</td>
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<td>Polymerase B2</td>
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<tr>
<td>PBLs</td>
<td>Peripheral Blood Lymphocytes</td>
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<td>Peptide Loading Complex</td>
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<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
</tr>
<tr>
<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
</tr>
<tr>
<td>SP</td>
<td>Spleen</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
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</tr>
<tr>
<td>SpA</td>
<td>Spondyloarthritis</td>
</tr>
<tr>
<td>TALEN</td>
<td>Transcription Activator-like Effector Nucleases</td>
</tr>
<tr>
<td>TAP</td>
<td>Transporter Associated With Antigen Processing</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal Deoxynucleotidyl Transferase</td>
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<tr>
<td>Tet</td>
<td>Tetracyclin</td>
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<tr>
<td>Tg</td>
<td>Transgenic</td>
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<tr>
<td>TM</td>
<td>Transmembrane</td>
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<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor Alpha (α)</td>
</tr>
<tr>
<td>TOP</td>
<td>Thimet Oligoendopeptidase</td>
</tr>
<tr>
<td>TPP II</td>
<td>Tripeptidyl Peptidase II</td>
</tr>
<tr>
<td>TReg</td>
<td>Regulatory T Cells</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded Protein Response</td>
</tr>
<tr>
<td>VDJ</td>
<td>Variable Diversity Joining</td>
</tr>
<tr>
<td>WT</td>
<td>Wild Type</td>
</tr>
<tr>
<td>WIV</td>
<td>Whole Inactivated Virus</td>
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Chapter 1

A part of this chapter describing the factors contributing to immunodominance was published in the journal of Clinical Immunology. The citation for this article is: Akram A and Inman RD. Immunodominance: a pivotal principle in host response to viral infections. Clin Immunol. 2012 May;143(2):99-115. Some minor parts of this chapter was also published as part of my MSc thesis.

1 MHC Molecules and Their Role in the Immune System

Major histocompatibility complex (MHC) class I molecules were discovered in studies of tumour rejection in inbred mice in the mid 1900s (1). Studies by Zinkernagel and Doherty established the physiological role of these molecules (1). They were able to show that T cells from a certain infected animal recognize virally infected cells only from genetically identical animals and not from genetically different animals. These findings defined the basis for the phenomenon of MHC restriction in which target cell recognition is restricted by the MHC genotype. Based on these results, subsequent studies concentrated on deciphering the details of MHC restriction and function in immune system. In subsequent sections below I describe the MHC molecules in detail.

1.1 MHC Class I and Class II

The MHC locus consisting of both class I and class II MHC genes is located on chromosome 6 in human and chromosome 17 in mice (2). The class I genes in mice are H2-K, D and – L while their human counterparts are HLA-A, -B and –C. Overall, each of these genes can exist in different allelic forms which contribute critically to antigen presentation. Due to the focus of my thesis, details about MHC class II molecules and genes will not be discussed, except to say
that there are two class II genes (i.e., I-A and I-E) in mouse and three class II genes (i.e., DP, DQ and DR) in human. Generally, class I gene products are found on almost all nucleated cell types, whereas those of class II are expressed only on antigen-presenting cells (APC) such as B lymphocytes, dendritic cells (DC) and macrophages, as well as cortical epithelial cells in the thymus (1).

MHC class I (i.e., MHC-I) genes encode cell surface proteins consisting of a highly polymorphic heavy chain (HC) and a light chain (LC) which is β2-microglobulin (β2m) (Fig. 1.1). While the HC is encoded in the MHC locus, the LC is not. The heavy chain is approximately 45 kDa and the LC is 12 kDa. The HC is made up of three extra-cellular domains (i.e., α1, α2 and α3), a transmembrane domain (TM) and a cytoplasmic domain. The α3 domain and β2m mainly consist of β sheets whereas the α1 and α2 domains are made up of both α helices and β sheets (Fig. 1.1). The eight β sheets in the α1 and α2 domains form a ‘floor’ on top of which two α helices are positioned in an anti-parallel orientation forming the antigen binding site (Fig. 1.1 B). This site consists of 6 “pockets” (named A to F) or “grooves” (3, 4). These “grooves” are oriented outwards from the cell membrane. Different peptides have been shown to interact with one or more of these unique pockets of the antigen binding site of different MHC-I molecules through their anchor residues (4-6). The chemical composition of the anchor residues, as well as the structural complementarity between these residues and the class I side chains within the pockets, determine the pattern of allele-specific peptide binding (4-6). Usually the length of peptides binding to class I molecules is 8-11 amino acids (aa).

The first three-dimensional structure of a MHC molecule, which was an HLA-A2, was resolved by Bjorkman et al. in 1987 (3). Subsequent studies by others have shown that all MHC class I molecules have the same overall general structure but with minor, unique differences that
differentiate each allele from the rest (4, 6-8). The bound peptide-MHC class I (i.e., pMHC-I) complex is expressed on the cell surface where it is recognized by T cells via specific T cell receptors (TCRs). The detailed description of antigen processing and presentation by MHC class I and T cell recognition will be discussed below.

**Figure 1.1 The structure of HLA-B2705**

*(A)* The different domains of HLA-B27 are labeled and color coded as shown. *(B)* The peptide binding cleft consisting of $\alpha_1$ (top) and $\alpha_2$ (bottom) domains is shown with its bound peptide (light blue) as seen from the top looking down at it. The numbers refer to different amino acids located within the peptide binding groove. Modified from *PLoS One* (2012): 7(3), e32865. (9)
1.2. Antigen Processing and Presentation

Successful clearance of a viral infection (e.g., influenza A) relies on the host’s ability to effectively present viral antigenic peptides to T cells in the context of self-MHC class I molecules. Following an infection, a complex multi-step process involving the action of several different proteins (i.e., antigen processing) leads to the generation of antigenic peptides which are then presented on the cell surface by MHC-I molecules. MHC-I molecules mostly present peptides derived from endogenous proteins (Fig. 1.2 A)(2, 10, 11) which are the products of intracellular synthesis. These undergo proteasomal processing before being bound and presented by MHC-I molecules. Viral proteins and tumour antigens provide important sources of foreign peptides that are presented by MHC-I molecules. Newly synthesised proteins failing to fold properly contribute to the misfolded and damaged protein group collectively referred to as defective ribosomal products (i.e., DRIPS). DRIPS presented by MHC-I molecules constitute about 30-70% of the newly synthesized proteins by ribosomes and are of cytosolic, nuclear, and mitochondrial origins (12, 13). In contrast to endogenous proteins, exogenous proteins such as bacterial proteins enter the host by phagocytosis, pinocytosis or endocytosis (Fig. 1.2 B). Exogenous protein-derived peptides are usually generated in endosomes or in other endocytic compartments and are presented on the cell surface by MHC class II molecules. While it was assumed for some time that endogenous peptides were only presented by MHC class I and exogenous peptides by MHC class II, recent findings have shown that class I molecules are also capable of presenting exogenous peptides under certain circumstances through a process called cross presentation (14-16). A number of different pathways have been suggested to explain how cross presentation occurs, and I will discuss some of these pathways briefly in Section 1.2.11. Overall, the steps and proteins involved in the classical pathway of peptide generation for MHC-I presentation described below are similar for both humans and mice.
Figure 1.2. Model of separate antigen-presenting pathways for endogenous and exogenous antigens.
Legend to Figure 1.2

Model of separate antigen-presenting pathways for endogenous and exogenous antigens.

Peptides generated through the endogenous pathway (A) are presented by MHC class I whereas those originating from the exogenous environment (B) are presented by MHC class II. The details of each pathway are not shown. Please refer to text for explanation of abbreviations. Modified from Nature Reviews Immunology 2012: 813-820. (17)
1.2.1 Antigen Processing and Presentation by MHC Class I

Following infection, viral proteins are degraded by proteasomes and the resulting peptides are transported by the transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER). In the ER, the newly generated peptides are loaded onto newly synthesized MHC class I molecules with the help of calnexin, calreticulin, tapasin, and ERp57. These class I molecules subsequently leave the ER and go through the Golgi before being presented on the cell surface (Fig. 1.3). Together, these processes are collectively referred to as antigen processing and presentation and the different components contributing to this process are described in detail below.

1.2.2 Calnexin

Immediately after synthesis, the MHC class I HC enters the ER lumen and becomes associated with a chaperone called calnexin (18, 19)(Fig. 1.3). Calnexin, discovered in 1991 (19), is a transmembrane protein which has been shown to bind to incompletely assembled and misfolded glycoproteins and to promote their proper folding (20). For MHC-I molecules, calnexin helps proper folding of the HC and association with β2m (21). Upon association of the HC with β2m and peptide, calnexin is released from the complex, while in the absence of β2m and peptide it promotes the retention of the HC in the ER (22-25). Thus, calnexin functions as a ‘quality control’ point preventing proteins such as MHC-I HC from reaching the Golgi in their non-native form (20). Phosphorylation of calnexin prevents the release of partially misfolded proteins to the secretory pathway (24, 25) indicating how calnexin is regulated in vivo. Beside these functions, calnexin is also involved in apoptosis (26) and plays an important role in controlling coronavirus’
ability to infect hosts in the severe acute respiratory syndrome (27). The details of calnexin’s role in ImDc will be discussed below (Section 1.5).

1.2.3 Antigen Processing and Presentation: ERp57

Another protein, a thiol-dependant oxido-reductase named ERp57, aids in disulfide bond formation of the HC and β2m proteins (28). Since association of the class I HC with β2m is non-covalent and unstable, this protein is thought to stabilize the HC/β2m complex (28). ERp57 was initially named ERp60, but in 1998 it was renamed ERp57 reflecting its actual molecular weight (29-31). ERp57 was shown to bind to newly synthesized glycoproteins (29-31). It binds covalently with tapasin via a disulfide bond and appears to work cooperatively with calnexin and calreticulin (see below) to assist in substrate folding (32). Also, the ability of ERp57 to carry out disulfide reduction/re-oxidation reactions has linked it to making the peptide binding groove more receptive to peptide loading (33). Studies with ERp57-deficient B cells (ERp57 knock-out mice do not survive) revealed reduced MHC-I expression, reduced pMHC-I complex formation due to suboptimal peptide loading, and lower antigen presentation (34). MHC class I molecules interacted briefly with the peptide loading complex when ERp57 was absent. This indicated ERp57 is essential for stabilizing the peptide-loading complex. Together, ERp57 contributes to formation of a stable peptide-bound MHC-I molecule.
Figure 1.3 The classical pathway of antigen processing and MHC class I biogenesis
The legend to Figure 1.3

The classical pathway of antigen processing and MHC class I biogenesis

Polypeptides containing MHC class I epitopes are tagged with a multiubiquitin chain (not shown) and are targeted to the 26S proteasome for degradation. Proteasomal processing results in the generation of various length peptides. Peptides larger than ~15 aa residues may undergo further trimming by aminopeptidases in the cytosol or in the ER to the correct length (~8-11aa). The newly synthesized class I heavy chain (HC) first binds to calnexin, which promotes its folding. Subsequently, β2m binds non-covalently to this class I HC forming an unstable heterodimer. This is followed by the joining of a preformed complex consisting of TAP, tapasin, ERp57 and calreticulin to this heterodimer. The resulting complex, called the peptide loading complex (PLC), is ready to receive peptides. Following peptide binding, the newly formed peptide-MHC class I complex (pMHC-I) is then transported through the Golgi to the cell surface. Refer to text for more details.
1.2.4. Antigen Processing and Presentation: Calreticulin

Studies with human cells have shown calnexin to be replaced by another chaperone called calreticulin during later steps in MHC class I biogenesis (Fig. 1.3)(35, 36). Mouse class I HC/β2m complexes, on the other hand, either remain attached to calnexin or are replaced by calreticulin (37). Calreticulin is a soluble luminal, Ca\(^{2+}\)-dependent binding protein with high amino acid sequence similarity to calnexin (37). Like calnexin, calreticulin binds to newly synthesized proteins and assists in their proper folding and subunit assembly. It associates with MHC-I prior to peptide binding and disruption of this association (e.g., by castanospermine)(35) prevents binding of class I/tapasin to TAP (see below). Calreticulin possibly functions by binding to peptides and chaperoning them onto MHC-I molecules (38, 39). In the absence of calreticulin, as shown by studies involving calreticulin-deficient cells (40), unstable/empty class I molecules are transported at a faster rate out of the ER and are expressed at a reduced level at the cell surface. This suggests that calreticulin affects the efficiency at which class I molecules exit the ER and are expressed on the cell surface. These findings also suggest that calreticulin is involved in retaining the assembly intermediates in the ER. In spite of similarities in their functions, calnexin and calreticulin have been shown to bind to different glycoproteins for reasons that are unknown (36, 41). Calreticulin also affects the WNT signaling pathway which is essential in embryonic stem cell pluripotency, growth and proliferation, cell differentiation and embryonic patterning. Calreticulin-deficient cells reveal disruption of the WNT signalling pathway presumably due to lack of Ca\(^{2+}\) release and activation affecting kinases and phosphatases involved in this pathway (42). Recent evidence implicated calreticulin in an unfolded protein response during tumor cell therapy. Tumor cells targeted by radiation therapy experienced enhanced antigen processing and surface expression of calreticulin inducing enhanced cytotoxic T lymphocyte (CTL) response (43). These
results collectively indicate calreticulin is an important component of the peptide loading complex. Its role in relation to ImDc will be discussed later on (See section 1.5).

1.2.5. Antigen Processing and Presentation: Proteasome and Immunoproteasome

The actions of these aforementioned proteins partially contribute to the formation of an empty, folded, peptide-receptive MHC class I heterodimer in the ER. This class I heterodimer becomes stable once a peptide has bound to it. Most of the peptides binding to these class I heterodimers are generated by proteasomes. Although proteasomes have been shown to function in protein turnover in many organisms, in mammals they have also been shown to be responsible for generating peptides presented by MHC class I molecules. Proteins destined for degradation are usually tagged with a 76 aa polypeptide called ubiquitin. These tagged proteins are recognized, unfolded and degraded by the proteolytically active 26S proteasome (Fig. 1.3)(44-46). The 26S proteasome consists of a 20S proteasome core and two 19S regulatory subunits. One 19S regulatory subunit is found at each end of the 26S proteasome. The 20S core complex is involved in the degradation of unfolded proteins while the 19S complex is responsible for substrate recognition and binding (44, 47, 48). The 20S core is a barrel-shaped structure of four stacked rings, each containing seven α (i.e., α1-α7) and β (i.e., β1-β7) subunits (49). The three β subunits (i.e., β1i, β2i, and β5i) are replaced following an immune response by induced subunits called low molecular weight proteins (LMP) 2, LMP10, and LMP7, respectively (50, 51). Proteasomes incorporating these subunits are called immunoproteasomes (discussed below). The 19S proteasome is made up of 17 different subunits forming a base and a lid (49, 52). The “base” contains the ATPase activity responsible for binding and unfolding of the substrate, while the lid is thought to be involved in de-ubiquitination (52). Studies where proteasome activity was
inhibited with specific inhibitors have shown impaired MHC-I assembly and peptide presentation (53-56), thus demonstrating the importance of the proteasome in antigen presentation.

Proteasomes can cleave proteins at the C-terminal side of basic and hydrophobic amino acids using trypsin-like activity and chymotrypsin-like activity, respectively (47, 55, 57). The generated peptides mostly have the correct C-terminus whereas the N-terminus is sometimes extended and needs further trimming by various aminopeptidases (see below). Peptides generated by proteasomes vary in length from ~3 to approximately 40 residues, many of which are too short (i.e., less than 8aa) to function in antigen presentation. TAP transporters are capable of transporting peptides of up to 40 residues long, although they preferentially transport shorter peptides (~8-16 residues) at a more efficient rate (58-60). Since about 25-30% of the newly generated peptides are 8-11 aa long, these are likely able to act directly as substrates for TAP transporters and MHC class I molecules (59-62), whereas longer peptides probably require additional processing (see below).

Interferon (e.g., IFN-α, -β, or γ) stimulation leads to the induction of immunoproteasomes (50, 63). Immunoproteasomes are also constitutively expressed in lymphoid organs such as the spleen, lymph nodes, and the thymus (64). Compared to 20S proteasomes, immunoproteasomes are ‘better’ at cleaving peptides after basic and hydrophobic residues, but weaker at cleaving after acidic residues (65, 66). Besides being involved in antigen processing and presentation, immunoproteasomes are also involved in generating biologically active proteins (e.g., cytokines) involved in inflammatory processes, thymocytes development, T cell differentiation and survival (67). It is interesting to note that immature DCs express equal amount of both proteasomes and immunoproteasomes while mature DCs predominantly express immunoproteasomes (68). Hence, it comes as no surprise that the identity of peptides generated by immunoproteasomes are mostly different than those generated by proteasomes. Following infection of HeLa cells with the vaccinia
virus expressing the hepatitis B virus core antigen, Kloatzel’s group showed the generation of the ImD hepatitis B virus 141-151 epitope was immunoproteasome-LMP7 dependent (69). Other studies showed DCs of mice lacking LMP2, -5- and 7 expression could not present several MHC-I epitopes normally expressed by WT DCs. The epitope repertoire in these KO mice was about 50% different than that of WT mice (70).

Other than its role in antigen processing and presentation, proteasomes also affect protein signaling and the induction of inflammatory cytokines. Studies where proteasome activity was inhibited by a chemical inhibitor (e.g., MG132) (46) showed a reduction in ocular inflammatory cytokine levels in patients with macular degeneration, the leading cause of blindness. Next, ERK (extracellular signal-regulated kinase 1) phosphorylation in human monocytes is essential in priming inflammasomes formed in response to pathogenic infections and tissue damage. Inhibition of the proteasome in LPS-stimulated human monocytes led to reduced inflammasomes indicating an important role for the proteasome in priming of inflammasomes (71). The role of the proteasome in ImDc will be discussed later on (see section 1.5). Collectively, these results show the importance of proteasomes in antigen presentation.

1.2.6. Antigen Processing and Presentation: Cytosolic Aminopeptidases

As mentioned previously, proteasomes can also generate N-extended precursors. These precursors are usually 2-25 (59) residues longer than the optimal 8-11 residues capable of binding to MHC class I molecules. These N-extended peptides are further trimmed to optimal size by aminopeptidases present in both the cytosol and the ER (Fig. 1.3) (72-78). Tripeptidyl peptidase II (TPP II) is a cytosolic aminopeptidase which has been shown to remove tri-peptides sequentially from free N-termini of peptides (79, 80). It has also been shown to generate peptides distinct from the ones generated by the proteasome, thus increasing the complexity of the MHC class I peptide
repertoire. Studies with TPP II deficient mice showed increased MHC-I surface expression indicating TPP II may also be involved in peptide destruction (81). Another cytosolic aminopeptidase is thimet oligoendopeptidase (TOP). Saric et al. (2001)(82) found that TOP can degrade peptides by endoproteolytic cleavage and that the preferred substrates are 6-17 residues in length. Using human cytomegalovirus Seifert’s group showed inhibition of TOP in Hela cells did not alter the overall generation- and the CD8+ T cell response to the pp65\textsubscript{495-503} epitope indicating that TOP is selective and does not degrade all cytosolic peptides with 6-17 residue lengths (83). Other cytosolic peptidases including leucine aminopeptidase (LAP), puromycin-sensitive aminopeptidase (PSA) and bleomycin hydrolase (BH) affect the overall pool of peptides generated in the cytosol too. Deficiency in LAP expression led to increased peptide presentation while no such change was observed when BH and PSA were absent (83-86). Dipeptidyl peptidase 9 is the first cytosolic aminopeptidase capable of removing dipeptides with a proline in the second position of the MHC-I ligand precursors (87). This is unique as peptides with proline at the first three amino acid positions usually are not processed within the cytosol or the ER (e.g., by ERAP, see below). A report by Kessler et al. (2011) showed, other than proteasome, TOP and nardilysin peptidases can also generate epitope precursors with the correct C-terminus (88). This was the first report of proteasome-independent cytosolic peptidase activities able to generate epitope C-terminals. In spite of the presence of cytosolic aminopeptidases, some N-terminally extended class I precursor peptides are still translocated into the ER in a TAP-dependent fashion (78, 82, 89, 90). Serwold et al. (2002)(72) were the first ones to show that the ER enzyme, ERAP (discussed in details in section 1.8), sequentially cleaves residues from the N-terminus of peptides but does not cleave peptides to less than 8 residues (Fig. 1.3). Recent data by Shen et al. (2011) showed some peptides with extended C-termini undergo carboxypeptidase trimming in the ER by angiotensin-
converting enzyme (ACE)(91). ACE was shown to both destroy and produce epitopes for presentation in association with major and minor histocompatibility molecules affecting the overall pMHC-I repertoire. Thus, these reports together indicate that there are several aminopeptidases residing in both the ER and the cytosol which likely contribute to the array of peptides available to be presented by MHC-I molecules. Some of these aminopeptidases likely also destroy certain peptides and thereby limit their presentation. Collectively, aminopeptidases and carboxypeptidases in the cytosol and the ER contribute to the overall pool of peptides generated in vivo.

1.2.7. Antigen Processing and Presentation: TAP

Newly generated peptides enter the ER lumen with the help of TAP and some of these become part of the peptide loading complex (PLC), whereas others are degraded. The PLC consists of the class I HC/β2m heterodimer, the chaperones calreticulin and calnexin, ERp57, TAP, and tapasin (Fig. 1.4). Translocation and binding of newly generated antigenic peptides influences class I HC folding, stability, and translocation to the cell surface, thereby affecting the level of HC/β2m expression on the cell surface. TAP is a heterodimer consisting of TAP1 and TAP2 which are both members of the ATP (Adenosine Triphosphate) binding cassette (ABC) family of transport proteins (92). The genes encoding these subunits are located in the MHC locus (92). Although the precise membrane topology of TAP1 and TAP2 is still under debate, most recent studies indicate that TAP1 and TAP2 monomers consist of a nucleotide binding domain (NBD) and six transmembrane helices forming the core of the transmembrane domain (Fig. 1.4B)(93-95). Both TAP subunits contain an N-terminal hydrophobic TM domain and a C-terminal ABC domain which contains the ATP binding site. The TM domains of TAP1 and TAP2 are thought to form a pore through which peptide substrates are translocated from the cytosol into the ER. Peptide
translocation starts with the binding of a peptide to the peptide binding site formed by both TAP1 and TAP2 between the putative pore and the ABC domains (92). The ABC domains hydrolyze two ATP molecules (Fig. 1.4A) to facilitate induction of conformational changes required for substrate transfer and subsequent translocation of peptides into the ER lumen (96). Studies with cell lines deficient in TAP (e.g., T2 or 0.174), as well as with TAP knock-out mice, have shown decreased levels of class I surface expression indicating the importance of TAP in antigen presentation and MHC cell surface expression (97-100).

1.2.8. Antigen Processing and Presentation: Tapasin

The other component of the PLC is tapasin (Fig. 1.4). Tapasin is a class I-specific accessory molecule thought to bridge an interaction between class I and TAP. It brings empty class I molecules into proximity of the TAP transporter which facilitates peptide loading. Tapasin, first discovered in 1994, is a 48 kDa type I ER membrane protein (101, 102). It consists of a single luminal region, a transmembrane domain, and a short cytosolic tail domain (101, 103, 104). Tapasin’s luminal domain is thought to be involved in class I binding (to the α2 and α3 domains of the HC)(105) and play an important role in ERp57 and calreticulin interaction. Indeed, tapasin is covalently linked via a disulfide bond to ERp57 (Fig. 1.4A)(106, 107). The TM domain and the cytosolic tail have been shown to be important in mediating interaction with TAP (101, 108-110). Recent data by Cresswell’s group indicate there are three tapasin docking sites on TAP (110). Manipulation of these sites indicated all three sites to be essential for TAP’s heterodimerization and stability. In 2012 Hulpke et al. (94) showed that the human PLC consists of two tapasin
Figure 1.4  Stoichiometry and molecular architecture of the MHC class I peptide-loading complex (PLC).

A)

B)
Legend to Figure 1.4

Stoichiometry and molecular architecture of the MHC class I peptide-loading complex (PLC).

(A) Model of PLC showing tapasin, calreticulin, ERp57, TAP, and MHC class I with a bound peptide. (B) 3D model of the PLC. The different members of the PLC consists mainly of alpha helices and beta sheets. Please refer to text for complete list of abbreviations. TMD, transmembrane domain. Modified from Hulpke S et al. (2012) FASEB J. 26: 5071-5080. (94)
molecules, with one tapasin molecule bound to each TAP subunit. Blocking of one tapasin molecule does not affect the overall antigen presentation indicating that one tapasin is sufficient enough for efficient MHC-I presentation (94). Tapasin’s ability to bind to different members of the PLC have led to its various suggested roles, including i) formation of a physical bridge between class I and TAP (101, 111), ii) retention of empty class I molecules in the ER (32, 112, 113), iii) TAP stabilization (101, 105), iv) peptide editing (114, 115), and v) enhancing peptide transport (108, 116-118) and peptide binding (106). Recent evidence from Shastri’s lab indicate tapasin influences the carboxy end of some epitopes (e.g., of LPS origin) affecting the overall peptide pool available for MHC-I presentation (119). Deficiency in tapasin altered the overall peptide repertoire leading to both loss and gain of pMHC-I complexes. The 3D structure of tapasin covalently bound to ERp57 was resolved in 2009 by Reinisch’s group (120)(Fig. 1.4B). Tapasin consists of two core domains linked by a flexible region. It interacts with the two catalytic domains of ERp57. Mutational analysis discovered a prominent conserved patch on the surface of the N-terminal domain of tapasin, absence of which affects the peptide loading and editing functions of the tapasin-ERp57 heterodimer (120).

1.2.9. Antigen Processing and Presentation: ERAP (Endoplasmic Reticulum Aminopeptidase)

Estimates are that about 50% of proteasome-generated fragments are too small for direct presentation by MHC class I molecules, ~25-30% are of the appropriate size and ~20% are too large (55, 61, 73). This implies that most of the longer peptides need to be trimmed before properly fitting MHC-I molecules. Peptides with extended N-terminals are further trimmed by ER aminopeptidase associated with antigen processing (ERAAP or ERAP). The details of ERAP’s role in peptide presentation and its structure will be discussed below (section 1.8). Briefly, humans
have two ERAP genes (i.e., ERAP1 and ERAP2) while mice only have ERAP1. ERAP trims peptides with extended N-terminal to the correct length suitable for MHC-I binding. The details of ERAP’s role in immunodominance will also be discussed in section 1.5.

1.2.10. Peptide Loading to MHC Class I and Transport from The ER to Golgi

A newly synthesized class I molecule achieves its 3D shape with the help of calnexin/calreticulin and associates with β2m to form an empty heterodimer. Subsequently, this heterodimer binds to the pre-PLC complex which leads to the formation of a ‘mature’ PLC capable of accepting peptides (Fig. 1.3 and Fig. 1.4). Thus, association of the MHC class I molecule with the pre-PLC complex appears to increase its efficiency of receiving peptides. Whether a peptide is loaded onto a MHC class I molecule randomly or is directed by various members of the PLC is not entirely clear. However, some studies suggest that tapasin (110, 121), calreticulin (40), ERp57 and TAP aid in loading peptides onto the binding site in the α1/α2 domains of class I molecules. It has been shown that four class I HC/β2m complexes and four tapasin molecules associate with a single TAP1/TAP2 heterodimer (120, 122). Aside from these known molecular interactions, it is possible that there may be additional molecules involved in MHC/peptide complex formation. This continues to be an active area of investigation.

The newly formed class I HC/β2m and peptide (i.e., pMHC-I) complex exits the ER and is transported via carrier vesicles, first to the Golgi apparatus and then to the plasma membrane where it is expressed on the cell surface and available for recognition by T cells (Fig. 1.3). The mechanisms of ER export are not fully known except that a set of proteins known as coatomer II (COP II) present in the carrier vesicles seems to promote transport of pMHC-I complexes from the ER to the Golgi. From the Golgi, these pMHC-I complexes are then transported via vesicles to
the cell surface where their membranes fuse with the plasma membrane allowing for cell surface expression.

1.2.11. Presentation of Exogenous Peptides by MHC Class I Molecules

Normally MHC class I molecules present peptides of endogenous origin (i.e., synthesized within the cell, discussed previously), but in certain instances they have been shown to present peptides of exogenous origin (i.e., not synthesized within the cell) through a process referred to as ‘cross-presentation’. Cross-presentation takes place with greatest efficiency in DCs, although macrophages and some other cell types have been shown to be capable of this activity too (123). Cross-presentation is one of the main ways tumour antigens and peptides from other microorganisms, which do not infect DCs or macrophages directly, are presented by MHC-I molecules. There is some controversy on the exact mechanisms and pathways leading to exogenous peptide presentation: some studies indicate that exogenous peptides can be presented by MHC class I molecules in a TAP-independent manner requiring no peptide transport to the cytosol (123, 124), whereas others suggest the opposite (i.e., in a TAP-dependent manner), meaning peptides have to be transported to the cytosol where they can be digested by proteasomes and loaded onto MHC-I molecules in a TAP-dependent manner. Whatever the mechanisms and the routes, antigens have to be first internalized by endocytosis, macro-pinocytosis or phagocytosis. Following peptide internalization, some of these peptides may escape into the cytosol because the endocytic-compartment is “leaky”. This leakiness has been suggested to be due to the presence of specific channels or translocators (e.g., Sec61) permitting peptides of certain sizes to escape from the endocytic compartments (124, 125). Once in the cytosol the same steps as outlined for endogenous peptides (Fig. 1.2) may take place leading to exogenous peptide presentation by MHC-I molecules.
Some other studies (123, 126, 127), however, have proposed the ER to be directly involved in cross-presentation. During phagocytosis, the ER membrane has been shown to fuse with the plasma membrane to form the phagocytic cup and the initial phagosome. Proteomic analysis of isolated phagosomes showed that several ER components, including Sec61, TAP and tapasin, were present in the phagosome membrane, suggesting that exogenous proteins may gain access to ER-based MHC class I loading machinery in the phagosomes. Once in the ER, these exogenous peptides can bind MHC class I molecules like their endogenous counterparts and are eventually presented on the cell surface. Taken together, these two pathways, in addition to the endogenous pathway described before (section 1.2), may provide further ways of presenting peptides for recognition by CD8⁺ T cells. Collectively, these pathways can affect the overall adaptive immune response elicited following an infection.

1.3. Recognition of MHC Class I/Peptide Complexes (pMHC-I) by CD8⁺ T lymphocytes and Memory T Cell Generation

Following a viral infection, the crucial first step in adaptive immunity is the activation of naive antigen-specific T cells by antigen-presenting cells in the lymphoid organs. Briefly, naive T cells respond to viral antigens only if they are presented by APCs in the context of self-MHC molecules (1). Both the MHC molecule and its bound peptide have to be recognized by the naive T cell’s TCR in order to achieve T cell activation (128). The type of TCR (i.e., αβ-TCR), the allele of the MHC class I molecule (e.g., HLA-B7 vs. HLA-B27), as well as the peptide origin (i.e., exogenous vs. endogenous), the sequence and the length of the peptide all play important roles in T cell activation (128-131). Generally, CD4⁺ T cells recognize exogenous peptides associated with class II molecules whereas CD8⁺ T cells recognize class I-associated endogenous peptide
complexes (128). However, due to the nature of this thesis, I will focus on the classical pathway of antigen processing, presentation and recognition by MHC class I-restricted CD8\(^+\) T cells. I will not deal directly with cross-presentation or with antigen recognition by CD4\(^+\) T cells.

CD8\(^+\) T cell activation is initiated by the interaction of a TCR-CD3 complex with a pMHC-I complex (Fig. 1.5)(132). TCR makes contact with both \(\alpha_1\) and \(\alpha_2\) domains of class I molecule as well as its bound peptide, whereas CD3 helps in signal transduction initiated by this interaction (71). Activation is further promoted by the interaction of the CD8 co-receptor molecule with the pMHC-I complex (128, 130-132). This interaction of TCR-CD3 complex with a pMHC-I complex enables the T cell and the APC to come closer, allowing for interaction of other molecules with their ligands. Interaction of co-stimulatory molecules and adhesive molecules with their ligands (e.g., CD80 or CD86 with CD28 or LFA-1 with ICAM-1), along with the interaction of CD8 co-receptors with TCR-pMHC-I complexes, initiate a cascade of biochemical events leading to gene activation and cell cycle induction of resting T cells. The induced gene products, such as IL-2, help in the differentiation and proliferation of peptide-specific T cells into either effector- (e.g., CTL) or memory cells, whereas other gene products, such as granzymes, help in the removal of antigen-infected cells by these activated, peptide-specific T cells (133). Activated CD8\(^+\) CTLs destroy infected target cells through release of membrane disintegrating proteins, such as perforins, or induction of apoptosis by activating the Fas/FasL pathway (134). After antigen elimination, the expanded antigen-specific T cell pool contracts substantially through apoptosis and only about 10\% of the antigen-stimulated T cells persist as memory cells (135).

Memory T cells provide enhanced protection after re-infection because of their increased precursor frequency compared with the naive repertoire, and their ability to proliferate and carry out effector functions at the site of infection. How memory T cells develop from the initial pool of
activated T cells is still not completely understood. Different models have been proposed as a result of investigation of responses to distinct viruses (e.g., influenza) (135-137). These models are not mutually exclusive. The divergent model predicts that early in the response, a population of memory cells is formed that persists even after the pathogen has been eliminated and the effector cells have died. The linear differentiation model, on the other hand, proposes that memory T cells develop from the effector T cell pool after the antigen load has decreased (138). Nevertheless, after their formation, memory cells may persist for extended time periods ranging from weeks to months (in mice) to years (in human) depending on the organism (139, 140). Re-infection with the same virus initiates clonal expansion of effector T cells from these memory pools and lead to an increased size of the antigen-specific T cell population in the memory state (135, 138, 140).

With the thematic focus of this thesis, the following sections will first introduce the influenza virus followed by a discussion of factors contributing to immunodominance in relation to a viral (e.g., flu) infection in general.
Figure 1.5 The structure of a TCR bound to the HLA-B2705 class I:peptide complex

The TCR binds to the MHC class I:peptide complex, straddling both the $\alpha_1$ and $\alpha_2$ domain helices. TCR, magneta; MHC heavy and light chains, green; bound peptide, orange. HIV-1 peptide, cyan. Bound $\beta_2m$ not shown. Modified from: Xia Zhen et al. (2014): Scientific Report. Feb 13; 4:4087. (132)
1.4. Influenza A

Influenza virus has been divided into three different types: A, B, C based on disease pathogenesis (141). Among these, influenza A is best characterized and poses the most serious threat to public health. According to World Health Organization, in North America alone more than 15,000 people die due to complications related to influenza infection. This number is even higher worldwide reaching well over 250,000 deaths per year. Recent emergence of avian influenza and bird flu strains reinforces the serious threat this virus poses to the human race. Influenza A virus belongs to the Orthomyxoviridae family of viruses consisting of negative RNA strands. Its genome consists of eight RNA segments encoding for ten different viral proteins (i.e., HA, NA, M1, M2, PB1, PB2, PA, NP, NS1 and NS2)(141). Most of these polypeptides are incorporated into virions and have known specific functions. Haemagglutinin (HA) and neuraminidase (NA) play important roles in cell cycle entry and exit, while matrix one (M1) is thought to act as an adaptor protein between the lipid envelope and the internal ribonucleoprotein (RNP) particles (141). Polymerase B1 (PB1), B2 (PB2), and A proteins (PA) act as RNA polymerases, while nucleoprotein (NP) is involved in virion RNA encapsidation (141, 142). The NS1 and NS2 (Non-structural Protein one and two)(also known as Nuclear Export Protein, NEP) proteins have been implicated in the export of influenza virus RNP complexes from the nucleus. A number of reports suggest these proteins may also be involved in the evasion of host immune response (141, 143, 144).

Viral infection begins with the entry of the virus into host cell through receptor-mediated endocytosis (Fig. 1.6)(145). After fusion of endosomal and viral membranes, the RNPs are released into the cytoplasm and then transported into the nucleus. Transcription and replication of the negative-sense RNA segments take place in the nucleus. The newly generated viral RNAs are
transported into the cytoplasm where translation occurs. The resulting viral proteins transit through the ER and Golgi and combine with the newly replicated RNA segments forming new virion particles. These virions eventually “bud out”, thereby killing the host cell and then go on to infect other cells (145). In addition, some of the newly synthesized viral proteins are processed to peptides by the proteasome (as previously discussed).

Infection with influenza A initiates both an antibody (Ab) response by B cells, and a clonal expansion of antigen-specific T cells (141, 146) in both humans and mice. T cells acquire various effector functions allowing them to eliminate infected cells by different means including apoptosis, while Ab responses are mainly directed against the HA and NA surface proteins. Antibodies help to block off further viral infection by preventing the binding of the viral particles to host cells (146) in addition to targeting invading viruses for destruction by either complement or Ab-dependent cytotoxicity (141, 146, 147). The composition of Abs produced following initial and subsequent flu infections differ: high levels of IgM make up for the majority of the Abs produced following first flu infection while IgG constitutes the majority of the Abs produced following subsequent flu infections. In both humans and mice, specific CTL activity arises within 3-4 days after infection, peaking by 7-10 days, and then declining after 12 days (141, 148, 149). Within 7-10 days of primary infection, most virions are eliminated by the viral-antigen-specific killer activity of CTLs, paralleling the developments of CTLs (148). A second round of infection with the same virus, will lead to a mainly CTL memory response. Memory CD8+ T cells confer enhanced protection after re-infection because of their increased precursor frequency compared with the naive repertoire, and their rapid ability to proliferate and carry out effector functions at the site of infection (141, 148).
Figure 1.6 Influenza A life cycle and host immune response following flu infection
Legend to Figure 1.6

Influenza A life cycle and host immune response following flu infection.

(A) Following influenza infection, the virus binds via its cell surface receptors (i.e., HA) to epithelial cell surface markers inducing endocytosis. Once inside the cell, the viral genome (i.e., RNA) enters the nucleus where replication and transcription take place. Newly transcribed RNAs move from the nucleus to the cytosol where translation occurs. Some of the newly translated viral proteins are degraded into peptides by the proteasome and some other cytosolic aminopeptidases whereas other viral polypeptides combine with the newly formed viral core to give rise to new viral particles. Peptides degraded within the cytosol are transported into the ER by TAP. Once in the ER, some peptides may be further trimmed by ERAP before being loaded onto MHC class I molecules whereas other peptides of the right length and sequence bind directly to newly synthesized MHC class I molecules. MHC class I molecules are synthesized and loaded with newly generated peptides within the ER with the help of tapasin, calreticulin, calnexin and ERp57. Bound peptide MHC class I complexes move along the ER and through the Golgi before being presented on the cell surface. (B) Thymocytes originating in the bone marrow travel to the thymus where they mature into functional T cells. Following negative and positive selections, T cells expressing CD4 and CD8 are generated. CD4⁺ T cells differentiate further into T_{H1}, T_{H2}, and T_{Reg} cells whereas CD8⁺ T cells differentiate into CTL following its activation via peptide-MHC class I-T cell receptor interaction. Infected host cells are destroyed by activated CTL. Subsequently, most of the activated CTL die while some differentiate into memory T cells persisting for a very long time. Newly formed viral particles may bud out of their host cell before they are destroyed by CTL thereby continuing its viral cycle. The numbers refer to different factors contributing to immunodominance in response to influenza infection (Section 1.5). Adapted from (145).
1.5. Introduction to Immunodominance (*The majority of this section was published in the journal of Clin Immunol. 2012 May; 143(2): 99-115*)

Following a viral infection, CD8+ CTL recognize viral antigenic peptides bound with a self-MHC class I molecule on the surface of an infected cell and are thereby activated to lyse that target cell (137, 150). A central feature of many anti-viral T cell responses is the phenomenon of immunodominance (ImDc). This refers to the observation that, despite the co-expression of 3-6 different MHC-I molecules on APC, and the potential generation of hundreds to thousands of distinct 8 to 11-mer viral peptides from the proteins of a typical virus for recognition, a large proportion of the anti-viral CTL population tends to be dominated by certain class I MHC alleles, and only a small number of viral peptides are presented by a given class I MHC (151). The peptides that dominate the recognition events with a particular MHC allele are referred to as the immunodominant (ImD) epitopes, whereas less-favored peptides are termed subdominant (SbD) (151).

Immunodominant epitopes have been shown to be critical in eliminating infected cells and in contributing to the memory T cell pool, thereby enabling the immune system to respond more rapidly following re-infection. For example, of the 10 different influenza A proteins generated during infection of H2b mice, one clearly preferred viral epitope recognized by CTL is the NP366-374 peptide in association with H2-Db (152). The predominant mouse TCR that recognizes this H2-Db/NP366-374 complex uses the Vβ8.3 chain (152, 153). Although this system seems to work well for responses to most infections, certain other viruses, like HIV-1 (Human Immunodeficiency Virus), are highly prone to mutation. As a result, such a focused CTL response against one or a few specific ImD epitopes may actually constitute a handicap in host response because of the potential mutation of the ImD epitope and consequent evasion of CTL recognition.
Although the mechanisms of ImDc are not completely understood, it appears that almost every step in the antigen processing and presentation pathway and factors listed in Table 1 may contribute to ImDc. Some of these factors influence ImDc directly (e.g., negative selection of potential immunodominant viral epitope specific T cell precursors) whereas others affect it indirectly (e.g., by inducing changes in the antigen processing and presentation pathways leading to changes in the overall immune response). There have been examples of ImDc in response to infections caused by the vaccinia virus (154-157), lymphocytic choriomeningitis virus (LCMV)(158-160), human cytomegalovirus (CMV)(161), hepatitis B virus (HBV)(162, 163), hepatitis C virus (HCV) (164), West Nile virus (165), Listeria (166), Epstein Barr virus (EBV)(167), and HIV-1 (168-173). Since the preponderance of my studies and studies dissecting the biologic basis of ImDc focus on influenza A infection, I will concentrate on the lessons learned from this infection. I will mention ImDc seen with other viral types (e.g., HIV-1) very briefly. Below is a detailed discussion of different factors contributing to ImDc.

1.5.1. Factors Influencing Immunodominance in Flu Infections

The dominant factors contributing to ImDc are listed in Table 1 and most of these factors are shown in Figure 1.6. The factors contributing to ImDc function either alone or in combination. These factors play an important role in ImDc regardless of viral type. A number of studies have shown evidence of ImDc or a shift of ImDc in flu-infected mice. Most of the anti-viral CTL responses are directed against the H2-D\(^b\)/NP366-374 peptide in flu-infected wild type (WT) mice (152, 153). This predominant CTL response has been shown to be due to the ability of DC and other APC to present this epitope (174). Other flu peptides, such as PA224-233, are presented only by DC and hence may be less recognized, compared to NP366-374, by CTL. Flu infection of HLA-
B7 or HLA-B27 transgenic mice recognizes ImD flu epitopes NP418-426 and NP383-391, respectively (152). These same epitopes are also recognized in allele matched humans (152, 175, 176). The individual factors contributing to ImDc are discussed below and are shown and numbered in no particular order in Figure 1.6.

**Table 1. Factors contributing to immunodominance following a viral infection.**

<table>
<thead>
<tr>
<th>Possible Factors</th>
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<tbody>
<tr>
<td>Prior viral infection</td>
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<tr>
<td>Route of viral infection</td>
</tr>
<tr>
<td>Viral mutation</td>
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<tr>
<td>Role of proteasome</td>
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<tr>
<td>TAP specificity</td>
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<tr>
<td>ERAP</td>
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<tr>
<td>Calnexin/calreticulin/tapasin</td>
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<tr>
<td>Co-expression of multiple MHC class I</td>
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<tr>
<td>Enzymes involved in TCR generation</td>
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<tr>
<td>Frequency in naive repertoire of T cells with appropriate TCR</td>
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<tr>
<td>TCR:MHC binding</td>
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<tr>
<td>Granzymes</td>
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<tr>
<td>Transport/stability of MHC/peptide complexes</td>
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<tr>
<td>Generation of memory CTL</td>
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<td>T_reg</td>
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<tr>
<td>Age</td>
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<tr>
<td>Ag processing specificity</td>
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<tr>
<td>Viral protein abundance</td>
</tr>
<tr>
<td>Degree of proliferative expansion of T cell following antigen recognition</td>
</tr>
<tr>
<td>Competition at the level of Ag processing and presentation</td>
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<tr>
<td>Polymorphism</td>
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</tbody>
</table>
1.5.1.1 Prior Infection History

Prior infection with a specific viral strain may influence the immune response to subsequent infection with a different viral strain of the same type. The immunodominant CTL response can be shifted as a result of prior flu infection or flu vaccination as shown by Bodewes et al. (177). This group showed that flu A/H3N2 infected mice with no prior vaccination with whole inactivated virus (WIV) had similar anti-viral CTL response against both NP366-374 and PA224-233 peptides following secondary infection with flu A/H5N1. Yet mice exposed to prior WIV vaccination or secondary infection with flu A/H5N1 had higher CTL responses directed at NP366-374 peptide. This demonstrates that prior vaccination with different flu strains may shift the repertoire of anti-viral CTL responses against a specific ImD epitope. Vaccination with WIV led to a larger pool of CD8+ CTL recognizing pMHC/NP366-374 complexes instead of pMHC/PA224-233 complexes. These results confirm previous findings shown by others (152) indicating the dominance of NP366-374 epitope recognition. Thus, prior viral infection influences T cell pools capable of recognizing both ImD and non-ImD epitopes.

1.5.1.2 Route of Viral Infection

Immunodominance is also influenced by the route of infection and the subsequent frequency of precursors capable of responding to ImD epitopes. Jenkins et al. (178) showed that mice infected intranasally with PR8/H1N1 and X31/H1N1 flu A viruses, which were engineered to express the ovalbumin OVA257-264 epitope in NA (i.e., PR8-OVA and X31-OVA), showed similar levels of anti-viral CTL responses to both ImD OVA257-264 and NP366-374 epitopes. However, the CTL responses to other ImD PA224-233 and PA1.703-711 epitopes were diminished. PR8-OVA primed mice infected intraperitoneally with X31-OVA led to reduced CTL
responses to all known ImD flu epitopes except for OVA256-264 (178). This indicates that increased frequency of CTL capable of recognizing a specific ImD epitope (i.e., OVA256-264) could influence the magnitude of CTL responses to other ImD epitopes. Furthermore, the route of infection seems to affect ImDc as well. Intraperitoneal infection leads to reduced antigen presentation whereas intranasal infection, which is the natural route of flu infection, allows for more effective antigen presentation. Our studies with flu infected transgenic mice (Chapter 3) and studies done by others with other pathogens, such as LCMV, have shown the important role of viral infection route in determining ImDc (160).

1.5.1.3 Viral Mutation

The influenza virus is prone to mutation due to the absence of proof-reading polymerase. Mutations within a viral genome may affect the overall anti-viral CTL responses in hosts following viral infections. Changes in the anti-viral CTL responses mostly, but not always, lead to changes in the overall ImDc following a viral infection. Whether a shift in the overall immune response and hence ImDc is observed depends on the type of mutation (e.g., point versus frame shift mutation), the viral type/strain and the host. To investigate the role of mutation to flu, Turner’s group (179) introduced a point mutation at position 6 of the ImD NP366-374 polypeptide in flu virus. WT mice infected with the mutated version of flu showed a narrower TCR repertoire. However, the magnitude of anti-viral CTL response to NP366-374 was similar in mice infected with both the native and the mutated flu strains. Even the level of CTL activity and cytokine production was similar among these two groups. These results indicate point mutations in flu sequences may not always lead to changes in the overall immunodominant response observed. But these may be exceptions to the norm, as shown by mutation affecting the flu HA and NA proteins.
Mutations affecting these proteins allow for viral evasion of host adaptive immunity. In this context, host immunity has adapted to respond to these viral changes by producing different kinds of antibodies as opposed to changing the overall anti-viral CTL response. Another example of this kind of mutation within an ImD flu epitope was reported by Berkhoff et al. (176). They generated two flu viruses, either with or without the B27-restricted flu NP383-391 epitope (i.e., with a mutation within the NP383-391 sequence). Peripheral blood monocytes (PBMC) infected in vitro with the mutated version of flu had an overall reduced level of anti-viral CTL response compared to PBMCs infected with the WT version of this virus. Thus, a change within the sequence of a specific viral protein seems to influence ImDc. These reports collectively show mutations as a result of replication and recombination influence the overall anti-viral CTL response. These reports also indicate that changes in the overall anti-viral CTL response on the most part, but not always, influence the overall pattern of ImDc following a viral infection.

1.5.1.4 Role of Proteasome and Immunoproteasome

The role of proteasome is critical in determining ImDc. Most of the peptides binding to MHC-I molecules are generated by proteasomes. The absence of proteasomal components has recently been shown to be detrimental on the overall pool of peptides generated following a viral infection (180). Although proteasomes have been shown to function in protein turnover in many organisms, in mammals they have also been shown to be responsible for generating peptides presented by MHC-I molecules as discussed previously (44, 47). A recent study has shown the absence of the 26S proteasome components lead to a different viral (e.g., LCMV) peptide pool than when these are present (180). Mice deficient in proteasomal catalytic core components, such as LMP2 and MECL1 (multicatalytic endopeptidase complex like 1), show overall reduced anti-
viral CTL responses to both ImD NP366-374 and PA224-233 epitopes following flu infection (181). The severity of diminished CTL response was more evident in LMP2 knock out (KO) mice (LMP2<sup>−/−</sup>) than in MECL1 KO mice (MECL1<sup>−/−</sup>). The absence of LMP2 in LMP2<sup>−/−</sup> mice led to reduced PA224-233 epitope generation compared to WT mice. Absence of other proteasomal components, such as LMP7, did not affect the overall presentation of PA224-233 epitope. The number of CD8<sup>+</sup> T cells was also lower (i.e., ~50%) in LMP2<sup>−/−</sup> mice compared to WT mice although the TCR Vß usage and PA224-233 peptide avidity was similar to that of WT mice (181). Thus, this report along with a recent report (180) indicates a direct correlation between the presence of some proteasomal components and the effects they would have on the overall number of peptides generated. Reduced number of peptides produced (e.g., PA224-233) can lead to a reduced number of peptide-specific T cells activated hence affecting the overall immune response to that particular virus. Other reports with different viruses (181-184) have also shown the importance of the proteasome in peptide generation. Thus, depending on the peptide some proteasomal components may affect its generation more than others. Further investigation into each of the proteasomal components should elucidate their specific roles in peptide generation and hence ImDc.

1.5.1.5 Role of Transporter with Antigen Processing (TAP)

The transporter associated with antigen processing also plays an important role in ImDc. Newly generated peptides enter the ER lumen with the help of TAP. As discussed above TAP is a heterodimer consisting of TAP1 and TAP2. Peptide translocation starts with the binding of a peptide to the peptide binding site formed by both TAP1 and TAP2 between the putative pore and the ABC domains (92). Studies with cell lines deficient in TAP (e.g., T2 or 0.174), as well as with
TAP KO mice, have shown decreased levels of class I surface expression indicating the importance of TAP in antigen presentation and MHC cell surface expression (185, 186).

There are no documented instances which demonstrate that TAP plays an important role in presentation of ImD flu epitopes in mice or humans. However, a recent study by Tenzer et al. (187) showed the importance of TAP in HIV-1 peptide presentation. This group followed HIV-1 infected individuals and found that peptide binding and affinity to TAP is critical in peptides being presented on the cell surface by MHC-I molecules. Peptides not having the correct C-terminal (i.e., basic and hydrophobic amino acid) composition and being longer than the desired length preferred by TAP (i.e., > 45aa in length) for translocation are omitted from being transported into the ER lumen. This affects the immune response to HIV-1 viral epitopes. Introduction of phenylalanine at position 79 of a polypeptide, from which many epitopes are generated, reduced its binding affinity to TAP leading to an overall reduced anti-viral CTL response to peptides that otherwise would have been ImD (187). This study along with other reports (188-191) with different viruses and bacteria show how important TAP is to the overall immune response and ImDc.

1.5.1.6 Endoplasmic Reticulum Aminopeptidase (ERAP)

The role of ERAP is becoming increasingly recognized as a key element in immunodominance. ERAP trims precursor polypeptides in the endoplasmic reticulum to epitopes of various lengths thereby generating (192) and destroying (193) potential peptides to MHC-I binding. The role of ERAP in peptide generation has been reviewed by us and others (125). Reports by two different groups (194, 195) suggest that absence of ERAP may not be detrimental in immunodominant CTL responses following flu infection. However, findings by others with ERAP-deficient mice challenged this view. For instance, a study by York et al. (196) showed that
even in the presence of T cells expressing TCR repertoires capable of responding to various viral peptides, the cytotoxic immune responses to LCMV epitopes in ERAP-deficient mice change. The anti-viral CTL responses to some ImD epitopes decreased while responses to some SbD increased following LCMV infection. The authors demonstrated that this was due to the absence of ERAP. The functional importance of ERAP is also evident in individuals infected with the HIV-1 virus. ERAP mediates the generation of ImD epitopes from p17 (i.e., HLA-A2-restricted gag77-85 and HLA-A30-restricted gag76-86 epitopes) and p24 (i.e., HLA-B27-restricted gag131-140) proteins. Different variants of the p17 epitopes, some with extended N-termini and some with point mutations at one or more positions within the epitope sequence, had different rates of digestion by ERAP. Only peptides which ‘fulfilled’ ERAP’s criteria for peptide modification were generated and subsequently recognized by specific anti-viral CTLs (187). These results indicate that ERAP importantly influences the overall immune response following viral infections.

1.5.1.7 Role of Tapasin

Following infection, viral proteins are degraded by proteasomes and the resulting peptides are transported by TAP into the ER. In the ER, the newly generated peptides are loaded onto MHC class I molecules with the help of calnexin, calreticulin, tapasin, and ERp57. Selected populations of these generated proteins may influence ImDc following a viral infection. Studies by Elliot’s group (158, 197) showed that tapasin deficient mice had altered immune response to LCMV ImD peptides rather than reduced immune response following infection. They were able to show that the altered immune response was due to tapasin’s editing ability, which quantifies peptide optimization as a function of peptide supply and peptide unbinding rates, instead of a lack of T cell stimulation or antigen presentation. Another study by Dalchau et al. (198) showed
tapasin’s role in ImDc in relation to gag and pol epitopes in HIV-1 infected individuals. They showed that tapasin influenced peptide binding to MHC-I molecules without affecting the overall MHC-I cell surface presentation. ImD epitopes seem to bind to their MHC molecules faster than other non-ImD epitopes. This in turn leads to more ImD peptides being presented on the cell surface, thus affecting the overall immune response. The role of the other three proteins, calnexin, calreticulin, and ERp57, in relation to ImDc is not well established although one study with HPV from Peng et al. (199) suggests that calreticulin is important in ImDc. Further investigation with calnexin, calreticulin and ERp57 in relation to flu infection will determine their roles in generating ImD flu epitopes.

1.5.1.8 Co-expression of Multiple MHC Class I Alleles

Expression of specific MHC-I allele combinations and their level of expression determine what epitopes CTLs will recognize. The absence of these MHC-I alleles may lead to reduced immune responses to well-known ImD viral epitopes. Thus, co-expression of multiple MHC-I alleles seem to be an important factor contributing to ImDc as seen in studies with monkeys, mice, and humans. In monkeys, such as the cotton-top tamarin for instance, co-expression of different types of MHC-I alleles determines what flu epitopes will be responded to following infection (200). As expected, only those peptides capable of binding to and being presented by specific MHC-I molecules induce an immune response. Similar studies by Day et al. (201) with mice co-expressing H2-K\(^k\) and H2-D\(^b\) showed that CD8\(^+\) T cell responses to the ImD epitope H2-D\(^b\)/PA224-233 diminished, whereas that of NP366-374 did not change following X31/H3N2 flu infection. They were able to show that this lower CD8\(^+\) T cell response was due to lower number of naive PA224-233 T cell precursors in H2-K\(^{k\&b}\) F1 compared to homozygous mice. Functional
studies showed impairment in PA224-restricted T cell expansion and differentiation. Our studies with flu infected transgenic mice co-expressing HLA-A2 with either HLA-B7 or HLA-B27 have shown similar anti-viral CTL responses to known ImD epitopes (Chapter 3). These results lead to different conclusions to the study of Turner’s group (201), but yet confirm others by Chamberlain’s group (152). Studies with flu infected human subjects expressing A2, B7, or B27 class I alleles showed CTL responses to the same ImD epitopes as in transgenic mice (152, 202). Thus, it seems that co-expression of certain allele combination determines whether one sees ImDc in host response to flu.

Not all combinations of allele co-expression lead to ImDc. This holds true also for HIV-1 infected subjects. Co-expression of HLA-B with HLA-A and HLA-C alleles shows ImDc of HLA-B epitopes over other alleles (168). Brander’s group showed that the magnitude of HLA-B restricted CTL epitope response was higher than those of HLA-A and –C as determined by ELISpot. Functional avidity and not the peptide binding affinity to MHC-I molecules accounted for the increased HLA-B restricted CTL responses. These results were recently confirmed by Friedrich et al. (203). This group was able to show that this increase in HLA-B epitope anti-viral CTL response was due to increased cell surface expression of HLA-B27 compared to HLA-B7, -14, -35, 57 and HLA-A2.

Expression of specific MHC-I alleles and their subtypes may also contribute to ImDc. Individuals positive for HLA-A3 or HLA-B35 mount greater anti-viral CTL responses to these peptides than individuals expressing HLA-B7 alone (173, 204). Individuals positive for HLA-B57 demonstrate restricted HIV progression and longer survival time compared to non-HLA-B57 individuals (205, 206). The ImD HIV epitope of HLA-B57 (i.e., TW10) is located in the gag p24 region, an integral protein essential for the HIV life cycle. Substitution mutation at this sequence
allows for viral escape from anti-viral CTL responses yet this escape comes with reduced viral ability to replicate (207). To date no one has been able to demonstrate why HIV-1 progression is reduced in HLA-B57 positive individuals. Furthermore, most of the CTL responses (i.e., ~ 75%) are directed against gag77-85 (i.e., SL9) in HIV-1 infected individuals expressing HLA-A0201 (208). In individuals expressing HLA-A2601 and HLA-A2603, but not HLA-A2602, the ImD CTL epitope responses is directed against gag169-177 even though this epitope binds with similar affinity to all HLA-A26 subtypes (171). Determining the reason why certain viral epitopes are recognized by flu and HIV-1 infected individuals more than others is difficult as humans can co-express up to six different MHC class I alleles. Nevertheless, it appears that co-expression of certain allele combinations in the same individual influences the overall anti-viral CTL response following viral infections.

1.5.1.9 Role of Enzymes Involved in TCR Generation

Enzymes involved in the generation of αβ-TCR repertoire contribute to ImDc. A study by Yewdell’s group (209) showed mice lacking expression of the terminal deoxynucleotidyl transferase (TdT), an important enzyme for VDJ rearrangement of TCR genes as well as antibody genes, shifted CTL responses to both ImD and SbD flu epitopes compared to WT mice. The absence of TdT enzyme led to ~30% reduction in CTL responses to ImD NP147-155 epitope while CTL responses to subdominant HA518-526 epitope were increased compared to WT mice. These findings were confirmed by Leon-Ponte et al. (210) again indicating the importance of TdT’s role in immune responses by T cells. Since HIV-1 infection leads to CTL responses directed at specific epitopes such as gag77-85 (208), the absence of TdT may lead to deletion of specific T cell subsets capable of recognizing specific ImD peptides. Although no direct studies investigating the role of
TdT in relation to HIV-1 have been carried out, studies with other viruses have shown that the absence of this enzyme predicts a relative depletion of T cells capable of recognizing specific ImD epitopes.

1.5.1.10 T Cell Precursor Frequency

T cell precursors are cells which have survived both negative and positive thymic selections and have matured into naive antigen-specific T cells. These cells can be activated and differentiated into effector T cells following antigen exposure. The number of T cell precursors and the ability of these precursors to proliferate and persist over time may also contribute to ImDc. There is experimental evidence for and against this notion. A study by La Gruta et al. (211) showed that infection of WT mice with flu PR8/H1N1 leads to primary CD8$^+$ T cell response dominated by both NP366-374 and PA224-233. However, secondary challenge with the same virus led to significantly (i.e., ~80-90%) increased NP366-374 epitope CTL response. This was shown earlier by this group (174) to be due to both DC and non-DCs presenting the NP366-374 epitope. Thus, higher number of APCs presenting the same peptide may have led to activation of increased numbers of CD8$^+$ T cells which in turn resulted in CTL dominance of NP366-374 over PA224-233. Yet a recent study contradicted these findings (212) by showing that it is not the number of T cell precursors available, but rather the rate of recruitment and expansion of those T cell precursors in response to flu which determines whether immunodominant responses will be elicited. This conclusion was subsequently challenged yet again by more recent findings from Tan et al. (213) and Schmidt et al. (164). Using flu and hepatitis C viral infection, these investigators were able to show that number of naive T cell precursors is indeed important in achieving ImDc. The same is valid following HIV-1 infection. Studies carried out with simian immunodeficiency virus (SIV),
which mimics HIV infection in monkeys, showed that a higher number of T cell clones responding to the ImD gag p11C epitope were present compared to T cells recognizing the pol p68A SbD epitope (214). The precursors for the ImD epitope were shown to be higher than those for SbD epitope. Thus, both T cell precursor number and their proliferation and differentiation in response to viral infection affect the overall anti-viral CTL response in individuals infected with flu and HIV-1.

1.5.1.11 TCR:pMHC Binding

The three dimensional aspect of structural binding of peptides to their MHC-I molecules may contribute to ImDc. MHC class I molecules and their peptides bind to TCR as the critical event in generating T cell responses. This overall MHC/TCR interaction influences ImDc as seen in flu and HIV-1 infections. The primary flu responses in WT mice include the ImD NP366-374, PA224-233, and Pb1.703-711 (152, 215). X-ray crystallographic analysis of pMHC/PA224-233 and pMHC/Pb1.703-711 complexes have shown a bulge at the C-terminus of these peptides while still bound to MHC-I molecule (215). This feature at the C-terminus is absent from SbD epitopes (e.g., HA468-477). This kind of bulge may allow for better TCR binding and hence may affect the overall TCR repertoire in these mice. DeMarse et al. (171) showed that peptide binding to HLA molecules plays an important role in HIV ImD peptides being presented. Mutations leading to reduced binding affinity of ImD epitopes to specific HLA alleles led to reduced ImD anti-viral CTL responses. In addition, HLA-A26 positive HIV-infected individuals respond to gag169-177 (171). Although this epitope binds with similar affinity to all HLA-A26 subtypes, the responses to this epitope in association with HLA-A2601 and HLA-A2603 is greater than those with HLA-A2602. Binding affinity did not influence the overall antiviral CTL responses to ImD epitopes in
HIV-infected individuals co-expressing HLA-B with HLA-A and HLA-C. The peptide binding avidity to MHC molecules was higher in individuals positive for HLA-B expression and negative for HLA-A and -C expression (168, 203). Thus, binding affinity may or may not influence ImDc seen following a viral infection.

1.5.1.12 Role of Granzymes

Following a viral infection, anti-viral CTL responses include activation of the apoptotic pathways and release of apoptotic enzymes. A study by Moffat et al. (216) demonstrated that following flu infection hierarchical production of granzymes, such as granzyme A and B, influences the order of CTL responses to ImD epitopes. Granzyme A is expressed by CTL about six days post-infection whereas granzyme B is expressed throughout the infection. Most CTL respond to ImD epitopes such as PB1.703-711 before NP366-374. These investigators showed these responses are dependent on the order, level, and the type of granzyme production. As in HIV-1 infection, individuals whose CD8\(^+\) T cells produce granzymes have different CTL profile in response to HIV-1 infection than those CD8\(^+\) T cells lacking granzyme production (217-220). These findings indicate a strong role for enzymes involved in the killing of infected target cells by CTL. Thus, CTL activation and its subsequent cytotoxic action seem to influence ImDc.

1.5.1.13 Role of Memory T Cells

Memory T cells are cells which have previously been exposed to antigen and have the ability to respond to a secondary viral infection of the same type and strain more vigorously than naive T lymphocytes. As mentioned above, following a viral infection the crucial first step in adaptive immunity is the activation of naive antigen-specific T cells by APC in the lymphoid
organs. Activated CD8+ CTL destroy infected target cells through several mechanisms, such as of membrane lytic proteins. After antigen elimination, the expanded antigen-specific T cell pool contracts substantially through apoptosis and only about 10% of the antigen-stimulated T cells persist as memory cells (135, 136). Memory CTLs and their location within the periphery and lymph node (LN) may influence their ability to recognize different types of DC as distinguished by the expression of CD11b, CD103, and CD8 markers. Different subsets of DC may or may not activate specific CTL memory cells. This in turn may determine whether ImDc will result. Cauley’s group (221) showed that flu memory NP-specific CTL proliferate sooner (i.e., within four days) than PA-specific CTL following secondary flu infection. Memory NP-restricted CTL recognize CD103+-expressing DCs in the medullar LN, which are well known for their involvement in peptide presentation in the early stages following an infection. Memory PA-restricted CTL remain dormant four days post-infection and recognize CD8+-expressing DC whose levels rise five days post-infection (221). These results indicate that the degree of CTL memory activation and proliferation along with APC ability to present the desired peptides to memory CTL contribute to ImDc. SIV vaccine studies in primates, such as Burmese rhesus macaques, have shown the importance of memory T cells in recognizing gag specific ImD gag241-249 and gag206-216 epitopes. Animals vaccinated with gag241-249, showed increased CTL responses to this particular epitope instead of showing any responses to gag206-216. Similarly, gag206-216 vaccinated macaques showed increased anti-viral CTL responses to this epitope only (222). This demonstrated that animals exposed previously to a particular antigen can respond to that specific antigen more rapidly than to other antigens. This study along with some others (223, 224) has shown that generation of memory T cells and their ability to recognize specific APCs could influence the overall ImDc.
1.5.1.14 Role of Regulatory T Cells (T\textsubscript{Reg})

Immuno-dominance can also be affected by the presence of regulatory T cells (T\textsubscript{Reg}). A mathematical model proposed by Levy’s group (225) suggested that T\textsubscript{Reg}s play an important role in ImDc. A detailed study by Yewdell’s group (209) looked at the role of CD4\textsuperscript{+}CD25\textsuperscript{+} T regulatory cells in flu infected mice. This group injected intraperitoneally anti-CD25 monoclonal antibody (also called PC61) and PBS (Phosphate Buffered Saline, as control) 4 days before flu infection. On day 7 post-infection, spleen and peritoneal cells were examined ex vivo for IFN-\gamma accumulation following re-stimulation with various flu peptides including well known ImD flu NP366-374, PA224-233, and PB1.703-711 epitopes. Antibody treatment with PC61 led to T\textsubscript{Reg}s depletion. Absence of T\textsubscript{Reg}s led to an increase in anti-viral CTL responses to ImD NP366-374, PA224-233, and PB1.703-711 epitopes (209). Following treatment of CD4-deficient mice with PC61 antibody (as described before) it was shown that CD4\textsuperscript{+}CD25\textsuperscript{+} T\textsubscript{Reg}s target a subset of CD4\textsuperscript{+} T cells and in the absence of T\textsubscript{Reg}s more CD8\textsuperscript{+} T cells can be activated by CD4\textsuperscript{+} T cells and hence can respond to viral epitopes. This study indicates T\textsubscript{Reg}s play an integral role in regulating the overall immune response following an infection by lowering the number of CD8\textsuperscript{+} T cells being activated by CD4\textsuperscript{+} T cells. Other recent flu (226-229) and HIV studies (230-233) confirm the importance of T\textsubscript{Reg}s in immune response. Following HIV-1 infection, T\textsubscript{Reg}s help to increase the number of CD4\textsuperscript{+} T cells and viral persistence over time eventually leading to a decrease in T\textsubscript{Reg} numbers thus reducing the overall immune response over time (231, 233). These studies indicate that T\textsubscript{Reg}s contribute to the overall immune response by regulating players such as CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells involved in ImDc.
1.5.1.15 Host Age

Another factor influencing ImDc is age of the host. There is evidence for and against the notion that with age the number of T cells produced decreases, thus influencing the overall immune response following an infection. A study by Yager et al. (234) showed that older mice had holes in their T cell repertoire compared to young mice. Following flu infection, younger mice responded more vigorously than older mice when their CD8+ T cell responses to ImD NP366-374, PA224-233, and PB1.703-711 epitopes were compared. The results from older mice were comparable to those of thymectomized mice indicating that decreased T cell production as a result of age influences the overall immune response. The authors were able to show that a lower frequency of T cell precursors to different flu epitopes in older mice accounted for the lower flu responses. These results are consistent with human studies of flu infection. Aged individuals (i.e., older than 50 years) and young children (i.e., up to 6 years old) are more susceptible to flu infection than adults (i.e., 18-50 years old) (235-239). At the same time, younger adults clear the virus faster compared to older individuals (237, 240). The same holds true for individuals infected with HIV. HIV-infected adults mount a stronger immune response and respond better to HIV treatment than young children and the elderly (241-245). These results indicate that the subject’s age may influence ImDc following an immune challenge.

1.5.1.16 Other Factors

Downstream steps following T cell activation may contribute to ImDc as well. Following a viral infection, activated CTL remove infected cells by several mechanisms including IFN-γ and perforin production. Using IFN-γ- and perforin-deficient mice, Remakus et al. (246) showed that the absence of IFN-γ and perforin did not affect the overall CTL response to ImD epitopes
following a viral infection. In this system the viral load seemed to be more important than the presence of IFN-γ or perforin. A study with HIV-1 infected individuals (247) showed that not all ImD CTL responses are mounted in the acute phase of disease progression (i.e., within four weeks) despite the presence of the virus throughout this period. Most of the specific CTL responses are mounted 18 weeks post infection. Another study by Berzofsky group (170) showed that peptide avidity to MHC class I molecules also influences ImDc. These investigators showed that peptide avidity is more important in CTL responses to ImD H-2D\textsuperscript{d} P18-I10 in HIV-1 infected individuals than peptide binding affinity and number of pMHC complexes available for CTL recognition.

Competition at the level of antigen processing and peptide presentation also affects ImDc. Certain peptides, based on their sequence, are generated faster than others. Both flu-infected individuals and flu-infected transgenic mice expressing the HLA-A2 allele respond predominantly to M1.58-66 peptide (202) although subdominant CTL responses to other epitopes, such as NS1.122-130 and PA46-55, have been observed (213). Using flu infection and epitope-based lipopeptide vaccination, Tan et al. (213) showed that DC were capable of expressing and stimulating M1.58-66-restricted CTL more (i.e., on average up to ~25-fold higher) than the SbD-restricted CTL. These kinds of anti-viral CTL responses were induced even when DCs were simultaneously exposed to A2-restricted SbD and ImD peptides. Thus, presentation of epitopes by APCs also contributes to the overall ImDc see following flu infection.

Immunodominance can also be influenced by temporal aspects of viral protein synthesis. Studies with individuals infected with EBV have shown that during the lytic cycle of EBV in infected individuals, three different types of EBV proteins are produced: ‘immediate early’-, ‘early’, and ‘late’ proteins. Most of the anti-viral CTL responses in these infected-individuals are directed against the ‘immediate early’ protein-derived epitopes (248). It is thought that epitopes
generated from the ‘immediate early’ proteins may have an advantage over epitopes generated from the ‘early’- or ‘late’ proteins. The mechanism of this is unknown although studies suggested that the immediate synthesis of these ‘immediate early’ proteins may give the antigen processing and presentation machinery more time to degrade and hence generate epitopes from the viral proteins. Subsequently, some of these newly generated epitopes could act as ImD epitopes.

Immunodominance can also exist at the level of antibody production. Andrabi et al. (249) showed that most of the antibodies produced following HIV-1 infection are directed against the third variable loop (i.e., V3) of gp120 compared to the membrane-proximal external region (MPER) of gp41. Lack of gp41 antibody persistence compared to gp120/V3 over time was shown to be the main reason for reduced gp41/MPER antibody number in HIV-1 infected individuals over time. Thus, overall these reports show that there are additional factors contributing to ImDc. The significance of these factors in affecting the overall immune response, immunodominance and viral control is still to be determined.

1.5.2 Immunodominance: Summary

Immunodominance plays a critical role in the ability to mount a specific immune response. Studies discussed above suggest that there are a number of different factors contributing to ImDc seen with flu and HIV-1 infected individuals. Some of these factors contribute to ImDc more (e.g., prevalence of peptide-specific T cell precursors) than others following a viral infection. An analogy to immunodominant responses following viral infection is a soccer team consisting of different players. By repeatedly targeting the most vulnerable players (e.g., defensive players) the offensive team can weaken the opposing team, and hence lead to more opportunities to score. Even after replacing that player with another one at the same position (i.e., a vulnerable position), it may
still lead to the same end result. By concentrating on a few vulnerable players, the whole team can be weakened more efficiently. Our host response to viral infection is similar to this team concept. The host’s ability to adapt to changing viral strains allows the immune system to mount specific adaptive responses to ever changing viral strains. Further studies need to be done to decipher the details of each one of the factors in relation to ImDc. New findings will enable the development of better viral vaccines using knowledge gained from detailed analysis based on immunodominant factors.

1.6. MHC Class I Genes and HLA-B Locus

Of the three major human MHC class I genes (i.e., HLA-A, -B, -C) located on chromosome 6, HLA-A is the most abundant followed by HLA-B and HLA-C (250, 251). So far about 2500 alleles have been identified for HLA-B locus followed by the identification of ~1900 HLA-A alleles and ~1400 HLA-C alleles. The three minor MHC class I genes (i.e., HLA-E, -F, -G), not discussed in this thesis, have significantly fewer alleles (i.e., about 11 alleles for HLA-E, 22 for HLA-F, and 49 for HLA-C) than the major class I genes (250, 251).

Expression of certain HLA-B subtypes have been linked to a number of different diseases including ankylosing spondylitis (AS), psoriasis, inflammatory bowel disease (IBD) and HIV-1 (252-255). There is some evidence suggesting that HLA-B57 expression is protective in HIV-1 infection while HLA-B35 and HLA-B53 expression renders an individual more susceptible to HIV-1 infection (256). A stronger linkage exists between AS and HLA-B27 expression. More than 95% of patients who have AS are HLA-B27 positive (257). Of the different HLA-B27 subtypes, only HLA-B2702, -B2704, -B2705, and HLA-B2707 are strongly linked to AS while expression of HLA-B2706 and HLA-B2709 subtypes are not associated with AS predisposition (258).
Antigen presentation or the lack of antigen presentation of certain epitopes in association with specific HLA-B genes have been suggested to play important roles in disease susceptibility and immunodominance. Due to the focus of this thesis, the discussion below will concentrate on HLA-B27 and HLA-B7. Here, I will describe the structure, the function, and the polymorphism of HLA-B27 and HLA-B7 in detail.

1.6.1. HLA-B27

HLA-B27 is a MHC class I molecule involved in peptide presentation. Here I used transgenic mice expressing HLA-B27 either alone or in combination with another MHC-I molecule (i.e., HLA-B7) to investigate the anti-viral CTL responses following flu infection in the presence or absence of ERAP expression (Chapter 3 and 4).

HLA-B27, as are all MHC class I molecules, is expressed on all nucleated cells. The first X-ray crystallographic structure of HLA-B27 was solved in 1991 by Madden et al., about two decades after it was first linked to AS. As shown in Figure 1.1 (259, 260) the HLA-B27 protein consists of a highly polymorphic HC and a monomorphic β2m LC. The HC is made up of three extra-cellular domains (i.e., α1, α2 and α3), a TM and a cytoplasmic domain. The α1 and α2 domains make up the peptide binding groove allowing for peptides of only 8-10 aa in length to bind. Physical constraints of the binding groove prevents binding of longer epitopes. Unlike other MHC I molecules, the B27 HC has a tendency to misfold following its synthesis and form homodimers within the ER. This is a unique feature of B27 molecules. These homodimers are subsequently transported to the cell surface where they are recognized by natural killer (NK) cells, DCs and macrophages. Overall, the structure of HLA-B27 is not any different than any other typical MHC I molecules.
As for all MHC class I molecules, the main function of HLA-B27 is to present peptides. Peptide presentation plays a critical role in both thymocyte development and antigen immunity. During T cell development thymocytes undergo a complex process involving both positive and negative selection. Presentation of self-peptides in association with MHC-I molecules plays a crucial role in thymocyte development. Mature naive T cells respond to foreign (i.e., viral or bacterial) antigens only if they are presented with peptides in the context of self-MHC molecules on the surface of APCs (1, 130). Both the MHC molecule and its bound peptide are recognized by TCRs in order to initiate CD8+ T cell activation (Fig. 1.5)(1). CD8+ T cell activation is initiated by the interaction of a TCR-CD3 complex with a pMHC class I complex (133). TCR makes contact with both α1 and α2 domains of the MHC-I molecule as well as its bound peptide, whereas CD3 helps in signal transduction initiated by this interaction (133). Interactions of co-stimulatory molecules with their ligands also contribute to the overall T cell activation following initial pMHC-TCR interaction. Overall, HLA-B27 functions to present peptides to CD8+ T cells to induce an immune response following an infection.

Like many class I molecules, different subtypes of HLA-B27 exist. So far 105 subtypes of HLA-B27 are known and these are encoded by 132 known B27 alleles (258). These subtypes are numbered as HLA-B2701 through to HLA-B27105. There are two alleles for HLA-B2702 (i.e., HLA-B270201 and HLA-B270202), 21 alleles for HLA-B2705, three alleles for HLA-B2704 and HLA-B2707 each, and two alleles for HLA-B2790. The most commonly encountered subtypes associated with AS and present in Caucasians, Chinese, and Mediterranean population are of HLA-B2705, -B2702, and –B2704 subtypes respectively. Of these the most widely encountered allele is HLA-B2705. It is interesting to note that AS predisposition increases when HLA-B27 is co-expressed with HLA-B60 (261). HLA-B2706 and HLA-B2709 are two subtypes that have no
known association with AS (258). Most of HLA-B27 polymorphism arises due to changes in the nucleotide sequence in exons 2 and 3, which encode the $\alpha_1$ and $\alpha_2$ domains of HLA-B27 molecules. Silent mutations may also occur within the intronic regions of some B27 alleles, which do not lead to any changes in the overall amino acid sequence of the protein. Due to the significance of HLA-B27 linkage to AS, a separate section will deal directly with the details of B27 association with AS (section 1.7).

HLA-B27 co-expression with other MHC-I alleles influences immune responses following antigen exposure (Chapter 3 and 4). Factors contributing to ImDc following viral infection in relation to MHC class I have been described in detail in Section 1.5.

1.6.2. HLA-B7

HLA-B7 is another MHC class I molecule that plays an important role in peptide presentation. HLA-B7 is expressed in about 10% of the general population (250). The overall structure of HLA-B7 is similar to other classical MHC class I molecules consisting of three alpha domains (i.e., $\alpha_1$-$\alpha_3$), $\beta_2$m, a TM, and a cytoplasmic tail. There are no major differences in the overall structure of HLA-B7 compared to other MHC-I molecules. The peptide binding groove and the anchor residue requirements of B7 is different from those of B27 molecules. Consequently different sets of antigenic epitopes bind to B7 molecules when compared to B27 molecules. For instance, as discussed in Chapter 3 below, the B7-specific ImD flu epitope is NP418-426 whereas that of B27 is NP383-391. The anchor residue differences determine which epitopes can bind to the B7 binding groove.

HLA-B7 plays an important role in antigen presentation to CD8$^+$ T cells. Like other class I molecules it binds to peptides of 8-10 aa in length and presents them to T cells. Unlike HLA-
B27, the B7 HC does not have a tendency to misfold within the ER. Hence, to date no B7 HC homodimers have been found on the cell surface.

Polymorphisms of HLA-B7 have not been investigated to the same extent as HLA-B27. Like B27 molecules, mutations within exonic and intronic regions of the B7 gene have been shown to contribute to the overall polymorphisms encountered in HLA-B7 molecules. The most common B7 subtypes are HLA-B702, -B704, and -B705. In United States, the proportion of individuals who are B7-positive varies in different ethnic groups with 11% for Caucasians and 8% for African-Americans (250).

Like HLA-B27, expression of HLA-B7 is also associated with predisposition to certain diseases. There are some reports indicating that expression of HLA-B7 increases the rate of cervical cancer in Chinese population (262). HLA-B7 seems to also contribute to faster progression to AIDS (Acquired Immunodeficiency Syndrome) in HIV-1 positive patients in the United States (256).

HLA-B7 expression contributes to the overall adaptive immune response following antigen exposure. The contextual co-expression of HLA-B7 with another class I allele, such as HLA-B27 or HLA-A2 (Chapter 3), likely dictates immunodominant anti-viral CTL responses. Details outlining factors contributing to ImDc in relation to HLA-B7 expression have been described above (Section 1.5).

1.7. MHC Class I genes and Spondyloarthritis

Spondyloarthritis (SpA) refers to a group of diseases that share several clinical features including association with HLA-B27, asymmetric oligoarthritis, axial involvement particularly of the sacroiliac joints, and characteristic extra-articular features including acute anterior uveitis
Certain MHC class I genes, such as HLA-B27, are strongly associated with SpA but it is unresolved how physiological expression or co-expression of multiple MHC-I alleles may influence susceptibility (255). Amongst clinical subsets of SpA, the role of infection as a triggering factor is best established in two of its subsets: reactive arthritis (264, 265) and AS (252, 253). Since the canonical role for class I MHC is peptide presentation to T cells, the B27 relationship to SpA implies a T cell response restricted by B27 following an environmental trigger (254). The means whereby HLA-B27 contributes to the immunopathogenesis of AS has proved difficult to resolve in the clinical setting due to expression of multiple MHC-I alleles by humans. Studies outlined in this thesis (Chapters 3 and 4) overcome this limiting factor by using HLA transgenic (Tg) mice, thus controlling the number of allele(s) each Tg mouse can express. Due to the overwhelming association of HLA-B27 to AS, the following section will briefly deal with AS in general.

1.7.1 Ankylosing Spondylitis (AS)

Ankylosing spondylitis (AS) is defined by inflammation of the sacroiliac joints, peripheral inflammatory arthropathy and the absence of rheumatoid factor. AS has a prevalence rate of 0.1 – 1.4% (266) and is an under-recognized form of chronic arthritis. It mainly affects men between the age of 20 and 40 years. AS was first described in the mid 1860s by an Irish Physician named Bernard Connor. This was followed by a detailed description of AS by Marie-Strumpell in the early 1870s (267, 268). AS is characterized by the fusion of spines and peripheral arthritis causing chronic back pain and a progressive spinal ankylosis (269, 270). AS can be detected early on during the onset of the disease by magnetic resonance imaging (MRI) technology. Changes in the spine structure in subsequent years can be visualized by radiographs. AS is accompanied by release of inflammatory cytokines (e.g., IL-17, TNF-α) by different cell types including monocytes and
macrophages and it can be treated by anti-inflammatory drugs and biologics targeting inflammatory cytokines, examples of which include inhibitors of tumor necrosis factor (271-273).

Although many of the main causal factors leading to AS are unknown, a number of different factors have been linked to AS. Based on family and twin studies, it has been well established that AS has a strong genetic component. The sibling recurrence risk of AS is 9.2% compared to 0.1% in the general population (266). The heritability of AS is estimated to be more than 95% (269, 274). Studies have shown only ~2% of B27+ individuals to develop AS suggesting that other genetic factors (e.g., ERAP), environmental factors (i.e., bacterial infection), and/or stochastic factors (e.g., development of specific immune cells) contribute to AS pathogenesis (269). All of these factors, either in mutually exclusive or non-exclusive fashion, contribute to AS. *Klebsiella pneumonia* for example, has been strongly linked to the pathogenesis of AS due to the sequence similarity of its nitrogenase and pullulanase D proteins with the HLA-B27 proteins (275). Molecular mimicry of cross-reactive antibodies may be the contributor to the role of *Klebsiella* infection in AS (275, 276). Cross-reactivity with HLA-B27 can lead to abnormal immune responses as evidenced by B27+ individuals possibly having more humoral response to the *Klebsiella* 60 kD heat shock protein (HSP60) and nitrogenase compared to B27- individuals (276, 277).

As mentioned above, other genetic factors including ERAP contribute to the overall pathogenesis of AS. In subsequent sections below I will first describe ERAP in detail in terms of structure and function, and this will be followed by a general discussion of evidence linking ERAP to AS.
1.8 Endoplasmic Reticulum Aminopeptidase (ERAP1)

Like MHC-I genes, ERAP is expressed throughout the body in all nucleated cell types. Recent evidence suggests it is expressed at a higher level in the gut than in any other site in the body (278). As the name suggests, ER aminopeptidase associated with antigen processing [ERAAP (mouse) or ERAP1 and ERAP2 (human)] is involved in peptide processing. In humans there are two ERAP genes (ERAP1 and ERAP2). ERAP2 has about 50% identity to ERAP1 (279). ERAP2 was discovered when loss of ERAP1 led to reduced trimming of the aminopeptidase substrate L-Amc (leucine-7-amino-4-methylcoumarin), but not Arginine-Amc (R-Amc)(73) indicating the presence of another ER-based aminopeptidase in humans. Human ERAP1 and 2 can co-localize into the ER and form a heterodimer (280, 281). These ERAPs have different peptide specificities resulting in unique peptides. Although the Human Genome Organization (HUGO) Nomenclature Committee has recognized and approved the name ERAP1 (and ERAP2) and ERAAP for humans and mice respectively, I will use ERAP1 for the remainder of this thesis to refer to ERAP1 and ERAAP unless specified otherwise. Furthermore, ERAP2 is not found in rodents and will not be discussed here further.

ERAP1 preferentially processes peptides that have a specific sequence and length (see below). ERAP1 processing occurs when peptides have a leucine located at the N-terminal end of the polypeptide. Based on this, ERAP1 was initially described as a Leucine Aminopeptidase (282). ERAP1 was initially known under different names including adipocyte 36 derived leucine aminopeptidase (A-LAP), aminopeptidase regulating TNF receptor I shedding (ARTS-1), and puromycin-insensitive leucyl aminopeptidase (PILS-AP). Recent data show that preference of ERAP1’s enzymatic activity to peptides with specific amino acids at specific locations has increased (see below)
ERAP1 is strongly associated with AS and to a lesser extent to cervical cancer (283), diabetes (284), and hypertension (285). Recent Genome Wide Association Studies (GWAS) have shown ERAP1 to have the second most influential effect after HLA-B27 (i.e., ~25% of AS patients have variations in ERAP1 expression)(269, 286) on AS predisposition. Gain or loss of ERAP1 function could be used in experimental settings to further decipher the mechanisms by which ERAP1 contributes to the overall pathogenesis of AS. The mechanisms by which ERAP1 and HLA-B27 contribute to AS are still under debate. In the sections below I will briefly describe the structure and the function of ERAP1 followed by a discussion of its role in relation to AS.

1.8.1. ERAP1: Structure

ERAP1 belongs to the oxytocinase subfamily of M1 zinc-metallopeptidases (287). ERAP1 is encoded on chromosome 5 (i.e., 5q21) and spans 54.61kb from 96149849 to 96095244 on the reverse strand. ERAP1 is composed of 20 exons and 19 introns (287). Human ERAP1 was originally isolated from HeLa cells and has been since shown to have about 85% sequence homology with mouse ERAP. The human ERAP1 mRNA consists of 2826 nucleotides and alternative splicing gives rise to two N-glycosylated isoforms of 941 and 948 aa residues (288). The mouse ERAP was initially isolated from liver and spleen and consists of 930 aa (72, 73). Unlike human ERAP1, rodent ERAP mRNA transcript is not spliced.

Two different groups have independently solved the crystal structure of human ERAP1 (288, 289)(Fig. 1.7 A) ERAP1 exists in two different conformations: “open” and “closed” (Fig. 1.7 B). ERAP1’s ‘open’ conformation assumes the ‘closed’ conformation upon peptide binding. Four different domains, labeled as domain I, II, III, and IV make up the final structure of ERAP1 (Fig. 1.7). Two of these domains entirely consist of α helices (i.e., domains II and IV) while the
Figure 1.7 The structure of ERAP1

A) Domain I (Residues 1-254)
   Domain II (Residues 255-527)
   Domain III (Residues 528-613)
   Domain IV (Residues 614-941)

B) Open Conformation
   Closed Conformation
The legend to Figure 1.7

The structure of ERAP1

(A) ERAP1 consists of four different domains made up of alpha helices and beta sheets as indicated and color coded. Dotted lines represent disordered loops. The residue numbers corresponding to different domains are indicated in brackets. (B) ERAP1 consists of two different conformations. Upon peptide binding ERAP1 domains reorient to assume a ‘closed’ conformation. Modified from Nature Structural & Molecular Biology 18, 604–613 (2011).
other two are made up of β sheets (i.e., domains I and III) only. The domain I of ERAP1 consists of residues 1-254. Domain II consists of residues 255-527 and contains the catalytic domain, the GAMEN and the HEXXHX18E motifs (288, 289). The N-terminal end of substrate peptides bind to this active site of domain II and are processed. Domain II is connected to domain IV by an interconnecting domain III. Domain III consists of residues 528-613 and acts to provide peptides with an increased cavity size for binding. Domain IV consisting of residues 614–941 is made of 16 alpha-helices arranged like a cup. In between domain II and IV lays a large cavity making up the ERAP1’s catalytic domain. The catalytic site of ERAP1 is the largest among known aminopeptidases (i.e., 36Å) to date and perhaps this is the basis for ERAP1 binding and processing even longer enzyme substrates.

1.8.2. ERAP1: Function

ERAP1 has two known functions. It is involved in shedding of membrane bound receptors, such as IL-1 receptor II and IL-16 receptor ligand, and processing of peptides. The former role allowed researchers to first discover ERAP1 as a membrane-bound cytokine receptor (278). This function has important implications for AS (see section 1.8.3). Its second function is to process peptides entering the ER. Proteasomes initially generate peptides of 2-25 residues with the correct C-terminus. Some of the N-terminally extended epitopes are degraded or trimmed further into shorter length peptides by cytoplasmic aminopeptidases while others that escape this cytoplasmic processing enter the ER through TAP and are processed further by ERAP1. TAP does not transport peptides when proline is located at the first three amino acid positions. Presence of proline and other hydrophilic amino acids at these positions in pMHC-I complexes led to the discovery of ERAP1 (90, 192).
ERAP1 has both sequence and length preference. In addition to its leucine preference at the N-terminal end, ERAP1 also prefers trimming peptides when these express hydrophobic residues (e.g., methionine, phenylalanine & alanine) at their N-termini. The presence of hydrophilic amino acids like lysine, threonine and arginine at this end reduces its enzymatic activity (290, 291). Other reports indicate a change in enzymatic activity when hydrophobic residues are present at the C-terminus of peptides (291, 292). These results indicate amino acids located in different peptide regions significantly alter the enzymatic activity of ERAP1.

ERAP1 acts as a molecular ruler and trims peptides of up to 16 residues entering the ER through TAP to peptides of 8-10 residues in length (292). As mentioned before ERAP1’s ‘open’ conformation assumes the ‘closed’ conformation once a peptide is bound to it. This process is facilitated by bound peptides with both N- and C-terminal ends containing the appropriate amino acid sequence and binding to the catalytic site and hydrophobic pockets respectively. Shorter peptides of less than 8 amino acid (293) in length with improper amino acid sequence do not extend from the catalytic site to the hydrophobic pocket. This may be the reason why ERAP1 remains in an ‘open’ conformation even after being exposed to such epitopes. Proper fitting and ideal epitope binding promotes the ‘closed’ conformation of ERAP1, and ERAP1 has its maximal activity in this conformation.

Due to ERAP1’s important function in peptide presentation, the following section will specifically deal with studies signifying the impact of ERAP1 in peptide processing and presentation.
1.8.2.1 ERAP1: Peptide Presentation

Abnormal function of ERAP1 or its deficiency can lead to generation and degeneration of peptides of incorrect length and sequence. Subsequent binding of these epitopes to MHC-I can lead to pMHC-I misfolding. Misfolded proteins can either be transported to the cell surface as Free Heavy Chains (FHC) (294) or accumulate in the ER causing ER stress (295-297). Both of these processes have been suggested to contribute to AS pathogenesis (see below).

Human cells deficient in ERAP2 expression are currently available, whereas ERAP1 deficient cells are not. Studies in these cells and ERAP-deficient mice have revealed the total peptide repertoire available for presentation to be different than those peptides encountered in ERAP-intact mice and cell lines. Studies with ERAP-deficient mice have shown reduced cell surface expression of MHC-I molecules, but not MHC class II (78, 298). These mice show no differences in the proportion of CD4 and CD8 compared to mice with intact ERAP. The peptide repertoire generated in ERAP-deficient mice following a viral infection differed from ERAP\(^{+/+}\) mice. Following infection, the CTL response to ovalbumin OVA257-264, LCMV NP396-404, mCMV YL9, and flu NP366-374, PA224-233, NS2.114-121 and PB2.198-206 epitopes was reduced, whereas that of LCMV GP33-41 and histocompatibility gene SVL9 epitopes increased (74, 193, 298, 299). In addition to viral infection, one study demonstrated that there was no CTL response to the immunodominant HF10 epitope of *Toxoplasma gondii* in ERAP\(^{+/+}\) mice, indicating the involvement of ERAP in the generation of this epitope (300). Expansion of HF10-specific CD8\(^{+}\) T cells was shown to be impaired in ERAP\(^{+/+}\) mice rendering these mice more susceptible to toxoplasmosis. A change in the level of ERAP expression also influences the peptide-MHC repertoire (192). Peptides not avidly bound to MHC-I molecules in the ER can be removed from these MHC-I complexes by ERAP (301). Overall, ERAP seems to be involved in both generation
and destruction of peptides. Peptides that are normally absent in the WT animals are present in ERAP-deficient animals and vice versa.

1.8.2.2. ERAP1: Unstable pMHC-I Complexes

IFN-γ induces ERAP1 expression (287). In addition, increased function of ERAP1 leads to enhanced peptide generation and/or increased peptide destruction depending on the polypeptide sequence. Abnormal peptide processing by ERAP1 can lead to unstable pMHC-I complexes that are prone to misfold. Misfolded proteins accumulate in the ER and can affect the overall process of a number of downstream processes including ER stress. A prime example of an MHC molecule that undergoes this sort of misfolding is HLA-B27. Improperly-folded HLA-B27 molecules accumulate in the ER and presentation of abnormally-processed epitopes can lead to an unfolded protein response (UPR), ER stress, and a pro-inflammatory cellular response (78, 295-297). Generation of abnormal peptides also contributes to the formation of unstable pMHC-I complexes. Unstable complexes do not last long within the ER and tend to form FHC, which bind to non-traditional immunoreceptors such as killer cell immunoglobulin-like receptors (KIR) and leukocyte immunoglobulin-like receptors (LIR) on NK cells resulting in abnormal immune responses (302-305).
1.8.3. ERAP1 and AS

The attributable risk factor for ERAP1 in AS is 26% (269, 306). This is the second highest attributable risk factor following HLA-B27 association (~50%) with AS. As mentioned earlier, abnormal function of ERAP1 can lead to peptide generation of incorrect length and sequence. Subsequent binding of these epitopes to MHC-I can lead to pMHC-I misfolding causing two downstream processes: accumulation of misfolded complexes within the ER leading to ER stress, and the transport of FHCs to cell surface. All three (i.e., pMHC I misfolding, ER stress, and FHC transport) events have been suggested as possible mechanism for AS, but the degree of their contribution to AS is speculative. Studies with ERAP-deficient mice (see below) have shown reduced MHC-I expression while MHC class II expression was not altered. Loss of function ERAP1 variants are protective for AS. There is a wide consensus among AS experts of the existence of “arthritogenic epitopes”. Although there are suggestions that an arthritogenic peptide or peptides plays a central role in AS (307-309), so far no such peptides have been discovered. The identity of this epitope is unknown and there are suggestions that ERAP may be involved in destruction/generation of this arthritogenic epitope(s)(281).

1.8.4. ERAP1: Other Diseases

ERAP1 is linked to a number of other diseases including psoriasis (293), Crohn’s disease (310), hypertension (285, 311), pre-eclampsia (312), hemolytic uremic syndrome (313), and osteoporosis (314). The uremic syndrome is caused by Shiga toxin and abnormal cleavage of this toxin by ERAP1 seems to contribute to the overall disease pathogenesis (313). Due to the focus of this thesis the details of ERAP1’s involvement in other disease will not be discussed further.
1.8.5. Development of ERAP Deficient Mice

Three different groups have used different techniques to knock-out ERAP to create ERAP-deficient (ERAP\(^{-/-}\)) mice (77, 299, 315). The ERAP\(^{-/-}\) mice described in this thesis were created by Shastri’s group in 2006 (77). As referred to previously, the ERAP gene consists of 20 exons distributed over 54 kilobases. The highly conserved ERAP active site is encoded by exons 4-8 and Shastri’s group deleted these exons by homologous recombination. In separate experiments, Niedermann’s group (315) and Luc Van Kaer’s group (299) deleted exon 4 and exon 4-8 respectively to create ERAP\(^{-/-}\) mice as well. The common features among these ERAP-deficient mice in comparison to WT mice are: an increase in peptide quantity in their original form (e.g., SVL9), an increase in N-terminally extended peptides (e.g., NOVAK), low MHC-I (i.e., H2-K\(^b\), -D\(^b\), -K\(^d\), -D\(^d\), -L\(^d\)) surface expression, and no alteration in MHC class II surface expression in the thymus, LN and the spleen of ERAP\(^{-/-}\) mice. ERAP\(^{-/-}\) mice do not exhibit any phenotypic abnormalities nor do they have any differences in the B and T cell percentages compared to WT mice. Peptide trafficking by ERAP\(^{-/-}\) mice does not differ from those seen with WT mice. Following flu (A/PR8/34) or LCMV infection, ERAP\(^{-/-}\) mice mounted strong immune responses similar to those seen with WT mice. These published studies suggest ERAP-deficiency may not modify the overall immune response to viral infection. However, studies described in this thesis (Chapter 4) and recent findings by Shastri’s group (316) following Toxoplasma gondii infection challenge this view. In our studies (Chapter 4) to investigate the effects of ERAP on immune response in HLA Tg mice following flu infection, ERAP\(^{-/-}\) mice obtained from N. Shastri were first crossed with H2-K/D double knock-out (DKO) mice to create ERAP\(^{-/-}\)/DKO mice. These mice were subsequently crossed with single Tg B7 and B27 (see below) to create HLA Tg
B27/ERAP$^{-/}$ and B7/ERAP$^{-/}$ still on a DKO background. Refer to Materials and Methods section of Chapter 4 for the full description of HLA Tg ERAP$^{-/}$ mouse creation.

1.9. Development of HLA Tg Mice

A major obstacle in carrying out research of human infectious diseases has been the lack of suitable animal models that accurately reflect the specificity of the immune response in humans. HLA transgenic mouse models developed by Chamberlain’s group have provided a powerful new tool to study human MHC-restricted T cell responses in vivo (152, 175, 202, 317-319). These models are based on expression of individual human MHC class I genes (i.e., A2, B7 and B27) in Tg mice that are also deficient for mouse H2-K/D MHC-I expression. Using these HLA Tg mice, Chamberlain’s group showed that i) introduction of different human class I alleles can restore development of CD8$^{+}$ T cells to a level comparable to that seen for a single expressed mouse class I molecule (152), ii) peripheral CD8$^{+}$ T cells of these Tg mice display a broad usage of TCR-V$\beta$ subfamilies, similar to that for H2$^{b}$ WT mice (152, 202), and iii) that influenza M1.58-66 and NP383-391 epitopes known to be recognized by A2$^{+}$- and B27$^{+}$-restricted CTLs in humans were also recognized in Tg A2 and B27 mice, respectively (152, 202). As the flu epitope recognized by B7$^{+}$-restricted CTLs was not known from human studies at the time, Chamberlain’s group used Tg B7 mice to identify flu/NP418-426 as a strong B7-restricted epitope and subsequently confirmed this was also recognized in B7$^{+}$ humans (152). These results demonstrate that these mouse models can be reliably used for identifying and characterizing viral CTL epitopes recognized with HLA alleles in humans.
Chapter 2

2.1. Aim:

The broad aim of my project was to identify the mechanisms responsible for HLA-mediated ImDc following influenza infection and how this is influenced by (a) class I allelic coexpression and (b) the presence or absence of ERAP. The specific aims are as follows:

1. Establish and characterize novel double HLA Tg mice (i.e., HLA-B7/B27) in the presence of ERAP expression. Are there any differences in T cell percentages and TCR Vβ expression in double Tg B7/B27 compared with single Tg B7 and B27 mice?
2. Does co-expression of multiple HLA transgenes alter the pattern of anti-flu CTL responses in B7/B27 Tg mice?
3. Establish and characterize novel HLA Tg mice in the absence of ERAP expression (i.e., HLA-B7/ERAP<sup>−/−</sup> and HLA-B27/ERAP<sup>−/−</sup>). Are there any differences in T cell percentage and TCR Vβ expression in these Tg mice in the absence of ERAP expression?
4. Does absence of ERAP expression in HLA Tg B7/ERAP<sup>−/−</sup> and B27/ERAP<sup>−/−</sup> mice alter the pattern of anti-flu CTL responses?
5. What light might HLA-B27/ERAP<sup>−/−</sup> mice shed on the pathogenesis of ankylosing spondylitis? Can flu infection in the B27/ERAP<sup>−/−</sup> mice define immune pathways with relevance to a human disease like AS in which both B27 and ERAP are intimately linked?
2.2. Hypothesis:

Allelelic co-expression can influence the pattern of anti-viral CTL responses in double HLA Tg mice. Change in the overall pattern of anti-flu CTL responses can lead to ImDc of one epitope over the other. Different factors including absence of epitope-specific naive CD8 T cells and/or lack of epitope generation in the absence of ERAP expression may contribute to flu ImDc in HLA Tg mice.
Chapter 3

This chapter describing the effects of dual HLA allele co-expression influencing the flu response was published in the European Journal of Immunology. I did all the experiments and analysis. I prepared the draft for publication. The citation for this article is: Akram A and Inman RD. Co-expression of HLA-B7 and HLA-B27 alleles is associated with B7-restricted immunodominant responses following influenza infection. Eur J of Immunol 2013 Dec; 43(12): 3254-67.

3 Co-expression of HLA-B7 and HLA-B27 alleles is associated with B7-restricted immunodominant responses following influenza infection

3.1 Introduction

The cellular immune response to a viral infection depends on the ability of CTLs to recognize, via their TCR, viral antigenic peptides in the context of major histocompatibility complex class I molecules (MHC-I) (320). Following a viral infection, viral peptides are bound by MHC class I molecules and transported to the cell surface where they are surveyed for recognition by the repertoire of αβ-TCR expressed by CTLs (151). In this context, immunodominance refers to the phenomenon whereby a large fraction of the anti-viral CTL population is directed against a limited number of MHC class I/peptide complexes (i.e., pMHC-I). The viral peptide which dominates CTL recognition with a particular MHC allele is referred to as the immunodominant epitope (ImD). As we recently reviewed (145), the mechanisms of immunodominance are not completely understood, but almost every step in antigen processing and presentation may contribute to determine which specific peptide sequences will be available for recognition by CTLs (321).
Immunodominance is very common in host responses to viral pathogens. Immunodominant responses have been observed to a range of pathogens including Hepatitis B virus (162, 163), EBV (167, 322), and CMV (161, 323). As analysis of anti-viral T-cell responses for humans is complicated by co-expression of multiple class I alleles, a series of single HLA Tg (HLA^hyb^ Tg) mice were generated (152, 319). The advantage of this hybrid class I human/mouse model is that Tg animals express the human peptide-binding cleft along with murine regions necessary for interaction with endogenous murine molecules such as CD8 and β2m. The HLA^hyb^ transgenes were introduced into MHC class I-deficient mice lacking H2-K^b^ and H2-D^b^ genes [i.e., double-knockout (DKO)]. Studies demonstrated that influenza A (flu)-infected PBMCs from HLA-A2, -B7, and -B27 expressing individuals and PBMCs from allele-matched single Tg mice respond to A2-restricted M1.58-66 (202, 324), B7-restricted NP418-426 (202) and B27-restricted flu NP383-391 epitopes (152, 175). Thus, these transgenic mice can be instructive for investigating factors leading to immunodominance.

The presence or absence of immunodominance following flu infection in HLA Tg mice expressing more than one HLA transgene has not been examined. In clinical studies, an earlier report (322) indicated reduced CTL responses to the HLA B8-restricted EBNA_{325-333} epitope following EBV infection in individuals co-expressing B8 and B44. Studies with flu-infected mice co-expressing H2-K^k^ and H2-D^b^ showed that CD8^+^ T-cell responses to the ImD epitope H2-D^b^/PA224-233 diminished, whereas that of NP366-374 did not change (201). These studies showed that lower CD8^+^ T-cell response was due to a lower number of naive PA224-233 T cells.

Here we show for the first time that following flu infection only certain combinations of HLA allele co-expression lead to immunodominant responses in HLA Tg mice. We established three new strains of double HLA^hyb^ Tg mice co-expressing A2/B7, A2/B27, and B7/B27 alleles.
We show that immunodominant responses are seen only in flu-infected B7/B27 Tg mice. We demonstrate that altered selection of specific B27-restricted NP383-391 naive T cells in B7/B27 Tg mice accounts for the lack of CTL response to this epitope.
3.2 Materials and Methods

3.2.1 Double Transgenic HLA<sup>hyb</sup>/H2-K<sup>-</sup>-D<sup>-</sup>- Double Knockout Mice (DKO) Generation

The generation of single Tg B27, B7 and A2 mice has been described (152, 202). Here appropriate crosses of single Tg mice were made to generate A2/B27, A2/B7 and B7/B27 mice. Double Tg A2/B7 and A2/B27 mice were distinguished by flow cytometry and PCR (Figure 1A). For PCR the following primer sets were used: for HLA-B7 the forward primer (P1) was 5’TACTACAACCAGCGAGGCCG3’, and the reverse primer (P2) was 5’CAGCGCGCTCCAGCTTGTCC3’ (band length = 540bp); for HLA-B27 the forward primer was P1 and the reverse primer (P3) was 5’GTAGGCGTCCTGGGTA3’ (band length = 350bp). All mice were housed in the specific pathogen-free animal facility at Toronto Western Hospital in Toronto according to the guidelines of the Canadian Council of Animal Care. All animal studies have been reviewed and approved by the University Health Network Research Committee.

3.2.2 Flow Cytometry Analysis

The monoclonal antibodies (mAbs) and detection reagents used for flow cytometry and their specificities are as follows: ME1 (specificities: HLA-B7, -B27, -Bw22, and –B14), BB7.1 (HLA-B7), B27M2 (HLA-B27 and HLA-Bw47) and MA2.1 (HLA-A2 and HLA-B17) were from the American Type Culture Collection (Manassas, VA); CD3-PerCP (0.5 mg/ml, diluted 1:150), CD8a-FITC (0.5 mg/ml, diluted 1:150), and TCR Vβ8.3-FITC (0.5 mg/ml, diluted 1:200) were from BD Pharmingen (San Diego, CA). FITC-conjugated F(ab’)2 goat anti-mouse IgG (Fc-specific, 0.5 mg/ml, diluted 1:150) and FITC-conjugated F(ab’)2 goat anti-rat IgG (Fc-specific,
0.5 mg/ml, diluted 1:150) were from Accurate Chemical and Scientific (Westbury, NY). FITC-conjugated F(ab’)2 goat anti-mouse IgM (μ chain specific, 0.5 mg/ml, diluted 1:200) was from Southern Biotechnology Associates (Birmingham, AL). The anti-TCR Vβ mAbs were from BD Pharmingen (San Diego, CA) and supernatants specific for Vβ2 (B20.6), Vβ6 (44.22.1), Vβ7 (TR310), Vβ8.1/8.2 (KJ16), Vβ8.2 (F23.2), Vβ11 (KT11), Vβ12 (MR11-1), Vβ14 (14.2) were obtained from Dr. J. Penninger.

3.2.3 Tetramer Staining and Enrichment of Antigen-specific CD8⁺ T Cells

Cell suspension from PBL, spleen, and LN were enriched for T cells using Pan T-cell Isolation Kit II Mouse (Miltenyi Biotec, Cat#:130-095-130). These cells were then stained with different influenza tetramers. Spleen and LNs were mashed through a cell strainer (Fisherbrand, 70μm Nylon Mesh) using a plunger, filtered, and lysed with ACK lysis buffer (NH₄Cl 8,024 mg/l, KHCO₃ 1,001 mg/l, EDTA. Na₂•2H₂O 3.722 mg/l). Cells were separated as outlined in the Miltenyi Biotec protocol. The bound cells were eluted from the columns and stained with anti-CD8, anti-CD4, anti-CD3, and PE- and APC-labeled pMHC-I tetramers for 45 min at 4°C. Flu-specific tetramers for NP366-374 (1.2 mg/ml, diluted 1:100), NP383-391 (1.2 mg/ml, diluted 1:100), NP418-426 (1.3 mg/ml, diluted 1:100), and M1.58-66 (1.5mg/ml, diluted 1:100) were synthesized by NIH (Atlanta, GA). Cells were washed and fixed with 1% PFA before being analyzed with a LSRII cytometer (Becton Dickinson). Data analysis was performed using Cell Quest and FlowJo softwares (BD Immunocytometry Systems, CA).
3.2.4 Influenza A/X31 (H3N2) Infection and IFN-γ ELISpot Assays

Mice between 7-10 weeks of age were used for infection with flu A/X31 virus (H3N2) (SPAFAS, North Franklin, CT) by either the i.n. or i.p. route as described (152). IFN-γ ELISpot assays were used to determine the frequency of peptide-specific IFN-γ-producing cells in spleens of flu-infected mice as described (152, 202). Influenza peptides at pre-specified concentrations were used to assess the anti-flu CTL response by ELISpot assay. All peptides were synthesized and purchased from Bio Basic Inc (Markham, ON, Canada). An experimental response was considered positive when the number of spot forming units (SFU) was at least 2-times greater than the number detected with the same spleen cell population incubated with either an irrelevant control peptide or no peptide.

3.2.5 In vitro Peptide Stimulation, Peptide and NP DNA Immunization

Spleen cells from mice infected i.p. with flu 3 weeks earlier were re-stimulated in vitro for two 7-day incubation periods with the indicated peptide. Spleen cells from flu infected mice were re-stimulated in vitro for two 7-day incubation periods with the indicated peptide in α-MEM (Life Technologies, Grand Island, NY) containing 10% FCS (Sigma-Aldrich, St. Louis, MO), 10mM HEPES, 5 x 10⁻⁵ M 2-ME, penicillin/streptomycin (Life Technologies), and 0.5 U/ml of mouse IL-2 (324, 325). The source of APC and T cells for the first period (i.e., Day 0) was autologous peptide-pulsed spleen cells. For the second period (i.e., Day 7), viable cells were harvested and stimulated with peptide-pulsed, irradiated (2600 rad) strain-matched spleen cells that served as APC. On day 14, the viable cells were harvested and stained with anti-CD8 mAb and with various anti-TCR Vβ mAbs.
Naive HLA Tg and non-Tg mice were immunized subcutaneously with CpG (10μg/ml, 50μg per mouse in 1 x PBS) two days prior to peptide immunization. Two days later the same mice were co-immunized subcutaneously with synthetic NP418-426 (10μg/ml, 75 μg/mouse) and NP383-391 (10μg/ml, 75 μg/mouse) peptides in incomplete Freund’s Adjuvant (IFA), or IFA alone as control, and 12 days post-immunization spleen cells were removed and tested by ELISpot. For the DNA vaccination, transgenic and non-Tg mice were immunized via the gastrocnemius muscle with 100μl of PBS containing 20μg of DNA vector alone and/or flu NP DNA vector. This was repeated three times at two-week intervals as described (326).

3.2.6 Generation and Identification of HLA Tg Chimeras

Different chimeric mice were generated using established protocols (327). Briefly, female mice were irradiated for 9 min (~900 cGy) and returned to the SPF housing unit at Toronto Western Hospital. Male BMs were harvested as described (327). These cells were washed and re-suspended in 1x PBS at a concentration of 3.0 x 10⁶ cells/ml. Twenty four hr post-irradiation 6 x 10⁶ male viable cells in 200μl 1x PBS were transfused intravenously into female recipient mice. Mice were left to recover for 60 days. PCR for the male SRY gene was performed on DNA extracted from PBLs of different chimeras. The following primer sets were used for SRY gene identification: Forwards primer (P1) CGCCCATGAATGCATTTAT and reverse primer (P2) CCTGTCCCACTGCAGAAGGT (expected band ~300bp).

3.2.7 Statistical Analysis

Data were analyzed by two way analysis of variance (two variables; naive vs. flu infected in GraphPad Prism 5.0, GraphPad Software Inc., La Jolla, CA) with a Bonferroni post test. All
values are expressed as mean (± SEM), P < 0.05 (adjusted P) was considered significant. Student t-test was used only in one instance as indicated.
3.3 Results

3.3.1 Characterization of a novel Tg HLA-B7/B27/H2 DKO mouse

A series of HLA class I Tg mice expressing HLA-A2 (i.e., Tg A2), -B7 (i.e., Tg B7) or –B27 (i.e., Tg B27) on a DKO background have been described (152, 319). Here we have generated three double HLA Tg strains, referred hereafter as A2/B7, A2/B27 and B7/B27 respectively. The Tg A2/B7 and -A2/B27 strains have been briefly described previously (152, 175, 319). This is the first description of the B7/B27 Tg strain. Genotyping of the B7/B27 was confirmed by PCR of tail DNA (Fig. 3.1A).

Characterization of the CD3+ T cells isolated from splenic tissues was performed by flow cytometry (Fig. 3.1B). The percentage of B7/B27 CD8+ T cells compared with single Tg B7 and B27 mice is not significantly different. Non-Tg DKO mice have low CD8 expression levels as expected whereas both single Tg B7 and B27 mice have comparable CD8 expression levels (Fig. 3.1B).

3.3.2 CD8+ response to influenza infection: B7/NP418-426 dominates the CD8+ response in B7/B27 Tg mice

To examine whether co-expression of A2 and B7 influences the specificity of the CD8+ T-cell responses to the respective immunodominant A2- and B7 flu epitopes, spleen cells from various A2 and B7 Tg mice were examined by IFN-γ ELISpot assay 11 days (i.n.) and 3 weeks (i.p.) post flu infection (Fig. 3.2A). Single Tg A2 and B7 mice showed strong responses to their respective immunodominant epitopes, but no response to NP366-374 (Fig. 3.2A). In contrast double Tg A2/B7 mice showed strong responses to both M1.58-66 and NP418-426 (Fig. 3.2A). The A2/M1.58-66 response was slightly
Figure 3.1 Characterization of Double Tg B7/B27 Mice.

A)

B)

i) Non-Tg WT

ii) Tg B7

iii) Tg B27

iv) Tg B7B27

v) Non-Tg DKO

CD4

CD8

% CD8 LNCs

*
Legend to Figure 3.1
Characterization of Double Tg B7/B27 Mice.

(A) Double Tg B7/B27 mice were identified by tail DNA PCR. (B) LNs from different Tg mice were stained for CD3, CD4, and CD8. The bar graph shows the percentage of CD3⁺CD8⁺ T cells in different Tg mice. Data are shown as mean ±SEM from n=6 mice per group and are pooled from three independent experiments. *, p<0.05 vs. Tg mice using student t-test.
Figure 3.2. ELISPOT analysis of the CD8⁺ CTL response to flu infections in single and double A2/B7 and A2/B27 Tg mice.
Legend to Figure 3.2

ELISpot analysis of the CD8* CTL response to flu infections in single and double A2/B7 and A2/B27 Tg mice.

(A-B) Mice were infected with flu i.p. or i.n. as indicated. After eleven days (i.n.) and/or three weeks (i.p.) post-infection the CTL response was tested by IFN-γ ELISpot for various flu peptides. Data are shown as mean ±SEM of n=6 mice per group and are pooled from six independent experiments. Significance was assessed using two way analysis of variance. ***, p<0.0001 vs. non-immunodominant epitopes for the given allele.
increased in the A2/B7 mice compared with A2 mice (p>0.05). Similarly, to determine the pattern of flu epitope CTL recognition when HLA-A2 is co-expressed with HLA-B27, comparable studies were carried out as above for flu-infected A2/B27, A2 and B27 Tg mice (Fig. 3.2B). The A2/M1.58-66 response was comparable in Tg A2/B27 and A2 mice. The B27/NP383-391 response was also comparable in single Tg B27 and double Tg A2/B27 mice. As the natural physiological route of flu infection is through the respiratory (i.e., intranasal) route, we repeated the above analyses following i.n. infection. Overall, while the magnitude of CD8+ T-cell response was higher following i.n. infection, the trend in responding to different allele-restricted ImD epitope was similar (Fig. 3.2, bottom panel).

To examine the anti-flu CTL responses restricted by two co-expressed HLA B alleles that present peptides from the same viral protein (i.e., nucleoprotein), double Tg B7/B27 mice were infected i.p. and tested by ELISpot (Fig. 3.3). For both single Tg B7 and double Tg B7/B27 mice, there was a strong response detected against the B7/NP418-426 peptide (Fig. 3.3Ai and Aiii). A strong response was detected also against the B27/NP383-391 peptide for single Tg B27 mice (Fig. 3.3Aii). However, this B27/NP383-391 response was significantly reduced (p<0.0001) for double Tg B7/B27 mice and was not detectable above background levels (Fig. 3.3Aiii). The non-Tg WT mouse (Fig. 3.3Aiv) served as an internal control showing CTL responses only to WT/NP366-374 flu epitope. The magnitude of the responses detected for the relevant peptides were 2-3 fold higher following i.n. infection than for i.p. infection confirming published results (Fig. 3.3B)(152, 202). With i.n. infection, clear immunodominance was still observed in B7/B27 Tg mice.
Figure 3.3 ELISpot analysis of flu-infected single and double HLA Tg B7/B27 mice.
Legend to Figure 3.3

ELISpot analysis of flu-infected single and double HLA Tg B7/B27 mice.

Mice were infected i.p. (A) or i.n. (B) and tested for various peptides. Data are shown as mean ±SEM of n=8 mice per group and are pooled from three independent experiments. Significance was assessed using two way analysis of variance. ***, p<0.0001 vs. non-immunodominant epitopes. #, p<0.0001 as indicated.
We then undertook the following experiments to investigate factors accounting for this immunodominance.

3.3.3 Differential class I expression level and antigen processing do not contribute to immunodominance

To investigate the role of HLA class I gene expression levels in immunodominance observed in flu-infected Tg B7/B27 mice, lymph node (LN) cells from various HLA Tg mice were analyzed by flow cytometry with HLA allele-specific mAbs (Fig. 3.4). Compared with the expression level of HLA-A2 detected with mAb MA2.1 on LN cells of A2/B7 Tg mice, expression for single Tg A2 cells was significantly (p=0.0449) different (Fig. 3.4Ai). Similarly, the level of HLA-B7 expression detected with mAb BB7.1 showed significant (p=0.0412) difference between single Tg B7 cells compared with double Tg A2/B7 cells (i.e., 1.6-fold; Fig. 3.4Aii). Similar analyses with single and double Tg A2/B27 mice showed that A2 expression levels were comparable in both strains (Fig. 3.4Bi), and there was a significant (p=0.0426) difference in B27 expression (detected with mAb ME1) in B27 and A2/B27 Tg mice (Fig. 3.4Bii). For the Tg B7/B27 mice, expression levels for both B7 (Fig. 3.4Ci) and B27 (Fig. 3.4Cii) were similar in double and single Tg LN cells.

To investigate the role of antigen processing and presentation in immunodominance observed in flu-infected Tg B7/B27 mice, we immunized Tg mice with synthetic flu peptides as indicated (Fig. 3.5A and 3.5B). Only single Tg B27, and not B7/B27, mice developed robust CTL response to NP383-391 epitope following NP383-391 immunization (Fig. 3.5A). In contrast, the NP418-426 CTL response was present in
Figure 3.4. The LN expression level of A2, B7, and B27 as detected by allele specific mAb in HLA Tg mice.
Legend to Figure 3.4

The LN expression level of A2, B7, and B27 as detected by allele specific mAb in HLA Tg mice.

(A-C) Representative plots of flow cytometric analyses for different mice are shown. To show the HLA-B27 expression with mAb B27M2 (C, ii), lymphocytes were stained for B cells (anti-B220), T cells (anti-CD3) and HLA-B27 (B27M2). The CD3+ T cells were gated and analyzed for the expression of HLA-B27. This experiment was repeated with 6 mice for each strain (n=6 per genotype).
Figure 3.5. Peptide and NP DNA immunization and *in vitro* stimulation of flu infected HLA Tg mouse splenocytes.
Legend to Figure 3.5

Peptide and NP DNA immunization and in vitro stimulation of flu infected HLA Tg mouse splenocytes.

(A-B) Different Tg mice were co-immunized subcutaneously with peptides and tested by ELISpot for various flu peptides 12 days post-immunization. Data are shown as mean +SED of n=6 mice per group and are pooled from two independent experiments. ***, p<0.0001 vs. non-immunodominant epitopes. (C) Mice were infected with flu i.p. and three weeks post-infection, splenocytes were stimulated (i.e., secondary (2°) stimulation) with strain-specific ImD peptides for two 7 day periods. Viable cells harvested following peptide stimulation on day 7 and day 14 as well as the splenocytes taken directly from mice three weeks post-infection (day 0) were stained for the expression of CD8. Data are shown as mean ±SEM of n=5 mice per group and are pooled from three independent experiments. (D-E) Mice were immunized with pCMVII vector alone (D) or with a flu NP DNA pCMVII vector (E) as described (328) and tested for various peptides as indicated in Fig. 2. n=3 per group pooled from three independent experiments. Each data point is the mean value ± SEM. Significance was assessed using two way analysis of variance. ***, p<0.0001.
both single B7 and double B7/B27 Tg mice following NP418-426 peptide immunization, mimicking the results with flu infection (Fig. 3.5B).

To examine whether the immunodominance is caused by the complete absence of NP383-391 specific CD8$^+$ T cells, flu-infected cells from Tg mice were stimulated with the known ImD peptides (Fig. 5C). The starting total splenocyte numbers was comparable for all mice and this total number decreased from Day 0 to Day 14 (data not shown). The level of CD8$^+$ T cells for infected Tg mice were similar at three weeks post-infection (Fig. 3.5C, white bars). After 7 days of peptide stimulation, the level of CD8$^+$ T cells in all mice increased (Fig. 3.5C, black bars). As the levels of B7/B27- and B27 cells, stimulated with NP383-391 increased in comparable manner (compare Fig. 3.5Cii and 3.5Cv, grey bars, p>0.05), this indicated that some B27/NP383-391 restricted CD8$^+$ T cells were detectable following flu infection in the B7/B27 Tg mice. Similarly, NP418-426 stimulated CD8$^+$ T cells from single and double Tg B7/B27 mice reached comparable levels of CD8$^+$ T cells following stimulation (compare Fig. 3.5Ci and 3.5Civ). There was no increase in the CD8$^+$ T levels of B7/B27 Tg cells in the absence of peptide stimulation (Fig. 3.5Ciii).

Further, to investigate the role of other flu proteins, such as hemagglutinin or neuraminidase, in the observed immunodominance in B7/B27 Tg mice, we immunized HLA Tg mice with a flu NP DNA vector (i.e., in the absence of all other flu proteins) (Fig. 3.5E) or DNA vector alone (Fig. 3.5D). The results revealed the same pattern of reduced B27/NP383-restricted CTL recognition previously seen following flu infection and peptide immunization.
3.3.4 Lower number of naive B27-restricted NP383-391 specific CD8$^{+}$ T cells in double Tg B7/B27 mice

A number of investigations have shown that certain T-cell populations are negatively selected when different MHC class I molecules are co-expressed (201, 322). Other investigators (178, 325, 329-332) have effectively used specific tetramer staining to determine the number of specific T cells under different circumstances. We undertook a similar approach as that outlined by Lefrancois’ group (330), and stained with flu-specific WT/NP366-374, B27/NP383-391 and B7/NP418-426 tetramers following T-cell enrichment. Cells from the splenic tissues (Fig. 3.6A-Ei), LNs (Fig. 3.6Eii), lungs, and PBLs (data not shown) were stained. There was a significant difference (p<0.001) in the number of naive B27/NP383-391 CD8$^{+}$ T cells between the single Tg B27 and the double Tg B7/B27 population (Fig. 3.6A left panel and Fig. 3.6B). Correspondingly, the number of B27/NP383-391 CD8$^{+}$ T cells in infected B27 mice increased compared with B7/B27 Tg population (Fig. 3.6A right panel and Fig. 3.6C). Similarly, the number of B7/NP418-426 CD8$^{+}$ T cells increased following flu infection in B7 and B7/B27 Tg mice (Fig. 3.6A right panel and Fig. 3.6C). Interestingly, no such significant difference was observed in the number of naive B27/NP383-391 and/or B7/NP418-426 CD8$^{+}$ T cells between the single and the double Tg A2/B27 and/or A2/B7 Tg mice, respectively (Fig. 3.7A). The number of naive Tet$^{+}$CD3$^{+}$CD8$^{+}$ T cells specific for M1.58-66 was similar in the single and the double Tg A2/B7 or A2/B27 mice (Fig. 3.7A and 3.7B).

To examine whether expansion of flu-specific Tet$^{+}$CD8$^{+}$ T-cell population varies among different Tg mice in vivo, we followed the kinetics of immune response following flu infection for 3 weeks (Fig. 3.6D). In B7/B27 Tg mice (Fig. 3.6Diii), Tet$^{+}$CD8$^{+}$ T cells specific for both NP418-426 and NP383-391 epitopes increased over time, although the latter expansion was significantly (p<0.001) lower than the Tet$^{+}$CD3$^{+}$CD8$^{+}$ T-cell expansion seen with the same population in single

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Tg B27 mice (*compare Fig. 3.6Di and 3.6Diii*). Contrary to B7/B27 Tg mice, Tet+CD3+CD8+ T cells specific for M1.58-66, NP383-391 and NP418-426 flu epitopes expanded in similar fashion following flu infection in both single and double Tg A2/B27 and A2/B7 mice (*Fig. 3.7C and 3.7D*). Thus, the reduction in B27/NP383-391 levels is present only in B7/B27 Tg mice.
Figure 3.6. Enumeration of naive and flu infected enriched T-cell populations in different Tg mice using B7/NP418-426, B27/NP383-391, and the WT/NP366-374 flu tetramers.
Figure 3.6 (Continued). Enumeration of naive and flu infected enriched T-cell populations in different Tg mice using B7/NP418-426, B27/NP383-391, and the WT/NP366-374 flu tetramers.
Legend to Figure 3.6


(A-E) T lymphocytes from naive and flu infected mice were enriched using magnetic beads (see Materials and Methods) from the spleen and LN. By first gating on the CD3⁺CD8⁺ positive cells (not shown), different T-cell populations corresponding to different flu tetramers were plotted. (A) Representative dot plots showing percentages of naive (left panel) and flu infected (right panel) enriched CD3⁺CD8⁺ T cells corresponding to different flu peptides. (B-C) Quantification of flu specific Tet⁺CD3⁺CD8⁺ T cells from the spleen of naive (B) and day 11 post i.n. flu (C) infected Tg mice. (D) Determination of Tet⁺CD3⁺CD8⁺ T-cell frequency at different times in i.n. infected HLA-B27 (i), -B7 (ii) and –B7/B27 (iii) Tg mice. (E) Mean Fluorescence Intensity (MFI) of different Tet⁺CD3⁺CD8⁺ T cells of flu-infected spleen (i) and LN (ii) on day 11 post infection. Data are shown as mean ±SEM of n=6 mice per group and are pooled from three independent experiments.
Figure 3.7. Enumeration and kinetics of naive and flu infected enriched T cell populations in single and double Tg A2/B27 and A2/B7 mice.
Figure 3.7 (Continued). Enumeration and kinetics of naive and flu infected enriched T cell populations in single and double Tg A2/B27 and A2/B7 mice.
Legend to Figure 7

Enumeration and kinetics of naive and flu infected enriched T cell populations in single and double Tg A2/B27 and A2/B7 mice.

(A) Quantification of flu specific Tet⁺CD3⁺CD8⁺ T cells from the spleen of naive Tg mice. (B) Representative naive A2/M1.58-66 tetramer staining in A2 Tg mice. (C-D) Tet⁺CD3⁺CD8⁺ T cell frequency in A2/B27 and/or A2/B7 Tg mice following flu infection. n=6 per group. The mean value ±SEM from six mice are shown.
Tet^+CD3^+CD8^+ T cells specific for NP418-426 expanded in similar manner in both single and double B7/B27 Tg mice (compare Fig. 3.6Dii and 3.6Diii). Determination of MFI of flu-specific Tet^+CD8^+ T cells of splenic and LN origin conclusively revealed that there were a reduced number of B27/NP383-391 CD8^+ T cells in B7/B27 mice compared with B27 mice (Fig. 3.6E). A similar trend was observed with lung and PBLs of these mice (data not shown).

3.3.5 Increased levels of peripheral TCR Vβ repertoires of flu-infected Tg B7/B27 mice compared to naive mice

To determine the relationship between TCR repertoire and immunodominance, we compared the TCR Vβ repertoires of naive and flu-infected Tg mice (Fig. 3.8). There was an overall similarity in the pattern observed for the various TCR Vβ chains tested for naive B7/B27 Tg mice compared with naive B7 and B27 Tg mice (Fig. 3.8A-D). The predominant TCR Vβ chain used for recognition of flu NP383-391 with Tg B27 is Vβ8.1, while that used for recognition of NP418-426 with Tg B7 is Vβ6 (152). T-cell populations expressing these Vβ6 are readily detected in both B7 and B7/B27 Tg mice (Fig. 3.8A and 3.8B). Comparison of Vβ8.1^+ CD8^+ T cells indicated a significant (p<0.001) reduction in the naive T-cell population expressing Vβ8.1 in the double Tg B7/B27 compared with B27 Tg mice (Fig. 3.8C and 3.8D). These results indicate that Vβ8.1^+ CD8^+ T cells may have been negatively selected in the naive B7/B27 Tg mice.

We next compared the TCR Vβ repertoires of flu-infected (Fig. 3.8) Tg mice following secondary stimulation with specific peptides in vitro. For most Vβ chains examined, the level of CD8^+ T cells expressing a given Vβ was slightly greater for the Day 0 samples compared with naive mice. Cells from infected double and single Tg B27 mice were stimulated with NP383-391
Figure 3.8. Analysis of TCR Vβ repertoire of naive and peptide-stimulated CD8+ T cells from flu infected Tg mice.
**Legend to Figure 3.8**

**Analysis of TCR Vβ repertoire of naive and peptide-stimulated CD8⁺ T cells from flu infected Tg mice.**

LN cells from naive mice were stained for the expression of CD3, CD8 and various TCR Vβs. By gating first on the CD3⁺CD8⁺ T cells (not shown), the relative abundance of cells expressing each TCR Vβ was examined. Three weeks post-flu-infection, splenocytes from different mice were stimulated with NP418-426 (A-B), NP383-391 (C-D) and NP366-374 (E) for 14 days. On day 0 and day 14, the relative abundance of cells expressing each TCR Vβ was determined. (F) The TCR Vβ8.1 expression shown at a larger scale for the indicated mice. The values were normalized as described (152). Data are shown as mean ±SEM of n=6 mice per group and are pooled from four independent experiments. Significance was assessed using two way analysis of variance.*, p<0.01 vs. naive mice; **, P<0.001 vs. single Tg B27 mice; ***, p<0.0001 vs. naive mice; # vs. single Tg B27 mice.
in vitro and analyzed after 14 days. As shown in **Figure 3.8C**, there was a large increase in the Vβ8.1+ CD8+ population from infected single Tg B27 mice. Similarly, the Vβ8.1+ CD8+ population increased for the infected B7/B27 Tg mice (**Fig. 3.8D**). There was an increase in the Vβ12+ CD8+ population on Day 14 from infected single Tg B27 mice compared with infected B7/B27 Tg mice (**Fig. 3.8C-D**). Similarly, NP418-426 stimulated cells from infected double and single B7 Tg mice revealed that CD8+ T cells expressing Vβ6 were the most abundant for both single and double Tg mouse samples (**Fig. 3.8A** and **3.8B**). As expected, there was an increase in the Vβ8.3+ CD8+ T-cell population from flu-infected WT mice confirming published results (**Fig. 3.8E**)(152).

3.3.6 Tetramer staining of HLA Tg chimeras: altered selection of naive B27/NP383-391 CD8+ T cells

To further address whether negative selection of B27/NP383 CD8+ T cells is indeed the basis for immunodominance seen with flu-infected B7/B27 Tg mice, we created different HLA Tg chimeras (327). The approach was to allow irradiated female mice to repopulate their immune responses from male donor bone marrow (BM) cells. After 60 days the mice were examined for the presence of male SRY gene by PCR. The SRY PCR (**Fig. 3.9A**) showed that male cells were the only source of cells developing within these female Tg chimeras and this was confirmed by showing that cells in the recipient Tg B7 and Tg B7/B27 mice, when reconstituted with B27 BMs, were negative for BB7.1 expression and hence were of donor origin (**Fig. 3.9B**). In addition, there was no difference in the expression level of MHC-I among chimeric and non-chimeric mice as examined by allele-specific markers (**Fig. 3.9C**).
Figure 3.9. Description and identification of HLA Tg chimeras.
Legend to Figure 3.9

Description and identification of HLA Tg chimeras.

(A) Female recipient mice were irradiated and 24 Hrs later received bone marrow (BM) cells of male origin. After 60 days, DNA extracted from PBLs of different recipient mice was examined for the presence of male SRY gene. (B) Single and double female Tg B7/B27 mice were reconstituted with male B27 Tg BMs (Donor BMs are indicated in the brackets). PBLs of non-irradiated mice (i.e., Day 0) and post-BM transfused mice (i.e., Day 30 and 60) were stained with mAb BB7.1. (C) LNs from single and double HLA Tg chimeras were stained with the indicated mAbs. Representative plots of flow cytometric analyses for different mice are shown. n=3 per group pooled from three independent experiments.
We next examined the CTL response of different Tg chimeras to i.n. flu infection. One mouse strain hypothesized to reflect negative selection of B27/NP383-391 T cells were B7/B27 mice transfused with single Tg B27 BM. By transfusing single Tg B27 BM we ensure that all cells developed in the host were of B27 origin. As controls we also transfused double Tg B7/B27 mice with single Tg B7 or B7/B27 BM (Fig. 3.10). Similar to ELISpot CTL responses seen in Figure 3B, there was a strong CTL response detected against the B7/NP418-426 peptide in B7 and B7/B27 chimeras reconstituted with B7 BM (Fig. 3.10i). Similar to non-chimeric mouse flu responses, this B7/NP418 response was augmented 1.3–fold (p<0.01) for the B7/B27 Tg chimeras (compare Fig. 3.3Bi and 3.3Biii with Fig. 3.10i). In double Tg B7/B27 mice reconstituted with B7/B27 Tg BM comparable levels of B7/NP418 CD8+ T-cell response, similar to double Tg B7/B27 mice reconstituted with single Tg B7 BM, was detected (p>0.05). Not surprisingly, Tg chimeras reconstituted with B27 Tg BM did not respond to NP418-426 peptide (Fig. 3.10i). The non-Tg WT reconstituted with WT BM served as an internal control showing no CTL response to NP418-426 flu epitope. Parallel to immunodominant results detected in Figure 3B, in B7/B27 Tg mice reconstituted with either B27- or B7/B27 BM we detected low B27/NP383-391 CTL response (Fig. 3.10ii). However, this B27/NP383-391 CTL response was about 6-fold more (p<0.0001) in B27 chimeras reconstituted with B27 BM (Fig. 3.10ii). We detected only H2-Db restricted NP366-374 CTL responses in non-Tg WT mice reconstituted with WT BM (Fig. 3.10iii) and no mice had any CTL responses in the absence of peptide (Fig. 3.10iv).

We next determined the mean fluorescence intensity (MFI) and the actual number of enriched splenic flu-specific Tet+CD8+ T cells in different Tg chimeras. As shown, the MFI (Fig. 3.11A) and the actual numbers (Fig. 3.11B) of B27/NP383-restricted T-cell tetramers was higher in B27 Tg mice reconstituted with B27 BM than double Tg chimeras reconstituted with either
Figure 3.10. ELISpot analysis of flu-infected HLA Tg chimeras.
Legend to Figure 3.10

ELISpot analysis of flu-infected HLA Tg chimeras.

Single and double female Tg B7/B27 mice were reconstituted with male HLA Tg BMs (Donor BMs are indicated in the brackets). Mice were infected with flu i.n. and eleven days post-infection the CTL response was tested by IFN-γ ELISpot for various flu peptides. Double HLA Tg B7/B27 chimeras reconstituted with the single Tg B27 BMs showed similar levels of NP383-391 CTL responses as seen with corresponding non-chimeras (ii)(compare with Fig. 3.3, iii). Similarly, the single Tg B27 chimera reconstituted with single Tg B27 BM showed high levels of NP383-391 restricted CTL responses, similar to CTL response levels seen with the corresponding single Tg B27 non-chimeras (ii)(compare to Fig. 3.3, ii). The CTL responses to NP418-426 in single Tg B7 and double Tg B7/B27 mice reconstituted with either single Tg B7 BMs alone - or with double Tg B7/B27 BMs alone induced similar levels of CTL responses as seen for NP418-426 in the corresponding non-chimeras (i)(compare with Fig. 3.3, i). Data are shown as mean ±SEM of n=4 mice per group and are pooled from two independent experiments. Significance was assessed using two way analysis of variance. *, p<0.01; ***, p<0.0001; n=4 per group pooled from two independent experiments.
Tg B27 and/or B7/B27 Tg BM. There was a 2-fold difference in the level of B27/NP383 T-cell MFI among the B27 and B7/B27 Tg chimeras reconstituted with B27 BM (Fig. 3.11A). Similarly, B7 and B7/B27 Tg chimeras reconstituted with Tg B7 BM showed significant (p<0.05) differences in MFI and the actual numbers of B7/NP418-restricted T cells. As seen with non-chimeric mice (Fig. 3.11E), double Tg mice reconstituted with B7/B27 BM had similar MFI of B7/NP418 T cells as seen with double Tg mice transfused with B7 BM (Fig. 3.11A, white bars). Furthermore, single Tg B27 chimeras expressed higher levels of Vβ8.1+ CD8+ T cells than their double Tg counterparts (i.e., 9.5% vs 3.9%, Fig. 3.11Ci and 3.11ii). On the contrary, comparison of B7/NP418 Vβ6+ CD8+ T-cell population among B7 and B7/B27 Tg mice reconstituted with B7 BM revealed no differences (p>0.05)(Fig. 3.11Biii and 3.11Biv). Together these results confirm deletion of most B27/NP383-391-specific, Vβ8.1+ CD8+ T cells in the double Tg B7/B27 mice.
Figure 3.11. Tetramer quantification of flu infected T-cell populations and determination of naive TCR Vβ repertoire of different chimeras.
Legend to Figure 3.11

Tetramer quantification of flu infected T-cell populations and determination of naive TCR Vβ repertoire of different chimeras.

(A) MFI of Tet⁺CD3⁺CD8⁺ T cells from the spleen of flu-infected chimeras. Bar graph showing MFI of flu-infected Tet⁺CD3⁺CD8⁺ T cells corresponding to different flu peptides. Significance was assessed using two way analysis of variance. ***, p<0.0001. (B) Transgenic chimeras were infected and the frequency of NP383-391 and NP418-426 Tet⁺CD3⁺CD8⁺ T cells from the spleen was quantified on day 11 post-infection (A,B). Data are shown as mean ±SEM of (A) n=4 mice per group or (B) n=3 mice per group and are pooled from/representative of three independent experiments. (C) LN cells from naive chimeras were stained as described in Figure 7 and the relative abundance of cells expressing TCR Vβ8.1 and Vβ6 was examined. Representative histograms are shown from two independent experiments (n=3 mice per group).
3.4 Discussion

The HLA Tg mouse models described here provide an informative in vivo approach for characterizing viral infection-associated CTL responses in the context of human MHC-I molecules. Our results show immunodominance only in flu-infected B7/B27 Tg mice but not in A2/B7 or A2/B27 mice. Co-expression of B7 with B27 led to altered selection of Vβ8.1\(^+\) B27-restricted, NP383-specific CD8\(^+\) T cells in naive B7/B27 Tg mice.

There are a number of different factors contributing to immunodominance, as we reviewed recently (145). Reduced cell surface MHC class I expression levels, prior infection history (333), route of viral infection (178), temporal protein synthesis (248), mutations within an ImD epitope sequence (176, 179), co-expression of different allele combinations (168, 201, 334), and role of different enzymes such as ERAP (74, 300, 301) in antigen processing and presentation all may contribute to immunodominance. None of these factors seem to be the major contributing factor to the immunodominance we observe in the present study. In our system the number of antigen-specific naive T-cell precursors is the determining factor. Results with flu-specific tetramer staining along with findings of different TCR Vβ chain utilization showed that there was a significant reduction in the number of Vβ8.1\(^+\) B27-restricted NP383-391 CD8\(^+\) naive T cells in B7/B27 Tg mice compared with B27 mice. In addition, the number of Vβ12\(^+\) B27-restricted CD8\(^+\) T cells decreased in flu-infected B7/B27 Tg mice possibly due to these high avidity T cells being negatively selected following infection. Our study with influenza infection confirm previous findings where high avidity T cells were deleted following, but not before, HIV-1 and EBV infection in individuals co-expressing various HLA-A, -B, and –C alleles (168). Furthermore, although it is possible that other B27-specific CD8\(^+\) T-cell subsets expressing Vβ chains other than those examined may have been deleted and thus contributed to the reduced level of B27/NP383-
restricted CTL response in B7/B27 Tg mice, this seems unlikely based on published results (152, 202, 335). Flu-specific tetramer staining with HLA Tg chimeras and non-chimeras confirmed that altered selection in the thymus of B7/B27 Tg mice is involved in deleting some B27/NP383-restricted Vβ8.1+CD8+ naive T cells and this deletion occurs only when B27 is co-expressed with B7.

A number of factors may also contribute to immunodominance seen in B7/B27 Tg mice. First, to explore the role of differential cell surface expression in Tg mice we showed that all single and double Tg mice have similar surface MHC class I expression. Next, since both NP383-391 and NP418-426 are derived from the same viral protein, discrete paths of protein synthesis do not seem to contribute to immunodominance. Further, the same stock of virus was used for all studies. The presence of B27/NP383 CTL responses with flu-infected B27 mice indicates that there is no mutation within the NP383-391 peptide sequence with this particular flu stock. Another possibility is that during antigen processing and presentation the B27/NP383-391 peptide is not effectively generated while the B7/NP418-426 peptide is effectively generated. We ruled out these possibilities when the same overall pattern of immunodominance of B7/NP418-restricted CTL response in B7/B27 Tg mice was detected following synthetic peptide immunization. Another possibility is that the pMHC-I complexes for B7/NP418 and B27/NP383 epitopes may have different stability leading to differential efficiency of surface expression. Studies have shown that the NP418-426 epitope is consistently present on the B7+ APC (336) and the stability of MHC-I-NP418 complex is one of the highest previously reported for any pMHC-I (337). Our findings indirectly confirm these reports suggesting that the presentation of B7/NP418, and not B27/NP383, is extremely efficient in B7/B27 Tg mice. This may also contribute to reduced B27/NP383 CTL responses in these Tg mice. Further, it may also be that during infection, other non-NP flu proteins,
such as hemagglutinin or neuraminidase, contribute to the observed immunodominance. However, studies of Tg mice immunized with a flu NP DNA vector (i.e., in the absence of all other flu proteins) or DNA vector alone revealed the same pattern of reduced B27/NP383-restricted CTL recognition previously seen following flu infection and peptide immunization.

Our observations bear an interesting comparison with findings made by McMichael’s group regarding flu infection. This group showed that co-expression of HLA-B8 with B27 leads to immunodominance of B27/NP383-391 epitope over the B8/NP380-388 epitope (338). The major difference between these peptides and those in our study is that the B8 and the B27 peptides overlap in terms of sequence whereas the B7 and the B27 peptides do not. These investigators argued since B8 and B27 peptides overlap then these alleles must compete for the presentation of the common peptide fragment. In our case, there is no competition between the different peptide fragments for presentation, and as such we regard peptide competition as an unlikely potential contributing factor to immunodominance seen with B7/B27 Tg mice. We also show that following peptide stimulation the number of Vβ6+ or Vβ8.1+ CD8+ T cells increased at similar levels in the B7 and B27 Tg mice, respectively. In addition, these cells were capable of responding to stimulation with the relevant peptide. These results suggest that the B7/NP418 CTLs do not increase much more rapidly than the B27/NP383 CTLs. Furthermore, as the level of peptide-stimulated Vβ6+ CD8+ T cells were similar in both single B7 and double B7/B27 Tg mice, this may account for the similar B7/NP418-restricted CTL recognition seen with these Tg mice. Moreover, another possibility is that co-expression of different allele combinations may or may not influence immunodominance seen with different double Tg mice. A number of studies have investigated this possibility in different contexts (200, 201, 203, 338). Our results with A2/B7 and A2/B27 mice show strong CTL responses restricted by both co-expressed HLA alleles, where the
peptides are derived from different viral proteins. The lack of competition between these peptides may explain the absence of immunodominance observed in flu-infected A2/B7 and A2/B27 Tg mice.

3.5 Conclusions

Identifying determinants contributing to immunodominance hierarchy plays a critical role in vaccine development. The transgenic mice described here provide appropriate controls and conditions to address the fundamental biology of the process. Deciphering mechanisms by which peptides are presented to patients when multiple class I MHC alleles are co-expressed will help in future vaccine development.
Chapter 4

This chapter describing the role of ERAP in flu response of HLA Tg mice has been published in the Journal of Immunology. The citation for this article is: Ali Akram, Aifeng Lin, Eric Gracey, Cathy Streutker, Robert D. Inman. HLA-B27, but not HLA-B7, Immunodominance to Influenza is Uniquely ERAP Dependent. J Immunol. 2014 Jun 15;192(12):5520-8

Aifeng Lin and Eric Gracey helped with mouse genotyping. Cathy Streutker helped with the scoring of the H and E slides. All authors approved the draft. I did all the described experiments and analysis. I prepared the draft for publication.

4 HLA-B27, but not HLA-B7, Immunodominance to Influenza is Uniquely ERAP-Dependent

4.1 Introduction

The immune system faces immunogenic challenges on a daily basis. Following a viral infection the host immune response is directed to prompt clearance of the virus. Elements of innate immunity act rapidly to contain the spread of the virus while providing elements of adaptive immunity sufficient time to mount a specific and sustained immune response. The adaptive immune response depends on the ability of CTL to recognize, via TCR, antigenic viral peptides in the context of major histocompatibility complex class I molecules (MHC I)(145). Proteasomes in the cytoplasm generate viral peptides of various lengths which are transported via TAP to the endoplasmic reticulum (ER). Proteasomes generate peptides of 15-26 amino acids in length with extended N-termini. The N-terminally extended peptides are further trimmed in the ER by
endoplasmic reticulum aminopeptidase (ERAP) to peptides of 8-10 amino acids in length which are appropriate for MHC I binding. These peptides are subsequently bound by MHC class I molecules and transported to the cell surface where they are surveyed for recognition by the repertoire of αβ-TCR expressed by CTL (145, 151).

Recent genome wide association studies have implicated an interaction of HLA-B27 and ERAP as a determining factor in the genetic predisposition to ankylosing spondylitis (AS)(286). The strong association of ERAP genetic variants with AS is seen exclusively in HLA-B27-positive AS patients, providing one of the clearest examples of gene-gene interaction in human disease. But it remains unresolved how physiological co-expression of multiple MHC I alleles with ERAP may influence susceptibility (255). In addition, the means by which HLA-B27 and ERAP contribute as cofactors to the immunopathogenesis of AS has proved difficult to resolve in the clinical setting. The co-dominant expression of multiple human class I alleles contributing to immunodominance (ImDc) has hindered detailed immune response analysis of clinical samples. A recent insight highlighting allele-specific events in AS comes from a new study showing that whereas B27 confers susceptibility to AS, B7 confers protection (339). These advances in the genetic basis of AS have provided strong indirect support for the concept that processing and presentation of arthritogenic epitopes likely play a central role in the pathogenesis of AS (340). However, the identity of such arthritogenic epitopes remains unknown at this time. These findings emphasize the need for a controlled experimental system in which the interaction of ERAP and different MHC I alleles can be systematically addressed.

To this end, we have developed single and double HLA transgenic (HLA^{hyb} Tg) mice (341). The endogenous mouse MHC I genes were deleted (i.e., H-2K^{−/−} and H-2D^{−/−}, double knock-out, DKO) and selected human HLA genes, specifically HLA-B7 and HLA-B27, were introduced to
these DKO mice. Following challenge with influenza virus, the HLA Tg mouse immune response was comparable to that of humans expressing similar HLA alleles (152, 341). These findings indicated that these Tg mice can be informative in dissecting genetic control of human immune response to infection. Concurrently there has been interest in addressing the role of ERAP in host immune response to infection. ERAP-deficient mice (77) have been generated to study the effects of ERAP on generation and presentation of immunogenic peptides following lymphocytic choriomeningitis virus (LCMV), mouse cytomegalovirus (mCMV), influenza (flu), and Toxoplasma gondii infections. We used these ERAP\textsuperscript{-/-} mice and crossed them with our HLA-B7 and HLA-B27 Tg mice (on a DKO background) to create HLA-Tg mice in the absence of ERAP. As identifying antigenic peptides is difficult in the clinical setting, these double Tg mice (i.e., B27/ERAP\textsuperscript{-/-} and B7/ERAP\textsuperscript{-/-}) would serve as an informative model on the mechanisms of host immune responses following an infectious challenge which may entail an interaction of HLA and ERAP. As the immunodominant epitopes for various human HLA alleles for influenza virus are well known (i.e., NP383-391 for HLA-B27\textsuperscript{+} and NP418-426 for HLA-B7\textsuperscript{+} individuals), we used flu as a model to investigate the effects of HLA-B27 and ERAP\textsuperscript{-/-} in vivo.

Studies with ERAP deficient mice have shown reduced cell surface expression of MHC I molecules, but not MHC II (78, 298). These mice show no differences in the profile of CD4 and CD8 compared to mice with intact ERAP. The peptide repertoire generated in ERAP deficient mice following a viral infection differed from ERAP\textsuperscript{+/+} mice. Following infection, the CTL response to ovalbumin OVA\textsubscript{257-264}, LCMV NP\textsubscript{396-404}, mCMV YL9, and flu NP\textsubscript{366-374}, PA\textsubscript{224-233}, NS2\textsubscript{114-121} and PB2\textsubscript{198-206} epitopes was reduced whereas that of LCMV GP\textsubscript{33-41} and histocompatibility gene SVL9 epitopes increased (74, 193, 298, 299). In addition to viral infection, one study demonstrated that there was no CTL response to the immunodominant HF10 epitope of
T. gondii in ERAP−/− mice, indicating the involvement of ERAP in the generation of this epitope (300). Expansion of HF10-specific CD8+ T cells was shown to be impaired in ERAP−/− mice rendering these mice more susceptible to toxoplasmosis.

Here we show for the first time that the generation and presentation of the immunodominant B27-restricted flu peptide NP383-391 is ERAP-dependent. This B27/NP383 epitope is likely made as an extended 14-mer which is subsequently trimmed by ERAP. Furthermore, we show B27/ERAP−/− mice to have reduced B27/NP383-specific naive Vβ8.1+-expressing CD8+ T cells in comparison with the ERAP-intact counterpart. Surface expression of HLA-B27 in naive and infected B27/ERAP−/− mice was also significantly reduced. HLA-B27/ERAP−− Tg mice had increased edema in lung tissues and low levels of inflammatory cytokines. These events were not paralleled in the HLA-B7 mice: there were no differences in the B7 surface expression nor in the number of B7/NP418-specific naive Vβ6+-expressing CD8+ T cells in B7/ERAP−/− compared to its B7/ERAP+/+ counterpart. These results indicate an important cohesive relationship by which HLA-B27 and ERAP play in host immunity.
4.2 Materials and Methods

4.2.1 Generation and Identification of HLA Tg ERAP<sup>−/−</sup> Mice

The generation of single Tg HLA-B27/DKO and HLA-B7/DKO has been described (152, 202). The generation of ERAP-deficient mice has also been described (77). These mice were a generous gift from Dr. N. Shastri. Appropriate crosses of ERAP-deficient and DKO mice were made to generate ERAP<sup>−/−</sup>/DKO (i.e., ERAP<sup>−/−</sup>). Subsequently, appropriate crosses of single Tg B27 and B7 were made with ERAP<sup>−/−</sup> Tg mice to generate HLA-B27/ERAP<sup>−/−</sup>/DKO (i.e., B27/ERAP<sup>−/−</sup>) and HLA-B7/ERAP<sup>−/−</sup>/DKO (i.e., B7/ERAP<sup>−/−</sup>) mice. B7/ERAP<sup>−/−</sup> and B27/ERAP<sup>−/−</sup> were analyzed by flow cytometry and PCR. For PCR analyses we used the following primers: (F1) GGAGTTTGTTTTATGGAGGGTTG, (F2) TTGTGTGCCATCTGTAGGG, and (R1) CGGCTTGATTTATCTTGTCTTG. All mice were housed in the specific pathogen-free animal facility at Toronto Western Hospital in Toronto according to the guidelines of the Canadian Council of Animal Care. All animal studies have been reviewed and approved by the University Health Network Research Committee.

4.2.2 Flow Cytometry Analysis

The monoclonal antibodies (mAb) and detection reagents used for flow cytometry and their specificities and sources are as follows: ME1 (specificities: HLA-B7, -B27, -Bw22, and -B14) and BB7.1 (specificity: HLA-B7)(22) were from the American Type Culture Collection (Manassas, VA); CD4-PE (0.5 mg/ml, diluted 1:100), CD3-PerCP (0.5 mg/ml, diluted 1:150), and CD8α-FITC (0.5 mg/ml, diluted 1:150) were from BD Pharmingen (San Diego, CA). The anti-TCR Vβ mAbs were from BD Pharmingen (0.5 mg/ml, diluted 1:100)(San Diego, CA). FITC-conjugated F(ab')<sub>2</sub> goat anti-mouse IgG (Fc-specific, 0.5 mg/ml, diluted 1:150) and FITC-
conjugated F(ab’)_2 goat anti-rat IgG (Fc-specific, 0.5 mg/ml, diluted 1:150) were from Accurate Chemical and Scientific (Westbury, NY).

4.2.3 Influenza A/X31 (H3N2) Infection and IFN-γ ELISpot Assays

Mice between 7-10 weeks of age were infected as described (341). Briefly, Tg mice were transiently anaesthetized with metofane (methoxyflurane, Pitman-Moore) and infected i.n. with 20 hemagglutinating units (HAUs) of influenza A/X31(H3N2) (SPAFAS, North Franklin, CT) in 30 μl of PBS. Eleven days post-flu infection IFN-γ ELISpot assays were used to determine the frequency of peptide-specific IFN-γ-producing cells in spleens of flu-infected mice. Both HLA-B27+ human CTL and Tg B27 mouse CTL recognize flu NP383-391 while the CTL response in HLA-B7+ humans and Tg B7 mice is directed primarily to flu NP418-426 (341). HLA-B27 mice may also respond to NS1.87-95, PB2.702-710, PB2.368-376, and PB1.571-579 flu epitopes (175). In Non-Tg WT C57Bl/6J mice, the anti-flu CTL response is directed at the NP366-374 in the context of H2-D^b. Each of these respective peptides at pre-specified concentrations was used as described (341). All peptides were synthesized and purchased from Bio Basic Inc (Markham, ON, Canada).

4.2.4 Tetramer Staining and Enrichment of Antigen-specific CD8^+ T Cells

Cell suspension from spleen and lymph nodes (LN) were enriched for T cells using Pan T Cell Isolation Kit II Mouse as directed by the manufacturer’s protocol (Miltenyi Biotec). The details of cell preparation and staining have been described (341). Briefly, spleen and LN were mashed using a plunger, filtered, and lysed with ACK lysis buffer. Cells were washed and counted before single-cell suspensions (up to 10^8 cells) were labeled and passed over a magnetized LS
column (Miltenyi Biotec). Columns were washed and the bound cells were eluted and stained with T cell markers. Tetramers specific for NP366-374 (1.2 mg/ml, diluted 1:100), NP383-391 (1.2 mg/ml, diluted 1:100), and NP418-426 (1.3 mg/ml, diluted 1:100) were synthesized by NIH (Atlanta, GA). Cells were washed and fixed with 1% PFA before being analyzed with a LSRII cytometer (Becton Dickinson). Data analysis was performed using Cell Quest and FlowJo softwares (BD Immunocytometry Systems, CA).

4.2.5 Peptide Immunization

The peptide immunization protocol used has been described (341). Naive HLA Tg mice were immunized subcutaneously with CpG (10μg/ml, 50μg per mouse in 1 x PBS) two days prior to peptide immunization. Two days later the mice were stratified into three different groups: one group of mice were co-immunized subcutaneously with synthetic NP418-426 (10μg/ml, 75 μg/mouse) and NP383-391 (10μg/ml, 75 μg/mouse) peptides in incomplete Freund’s adjuvant (IFA), or IFA alone as control; the second group was immunized with N-terminally extended 14-mer version of NP383-391 (10μg/ml, 75 μg/mouse)(TLELRSRYWAIRTR) and NP418-426 (10μg/ml, 75 μg/mouse) (SVQRNLPFDRTIM), and the last group was immunized with the N-terminally extended 18-mer of NP383-391 (10μg/ml, 75 μg/mouse)(MESSTLELRSRYWAIRTR) and NP418-426 (10μg/ml, 75 μg/mouse) (OPTFSVQRNLPFDRTIM). Eleven days post-immunization spleen cells were removed and tested by ELISpot.

4.2.6 Body Weight Loss and H and E Staining

Following flu infection mice were weighed on a daily basis for 12 days. On day 12 flu-infected HLA Tg mice along with allele- and age-matched naive counterparts were sacrificed, the
lungs excised and fixed in 10% formalin. The fixed lung samples were embedded and stained with haematoxylin and eosin (H&E) dyes. Pictures were generated using a Nikon Eclipse TE2000-U microscope equipped with a Nikon Digital Sight DS-U2 camera and NIS-Elements BR3.10 acquisition software. This microscope was equipped with a 10x objective (NA 0.3) and 20 x objective (NA 0.45). The lung slides were scored in a blinded fashion by Dr. Cathy Streutker.

4.2.7 Cytokine Analysis

Serum was prepared as per Eve Technologies direction (Calgary, AB, Canada). Briefly, naive and flu infected mice were sacrificed and blood was immediately removed by cardiac puncture. Blood was allowed to clot for 2 hrs before spinning at 1000 RPM for 10 minutes at 4°C. Supernatants were aliquoted and stored at -20°C before analysis. Lung tissue homogenates were prepared as described (342). Lung tissues were homogenized in PBS-based buffer (20mM Tris.Cl pH7.5, 1% Triton-x, 0.05% SDS, 5mg/ml Deoxycholic acid, 50mM NaCl, and1mM PMSF) containing various protease inhibitor (Roche, Germany). Lung homogenates were filtered (0.22µm), spun at 12000 RPM for 4 minutes, and stored at -20°C before analysis by Eve’s Technologies.

4.2.8 Statistical Analysis

Data were analyzed by two way analysis of variance (two variables; naive vs. flu infected and ERAP\(^{+/+}\) vs. ERAP\(^{-/-}\) in GraphPad Prism 5.0, GraphPad Software Inc.,La Jolla, CA) with a Bonferroni posttest correction. All values are expressed as mean (± SEM), P < 0.05 (adjusted P) was considered significant. Student t-test was also performed as indicated.
4.3 Results

4.3.1 Characterization of novel B7/ERAP\textsuperscript{−/−} and B27/ERAP\textsuperscript{−/−} Mice

ERAP-deficient mice were identified by PCR and flow cytometric analysis as described (77). Consistent with previous findings we saw no significant differences in the percentage of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells in LN (Fig. 4.1A) or spleen (not shown) between the ERAP\textsuperscript{+/+} (341) and ERAP\textsuperscript{−/−} HLA Tg mice. Non-transgenic wild type (Non-Tg WT) mice had the highest level of CD8\textsuperscript{+} T cells compared to HLA Tg ERAP\textsuperscript{−/−} mice. As expected, double-knock out (DKO) mice had the lowest CD8\textsuperscript{+} T percentage confirming previous results (341). The absence of ERAP did not significantly alter the cell surface expression of HLA-B7 (Fig. 4.1B, left panel). In contrast, the expression of HLA-B27 as detected by ME1 antibody was significantly (p=0.0032 for MFI) reduced in B27/ERAP\textsuperscript{−/−} mice (Fig. 4.1B, right panel) in comparison with B27 Tg mice.

4.3.2 Reduced B27/NP383-391 CD8\textsuperscript{+} T cell response in flu-infected B27/ERAP\textsuperscript{−/−} Tg mice

Previous studies of ERAP\textsuperscript{−/−} mice did not address the “interaction” of MHC-I, either endogenous or transgenic, with ERAP, whereas our current mouse constructs allow a specific analysis of possible roles of HLA alleles and ERAP on CD8\textsuperscript{+} T cell responses following influenza infection. We infected B7/ERAP\textsuperscript{−/−} and B27/ERAP\textsuperscript{−/−} Tg mice along with the corresponding ERAP\textsuperscript{+/+} HLA Tg controls with flu as described (Fig. 4.2)(341). Spleen cells were examined by IFN-\gamma ELISpot assay 11 days post intranasal (i.n.) flu infection. B7/ERAP\textsuperscript{−/−} and B7/ERAP\textsuperscript{+/+} mice both showed strong CTL responses to B7-restricted NP418-426 immunodominant epitope (Fig. 4.2A and B). There was no significant difference in the B7/NP418-426 CTL response among these
two mouse strains. Controls indicated no CTL response in the presence of irrelevant peptide or in the

**Figure 4.1. Characterization of HLA Tg ERAP<sup>−/−</sup> mice.**

(A) LN from different Tg mice were stained for CD3 and CD8. The bar graph shows the percentage of CD3<sup>+</sup>CD8<sup>+</sup> T cells in different Tg mice. Data are shown as mean ±SEM from n=6 mice per group and are pooled from three independent experiments. *, p<0.05 vs. Tg mice using student t-test. (B) The expression level of HLA-B7 (as detected by BB7.1 mAb) and HLA-B27 (as detected by ME1 mAb) in single and double HLA Tg mice. Representative plots of flow cytometric analyses for different mice are shown. This experiment was repeated three times with n=6 mice per group. Significance was assessed using two way analysis of variance.

**Legend to Figure 4.1**

**Characterization of HLA Tg ERAP<sup>−/−</sup> mice**

(A) LN from different Tg mice were stained for CD3 and CD8. The bar graph shows the percentage of CD3<sup>+</sup>CD8<sup>+</sup> T cells in different Tg mice. Data are shown as mean ±SEM from n=6 mice per group and are pooled from three independent experiments. *, p<0.05 vs. Tg mice using student t-test. (B) The expression level of HLA-B7 (as detected by BB7.1 mAb) and HLA-B27 (as detected by ME1 mAb) in single and double HLA Tg mice. Representative plots of flow cytometric analyses for different mice are shown. This experiment was repeated three times with n=6 mice per group. Significance was assessed using two way analysis of variance.
absence of peptide in all mice. In contrast, B27/ERAP\textsuperscript{+/-} mice unexpectedly showed reduced CTL responses to the B27-restricted NP383-391 flu epitope compared to CTL responses seen in B27/ERAP\textsuperscript{+/+} mice (Fig. 4.2C and D). The B27/NP383-391 CTL response was significantly (p<0.0001) reduced in the B27/ERAP\textsuperscript{+/-} Tg mice. The PB2.702-710 CTL response appeared higher (p=0.0681) in B27/ERAP\textsuperscript{+/-} Tg mice than in B27/ERAP\textsuperscript{+/+} mice. Overall, the CTL response to other subdominant B27 flu epitopes did not reach significance above the background in both ERAP\textsuperscript{+/+} and ERAP\textsuperscript{+/-} B27\textsuperscript{+} mice (Fig. 4.2C and D).

4.3.3 Increased weight loss and pathology in B27/ERAP\textsuperscript{+/-} Tg mice

The ELISpot results above prompted us to assess the overall response to flu in these mice (Fig. 4.3). Others have used fluctuations in the body weight following a viral infection as a viral clearance index (343, 344). We used the same index following flu infection of ERAP\textsuperscript{+/-} and ERAP\textsuperscript{+/-} HLA Tg mice (Fig. 4.3A and B). We followed the changes in the body weight on a daily basis for twelve days post-infection. There were significant (p<0.01) differences in the body weight between the B27/ERAP\textsuperscript{+/-} and B27 Tg mice 5-9 days post-flu infection (Fig. 4.3A). However, such differences were neither observed in the initial stages of flu infection (i.e., between Day 1-5) nor in the latter parts of recovery (i.e., between Day 11 and Day 12)(Fig. 4.3A). Both B27/ERAP\textsuperscript{+/-} and B27 Tg mice recovered from flu infection and ended up with similar profile of body weight change 12 days post-flu infection (Fig. 4.3A). Such significant differences in the baseline body weight were not observed in the B7/ERAP\textsuperscript{+/-} and the B7 Tg mice (Fig. 4.3B). Although the body weight profile of flu infected B7/ERAP\textsuperscript{+/-} and the B7 Tg mice were similar, it is noteworthy to notice that the B7/ERAP\textsuperscript{+/-} Tg mice seemingly (but not significantly) lost less weight overall during the course of infection compared to B7 Tg mice (Fig. 4.3B).
Figure 4.2. ELISpot analysis of the CD8\(^{+}\) CTL response to flu infections in single 
(A & C) and double (B & D) HLA Tg ERAP\(^{+/-}\) and ERAP\(^{-/-}\) mice.
ELISpot analysis of the CD8⁺ CTL response to flu infections in single (A & C) and double (B & D) HLA Tg ERAP⁺/+ and ERAP⁻/⁻ mice. Mice were infected with flu i.n. as described in the Materials and Methods. After eleven days post-infection the CTL response was tested by IFN-γ ELISpot for various flu peptides. Data are shown as mean ±SEM of n=6 mice per group and are pooled from six independent experiments. Significance was assessed using two way analysis of variance. ***, p<0.0001 vs. NP383-391 CTL response seen in Tg B27 mice.
Figure 4.3. Differences in the profile of HLA Tg ERAP$^{+/+}$ and ERAP$^{-/-}$ Tg mice following flu infection as examined by body weight baseline change, cytokine analysis and H&E staining of lung sections.

A) Tg B27 vs. Tg B27/ERAP$^{-/-}$

B) Tg B7 vs. Tg B7/ERAP$^{-/-}$

C) Cytokine analysis and H&E staining of lung sections.
Figure 4.3 (continued). Differences in the profile of HLA Tg ERAP$^{+/+}$ and ERAP$^{−/−}$ Tg mice following flu infection as examined by body weight baseline change, cytokine analysis and H&E staining of lung sections.
Legend to Figure 4.3.

Differences in the profile of HLA Tg ERAP\(^{+/+}\) and ERAP\(^{-/-}\) Tg mice following flu infection as examined by body weight baseline change, cytokine analysis and H&E staining of lung sections.

(A-B) Percent change in baseline body weights of B27 and B27/ERAP\(^{-/-}\) (A) and B7 and B7/ERAP\(^{-/-}\) (B) Tg mice. Mice were infected i.n with 20 HAUs of influenza A/X31(H3N2) and the body weight change was tracked for 12 days post-flu infection. Data are shown as mean ±SEM of n=6 mice per group and are pooled from three independent experiments. (C) Cytokine profiles of pro-inflammatory cytokines in naive and infected (Day 11 post-infection) Tg mice. Lungs were homogenized and examined for the expression of different cytokines. Data are shown as mean ±SED of n=4 mice per group and are pooled from three independent experiments. (D) Histopathological characteristic of naive and flu infected Tg lung sections. Lung sections were stained with H&E (original Magnification, x 10). Bar graph indicates the presence or absence of edema in different Tg mice (right panel). Histopathology scores of lung sections were scored in a blinded fashion by a lung pathologist. Data are shown as mean ±SEM of n=6 mice per group. Significance was assessed using two way analysis of variance. **, p<0.001 as indicated.
4.3.4 Flu infected B27/ERAP\textsuperscript{−/−} Tg mice have reduced levels of inflammatory cytokines and increased lung edema

Differences seen in body weight 5-9 days post-flu infection indicative of differential viral clearance and/or increased cytokines between the B27/ERAP\textsuperscript{+/−} and B27 Tg mice suggests ERAP influences adaptive immune response in a time-dependent manner. To investigate whether there is any difference in the level of inflammatory cytokines produced in ERAP\textsuperscript{+/+} and ERAP\textsuperscript{−/−} Tg mice, we determined the cytokine profile in serum and lung homogenate of naive and flu infected mice (Fig. 4.3C)(342). As expected, cytokine level of flu infected animals was higher than its naive controls confirming previous results (345, 346). There was a significant (p<0.001) difference in the levels of IL-1a, IL-6, and MIP-1a produced in the lung of B27/ERAP\textsuperscript{+/+} and B27/ERAP\textsuperscript{−/−} Tg mice (Fig. 4.3C). Similar trend of IL-6 and MIP-1a, but not IL-1a, production was observed in infected serum samples of B27/ERAP\textsuperscript{+/+} and B27/ERAP\textsuperscript{−/−} mice. The effect of ERAP on differential expression of IL-6 and MIP-1a in the lung seen in B27 Tg mice was not seen in B7 Tg mice. Next, to determine whether there are any differences in the pathology of the target organ, we stained naive and flu-infected fixed lung sections with H&E (Fig. 4.3D). The slides were subsequently scored by a lung pathologist in a blinded fashion. Overall, as evident in Figure 3D, there is an increase in edema in lungs of flu infected ERAP\textsuperscript{+/+} and ERAP\textsuperscript{−/−} Tg mice compared to naive mice regardless of mouse strain. The alveolar membranes showed significant damage following flu infection. The total edema score for infected B27/ERAP\textsuperscript{−/−} was significantly (p<0.001) higher than that of B27/ERAP\textsuperscript{+/+} Tg mice (Fig. 4.3D, bar graph). No differences in the edema score were observed between the B7 and B7/ERAP\textsuperscript{−/−} Tg mice (Fig. 4.3D).
4.3.5 Reduced number of B27/NP383-391 CD8$^+$ T cell in B27/ERAP$^{-/-}$ Tg mice

We recently showed that double Tg B7/B27 mice (i.e., ERAP-intact mice) have a reduced number of naive B27/NP383-391 CD8$^+$ T cells compared to single Tg B27 mice (341). Differences in the naive B27/NP383-391 CD8$^+$ T cell number accounted for the decreased NP383-391 CTL response following flu infection in B7/B27 Tg mice. To investigate whether the difference in the B27/NP383-391 CTL response (compare Fig. 4.2C and 4.2D) seen between the B27/ERAP$^{-/-}$ and B27 Tg mice is due to a reduced number of B27/NP383-391 naive CD8$^+$ T cells in B27/ERAP$^{-/-}$ Tg mice, we stained T cell-enriched splenocytes with flu NP383-391 tetramer as described in the Materials and Methods (Fig. 4.4A). The percentage of CD3$^+$CD8$^+$NP383$^{+}$Tet$^+$ T cells in naive and flu-infected B27/ERAP$^{-/-}$ Tg mice is lower (i.e., 0.601\% and 1.31\%) than our previous results for naive and flu-infected B27 Tg mice (i.e., 1.15\% and 6.82\%)(341) respectively (Fig. 4.4A). These percentage differences reflect the variance seen in the actual numbers of B27/NP383-specific CTL. The number of naive and flu-infected B27/NP383-391 CD8$^+$ T cells is significantly (p<0.0001) lower in B27/ERAP$^{-/-}$ Tg mice compared to B27 Tg mice (Fig. 4.4B and 4.4C). Tetramer staining with NP418-426 flu epitope did not reveal any major differences in the CD3$^+$CD8$^+$NP418$^{+}$Tet$^+$ T cell number in naive and flu-infected B7 and B7/ERAP$^{-/-}$ Tg mice (Fig. 4.4B and 4.4C). We used the Non-Tg WT/NP366-374 flu tetramer as a negative control to show the specificity of our tetramer staining to different HLA alleles (Fig. 4.4).
Figure 4.4. Enumeration of naive and flu infected enriched T-cell populations in different Tg mice using B7/NP418-426, B27/NP383-391, and the WT/NP366-374 flu tetramers.

A) Naive Mice

B) Naive

C) Infected
Legend to Figure 4.4.


(A-C) T lymphocytes from naive and flu infected mice were enriched from the spleen and LN. By first gating on the CD3⁺CD8⁺ positive cells (not shown), different T-cell populations corresponding to different flu tetramers were plotted. (A) Representative dot plots showing tetramer percentages of naive (left panel) and flu infected (right panel) enriched CD3⁺CD8⁺ T cells corresponding to different flu peptides. Quantification of flu specific Tet⁺CD3⁺CD8⁺ T cells from the spleen of naive (B) and day 11 post i.n. flu (C) infected Tg mice. Data are shown as mean ±SEM of n=6 mice per group and are pooled from five independent experiments. Significance was assessed using two way analysis of variance ***, p<0.0001 as indicated.
4.3.6 Deletion of Vβ8.1+CD8+ T cells in B27/ERAP−/− Tg mice

We have previously shown that TCR Vβ6+CD8+ T cells recognize NP418-426 flu epitope in B7+ human and B7 Tg mice (341). The recognition of NP383-391 flu epitope in both B27+ humans and B27 Tg mice is dependent on TCR Vβ8.1-expressing CD8+ T cells. Our recent published data showed co-expression of B7 with B27 led to negative selection of B27/NP383-391 flu specific naive Vβ8.1+-expressing CD8+ T cells in B7/B27 Tg mice (341). Here we identify a significant difference (p<0.001) in the expression of Vβ8.1 CD8+ T cells in naive (compare Fig. 4.5A and B) and flu-infected (Fig. 4.5E) B27/ERAP−/− Tg mice compared to B27 Tg mice. Unlike Vβ8.1 expression, there was no drastic change in the overall expression of TCR Vβ6+-expressing CD8+ T cells in naive (compare Fig. 4.5C and D) and flu-infected (Fig. 4.5F) B7 Tg and B7/ERAP−/− mice. Overall, significant differences were observed for Vβ8.1 expression in naive B27 Tg and B27/ERAP−/− mice (Fig. 4.5E), and the lack of differences present for Vβ6 expression in naive B7 Tg and B7/ERAP−/− mice (Fig. 4.5F) confirm the above results. There were no major deviations in the expression of other TCR Vβ markers between the naive and flu-infected ERAP+/+ and ERAP−/− B7 or B27 Tg mice (Fig. 4.5E and F).
Figure 4.5. Analysis of TCR Vβ repertoire of ERAP\textsuperscript{+/+} and ERAP\textsuperscript{−/−} HLA Tg mice.
Legend to Figure 4.5.

Analysis of TCR Vβ repertoire of ERAP<sup>+/+</sup> and ERAP<sup>−/−</sup> HLA Tg mice.

Spleen cells from naive mice were stained for the expression of CD3, CD8 and various TCR Vβs. By gating first on the CD3<sup>+</sup>CD8<sup>+</sup> T cells (not shown), the relative abundance of cells expressing each TCR Vβ was examined. Representative graphs showing the relative abundance of (A) naive B27 and (B) B27/ERAP<sup>−/−</sup> cells expressing TCR Vβ8.1. Representative graphs showing the relative abundance of (C) naive B7 and (D) B7/ERAP<sup>−/−</sup> cells expressing TCR Vβ6. (E-F) Bar graphs of TCR Vβ expression in naive and flu-infected HLA (E) B27 and (F) B7 Tg mice in the presence and absence of ERAP. The values were normalized as described in (341). Data are shown as mean ±SEM of n=6 mice per group and are pooled from three independent experiments. Significance was assessed using two way analysis of variance. **, p<0.001.
4.3.7 Generation and presentation of B27/NP383-391 flu epitope is ERAP-dependent

ERAP is involved in generation and trimming of different peptides for recognition by CTL (145). To directly addresses the role of ERAP in NP383-391 and NP416-426 peptide generation we immunized HLA Tg ERAP\(^{+/+}\) and ERAP\(^{-/-}\) mice with canonical B27 and B7 flu epitopes and examined the CTL response by IFN-\(\gamma\) ELISpot assay as described (Fig. 4.6A and B)(341). The B27/NP383 CTL response in B27/ERAP\(^{+/+}\) mice was significantly lower (p<0.001) than CTL response in B27 Tg mice (Fig. 4.6A). There were no significant differences in the CTL response to NP418-426 flu epitope in B7 Tg and B7/ERAP\(^{+/+}\) mice (Fig. 4.6B). Since ERAP has been shown to trim some N-terminally extended peptides in the ER in a sequential manner, we immunized the HLA Tg mice with N-terminally extended modifications of B7 and B27 flu epitopes and examined the CTL response by IFN-\(\gamma\) ELISpot assay (Fig. 4.6C and D). Surprisingly, following 14-mer NP383 peptide immunization, there was no CTL response to NP383-391 flu epitope in B27/ERAP\(^{-/-}\) mice while the CTL response in B27 Tg mice to the same epitope was significantly (p<0.0001) higher (Fig. 4.6C). Immunization with an 18-mer version of NP383-391 flu epitope did not result in any CTL responses in any HLA Tg mice regardless of ERAP status (Fig. 4.6E). Immunization with a 14-mer version of the NP418-426 flu epitope demonstrated no significant differences in the CTL response between B7 Tg and B7/ERAP\(^{+/+}\) mice (Fig. 4.6D). As was the case with the 18-mer version of NP383-391 vaccination, immunization with 18-mer version of NP418-426 was not associated with a detectable CTL response in any mouse strain (Fig. 4.6F). The Non-Tg WT/NP366-374 flu epitope was included as a negative control for our peptide immunization experiments.
Figure 4.6. Peptide immunization of HLA Tg mice in the presence or absence of ERAP with canonical and N-terminally extended flu epitopes.
Legend to Figure 4.6.

Peptide immunization of HLA Tg mice in the presence or absence of ERAP with canonical and N-terminally extended flu epitopes.

(A-D) Different Tg mice were immunized subcutaneously with canonical (A & C) and N-terminally extended 14-mer (B & D) and 18-mer (E & F) version of flu epitopes as indicated and tested by ELISpot for various flu peptides 11 days post-immunization. Data are shown as mean ±SEM of n=6 mice per group and are pooled from four independent experiments. Significance was assessed using two way analysis of variance. **, p<0.001 as indicated; ***, p<0.0001 as indicated.
4.4 Discussion

The results presented here signify the importance of HLA-B27 and ERAP collaboration in peptide generation and presentation. This is the first report where the absence of ERAP has led to altered selection of allele-specific naive CD8+ T cells. Our results complement a number of previous findings with ERAP−/− mice while adding new knowledge about the role of ERAP in the adaptive immune response. These results suggest that the function of ERAP is dependent on its MHC-I context: the presence or absence of ERAP in combination with HLA-B7 had no effects on host immune responses, while the absence of ERAP significantly altered the immune response to infection when co-expressed with HLA-B27. These in vivo studies complement recent genetics studies (286) which have implicated class I allele-specific interactions with ERAP not only in AS but also in psoriasis and Behcet’s disease (269). Discovery of pathogenic peptide epitopes has been very difficult in these diseases and the current study provides a proof-of-principle demonstration of specific ERAP-MHC I “interactions” influencing host response to infection.

ERAP has been shown to influence the overall peptide repertoire available for presentation (74, 193, 347). Our findings demonstrate that generation and presentation of B27-restricted NP383-391 flu epitope is critically dependent on ERAP. The absence of ERAP in B27/ERAP−/− mice lead to a significant reduction in CTL response to NP383-391 epitope following flu infection and peptide vaccination. Our peptide immunization studies suggest that the NP383 epitope is most likely generated as an N-terminally extended multimer which is subsequently trimmed in the ER by ERAP before being loaded into the MHC I peptide-binding groove. ERAP has been shown to cleave polypeptides at specific sites expressing leucine (L), methionine (M), phenylalanine (F) and tyrosine (Y) in the amino acid sequence (75, 290). The natural 14-mer sequence of NP383-391 (i.e., TLELRSRYWAIIRTR) contains two leucines upstream of NP383-391 at position 11 and 13,
which provide potential cleavage sites for ERAP. The absence of NP383-specific CTL response following 14-mer and 18-mer NP383-391 peptide immunization in B27/ERAP−/− Tg mice indicates that this epitope is indeed trimmed by ERAP. On the other hand, TAP has been shown not to transport peptides containing proline (P) at position 2 within its sequence (192). The presence of proline at position 2 of NP418-426 (i.e., VQRNLPFDRTTIM) strongly suggests that this epitope is initially generated as an N-terminally extended species before being transported into the ER by TAP. Lack of B7/NP418 CTL response difference between the B7 Tg and B7/ERAP−/− mice following flu infection and peptide immunization argues against a specific role for ERAP in the final generation of the B7 epitope. The absence of ERAP’s preferred cleavage amino acids upstream of the NP418-426 flu sequence seems to confirm this. It is worth noting that immunization with exogenous peptide precursors does not always guarantee ER trimming. Aminopeptidases located at the cell surface or in the endosome (e.g., IRAP) can also contribute to the overall peptide trimming. Given that the only difference between the B27/ERAP and the B27/ERAP−/− Tg mice is absence of ERAP expression, these other possibilities were ruled out as contributing factors. Our B7 findings confirm previous observations with ERAP−/− mice (194, 348). These studies showed there was no significant difference in the CTL number and CTL response to flu NP366-374, PA224-233, NS2114-121, PB1F262-70, PB2198-206 epitopes. It is known that ERAP can influence the CTL response to one epitope but not another originating from the same virus. Previously Niedermann’s group showed that absence of ERAP expression leads to diminished LCMV CTL responses to immunodominant NP396-404, but not to immunodominant GP33-41 (348). Our findings are the first to show that the B27-restricted flu CTL response, but not the B7-restricted flu CTL response, are influenced by ERAP.
ERAP when co-expressed with HLA-B27 plays multiple critical functions in host immunity. First, as discussed above, it trims the N-terminally extended version of the immunodominant flu NP383-391 epitope to the appropriate length before it is being presented by HLA-B27. Secondly, ERAP may figure critically in determining the T cell repertoire in B27/ERAP−/− mice. It is notable that the reduction in T cells reactive with NP383-391 is detected in naive as well as flu-infected mice. During thymic selection in B27 Tg mice, ERAP may contribute to destruction of self-reactive antigens, some of which might mimic flu NP383-391 epitopes, thus allowing positive selection of those thymocytes capable of recognizing the NP383-391 flu epitope later on in life. When ERAP is absent, as is the case in B27/ERAP−/− mice, deletion of self-reactive T cells recognizing antigens which mimic flu NP383-391 epitope does not take place, leading to eventual negative selection of B27/NP383-specific thymocytes expressing TCR Vβ8.1+. Partial deletion of a specific T cell population may occur for different reasons. We have recently shown that co-expression of HLA-B7 with HLA-B27 in double Tg B7/B27 mice (i.e., ERAP-intact) leads to negative selection of B27/NP383-reactive T cells (341). Thus, both MHC I allelic co-expression and ERAP-mediated peptide trimming contribute in an allele-specific manner to selection of T cells. Lastly, surface expression of HLA-B27, but not HLA-B7, is significantly reduced when ERAP is absent. Our B27 results are consistent with published results of others showing reduced endogenous MHC class I expression in ERAP−/− mice. These results suggest that ERAP normally stabilizes the B27 heavy chain (HC) by presenting it with appropriate B27-specific peptides during assembly of MHC I. When ERAP is absent, such peptide presentation to newly synthesized B27 molecules is impaired. This may contribute to two of the proposed mechanisms whereby HLA-B27 contributes to disease pathogenesis, namely, misfolding of the HC of B27 within the ER resulting in an unfolded protein response (294) phenomenon and homo-dimerization.
of B27 HC leading to altered interaction with NK cells (349, 350). These two phenomena associated with B27 may lead to slower transport of a diminished number of mature B27 molecules to the cell surface. Taken together, altered selection of B27/NP383-specific CD8⁺ T cells and reduced B27 surface expression in B27/ERAP⁻/⁻ mice likely account for both the reduced number of NP383-391 CD8⁺ T cells and the reduced NP383-391 CTL response following flu infection and peptide immunization in these mice.

To date this is the first report, to our knowledge, directly linking ERAP as a determining factor in T cell repertoire generation during thymic development. ERAP appears not to influence presentation of other B27-restricted subdominant epitopes (Fig. 2). Increased weight loss 5-9 days post-flu infection in B27/ERAP⁻/⁻ Tg mice is reflective of partial impaired viral clearance, likely secondary to lower numbers of NP383-391-specific CD8⁺ T cell number. Increased edema in the lung in the same mice reflecting enhanced direct lung injury recapitulated this trend. Lower levels of pro-inflammatory cytokines in the lung of B27/ERAP⁻/⁻ Tg mice is consistent with an impaired local response to the virus. Blinded scoring of the lung pathology revealed lower number of acute and chronic inflammatory infiltrates in B27/ERAP⁻/⁻ mice compared to B27/ERAP⁺/+ Tg mice, accounting for the lower inflammatory cytokine levels in B27/ERAP⁻/⁻ Tg mice. We have previously shown and confirmed here that the predominant CTL response in B27 Tg mice is directed at NP383-391. In addition to this specific NP383 CTL response, it is possible other B27-specific flu epitopes, not investigated here, contribute to the overall viral clearance. Slight increases in the level of CTL responses to other subdominant B27 flu epitopes (e.g., PB2.702-710, Fig. 2D) in B27/ERAP⁻/⁻ Tg mice may partially make up for the reduced B27/NP383-391 CTL response aiding in overall viral clearance.
4.5 Conclusion

We propose that in the absence of ERAP there is a relative inability to trim N-terminally-
extended NP383-391 peptide and that this accounts for the reduced number of B27/NP383-specific
CD8\(^+\) V\(\beta\)8.1\(^+\) T cells seen in naive and flu-infected B27/ERAP\(^{-/-}\) mice. Utilization of a well-
controlled \textit{in vivo} animal model can enhance our ability to resolve the cohesive ERAP-B27
“interaction” in disease pathogenesis. Such a system will shed more light into the mechanisms
behind the genetic basis of AS.
Chapter 5

5 Conclusions and Future Directions

5.1 Conclusions

The work presented here illuminates the importance of MHC-I allele co-expression on the overall immune response following influenza infection. Co-expression of certain MHC-I combinations (i.e., A2/B7, A2/B27, and B7/B27) determines whether ImDc of one allele over the other is observed following flu infection. Expression of ERAP influences ImDc as well. The in vivo studies with flu-infected ERAP-deficient B27 Tg mice presented here showed for the first time, to our knowledge, that the B27/NP383-restricted CTL response is ERAP-dependent.

Using single Tg A2, -B7, or -B27 mice I established that the anti-flu CTL responses are mainly directed at the respective ImD epitopes. However, only co-expression of B7 with B27 led to B7/NP418-restricted immunodominant CTL responses in flu-infected B7/B27 Tg mice. Using chimeras I showed this was likely due to negative selection of B27/NP383-restricted Vβ8.1+CD3+CD8+ naive T cells in B7/B27 Tg mice. No such dominance of allele-based epitope was observed in flu-infected A2/B7 or A2/B27 Tg mice suggesting that certain combinations of allele co-expression influence ImDc more than others. This investigation proved that my initial hypotheses were correct.

Studies with B27 ERAP deficient mice revealed for the first time, to our knowledge, that the anti-flu CTL response to B27-restricted NP383-391 epitope is ERAP-dependent. Peptide
immunization of B27/ERAP<sup>−/−</sup> mice with N-terminally extended synthetic B27 flu epitopes showed that the absence of ERAP prevents the generation and subsequent presentation of B27/NP383 epitope to B27<sup>+</sup>Vβ8.1<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> T cells. Indeed, lack of ERAP expression may have contributed to the partial negative selection of naive Vβ8.1<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> T cells in B27/ERAP<sup>−/−</sup> mice. This investigation agreed with my hypotheses as well.

Immunodominance is a phenomenon influenced by multiple factors. Deciphering individual components contributing to ImDc will enhance our overall ability to develop improved i) vaccines (e.g., for flu) and ii) treatment for AS patients. Studies outlined here revealed new parameters that are critical in our overall knowledge of ImDc.

### 5.2 Future Directions

The results shown in the previous chapters answer several important questions, while raising new ones. Specifically the interplay between B27 and ERAP is relevant to AS, since genetic variants of ERAP with less enzymatic activity have been shown to be protective in AS. I would like to take this correlation to next the step. Studies outlined below will deal directly with ERAP in relation to AS. These studies will address some currently unanswered questions which could be critical in advancing our understanding of AS.

#### 5.2.1 Studies of ERAP Expression in B27/ERAP Tg Mice

#### 5.2.2 What is the length of the N-terminally extended B27/NP383-391 epitope before it is trimmed by ERAP?
Studies outlined in chapter 4 suggest that NP383-391 is made as an N-terminally extended epitope. Studies with 18-mer version of N-terminally extended NP383-391 did not induce any CTL response while peptide immunization with the 14-mer version of the same epitope did. What is the exact length of the N-terminally extended NP383-391 epitope?

To address this question I would synthesize different versions of N-terminally extended NP383-391 epitope as follows: 10-mer (RSRYWAIRTR), 11-mer (LRSRYWAIRTR), 12-mer (ELRSRYWAIRTR), and 13-mer (LELR5RYWAIRTR) (Bio Basic INC, ON, Canada). Two days prior to peptide immunization B27/ERAP+/+ and B27/ERAP−/− Tg mice will be subcutaneously injected with CpG (10μg/ml, 50μg per mouse in 1 x PBS). Two days later these mice will be immunized with different peptides (10μg/ml, 75 μg/mouse) in IFA as outlined in Chapters 3 and 4 Material and Methods section. Eleven days post-immunization spleen cells will be removed and tested by ELISpot. Results with B27/ERAP+/+ mice will reveal what is the exact length of the N-terminally extended B27/NP383-391 epitope before it is trimmed by ERAP. Results seen with B27/ERAP−/− splenocytes will act as controls. The identification of the actual length of NP383-391 epitope is important as it may give a hint on the length of the arthritogenic peptide that may be generated by ERAP in AS patients. Ideally we would expect the length of the arthritogenic peptide to be a 9-mer, but it is also possible the arthritogenic epitope first may be generated as an N-terminally extended species which is subsequently trimmed by ERAP. This study aids in the identification of arthritogenic epitope.
5.2.3 Introduction of human ERAP-variants linked to AS to B27/ERAP\textsuperscript{\textminus}\textsuperscript{\textminus} mice. Does the introduction of human ERAP-variants associated with AS (e.g., 30187) rescue the B27-directed T cell response in B27/ERAP\textsuperscript{\textminus}\textsuperscript{\textminus} Tg mice following flu infection?

Studies outlined in Chapter 4 revealed reduced B27/NP383-restricted CTL response following flu infection in B27/ERAP\textsuperscript{\textminus}\textsuperscript{\textminus} mice. Can we rescue this response back to levels seen with B27/ERAP\textsuperscript{\textplus}\textsuperscript{\textplus} Tg mice? I am proposing to introduce human ERAP-variants associated with AS back into ERAP deficient B27 Tg mice. Lentiviral vectors (Santa Cruz, CA) expressing full length human ERAP gene will be inserted into B27/ERAP\textsuperscript{\textminus}\textsuperscript{\textminus} mice as described by Taurog’s group (1990)(351). Briefly, lentiviral constructs expressing genetic variants of the human ERAP gene will be inserted into embryonic stem cell (ES) lines isolated from the inner cell mass of B27/ERAP\textsuperscript{\textminus}\textsuperscript{\textminus} blastocysts. The targeted ESs are subsequently microinjected into host embryos, which are then re-implanted into pseudopregnant foster B27/ERAP\textsuperscript{\textminus}\textsuperscript{\textminus} mothers. Embryos develop chimeric animals which will be tested by PCR (i.e., with B27- and ERAP specific primers described in Chapter 3 and 4) and FACS (i.e., by B27-specific ME1 mAb) to identify animals positive for B27 and human ERAP gene expression. “Rescued” B27/ERAP Tg mice will be infected with flu and 11 days post-infection their splenocytes will be analyzed by ELISpot as described previously. It is expected that the re-introduction of the WT ERAP gene would lead to higher B27/NP383-391 restricted CTL response in these “rescued” B27/ERAP animals. Analysis of gain-of-function variants of ERAP (associated with susceptibility to AS) and of loss-of-function variants (associated with protection against AS) will be analyzed for generation of protective T cell responses to influenza. This experiment would convey the critical point that variants of human ERAP are involved in the generation of the B27-specific flu epitopes in the same way it may be involved in the overall generation of the arthritogenic peptide linked to AS.
5.2.4 Overexpression of ERAP in B27/ERAP\textsuperscript{−/−} Tg mice. Does overexpression of ERAP lead to AS-like symptoms in single Tg B27/ERAP\textsuperscript{−/−} mice?

Rats expressing multiple copies of HLA-B27 and human β2m develop similar symptoms as seen in AS patients (351). However, to date there are no studies looking at the possible role of overexpressed ERAP in relation to AS in mice. Since HLA-B27 Tg mice described here do not exhibit any AS symptoms, overexpression of ERAP would reveal new insights on the role of ERAP to AS in these Tg mice. In order to create overexpressed ERAP we would make use of the Tetracyclin (Tet)/Doxycycline (Dox) on-and-off system. The Tet On-Off Gene Expression System is controlled by a Tet/Dox-inducible vector. Tet-Off system gets activated in the absence of Dox, whereas Tet-On gets activated in the presence of Dox (352). ERAP-expressing lentiviral vector (Santa Cruz, CA) and Tet/Dox vectors will be cut with the same restriction enzyme(s). The cut fragments will be ligated in the presence of ligases to create Tet/Dox-controlled ERAP-expressing vector as per manufacturer’s directions (Clonetech, CA, Cat#PT3001-1). Similar steps as outlined above (section 5.2.4) will be used to generate B27/ERAP\textsuperscript{−/−} Tg mice capable of overexpressing ERAP in the presence of doxycycline (e.g., Dox can be added to the mouse water bottle). Overexpression of ERAP will be examined by western blot of tissues. Subsequently, these Tet/Dox-dependent B27/ERAP\textsuperscript{−/−} mice will be exposed for different time periods (e.g., ranging from 1 month to 24 months) to Dox and the joints will be examined by X-rays while also checking for changes in the peripheral gastrointestinal symptoms (e.g., weight loss, diarrhea). When, and if, joint changes suggestive of AS are evident these animals will be sacrificed and examination of the joints and the gut histology will reveal more about the effects of ERAP over-expression in the mice. This experiment is important to establish that ERAP is involved in the generation and trimming of the arthritogenic peptide. Although the identity of the arthritogenic epitopes are
unknown, the fact that ERAP is involved in antigen processing and presentation would strongly suggest the existence of an arthritogenic epitope in AS patients.

5.2.5. Presence of HLA-B27 homodimers in B27/ERAP\(^{-/-}\) Tg mice. Does absence of ERAP lead to increased homodimerization of B27 molecules?

As discussed previously three different mechanisms have been suggested to contribute to the overall manifestation of AS; i.e., presentation of an arthritogenic epitope, ER stress leading to UPR, and MHC-I homodimerization. Experiments described so far dealt with the presence/generation of an arthritogenic epitope in relation to AS. But this leaves unanswered the possible role of B27 homodimerization in B27/ERAP\(^{-/-}\) and B27/ERAP\(^{+/+}\) Tg mice. To examine whether there is any difference in the expression profile of B27 dimers in these aforementioned Tg mice, the HD6 mAb (a gift from Dr. Paul Boweness, University of Oxford, UK), which specifically binds to B27 homodimers, will be used in FACS experiments to determine the cell surface expression of B27-specific dimers in B27/ERAP\(^{-/-}\) and B27/ERAP\(^{+/+}\) mice. As loss of ERAP function is protective in AS patients, I am expecting to see less B27 homodimers in B27/ERAP\(^{-/-}\) mice compared to B27/ERAP\(^{+/+}\) mice. If this is the case, this would validate the clinical findings with our in vivo animal outcomes.

5.3. Studies of ERAP Expression in B27 Tg Rats

5.3.1. Down regulation of ERAP in the rat AS animal model. Does down regulation of ERAP lead to decreased AS symptoms?
Studies by Hammer et al. (1990)(351) with Lewis rats expressing multiple copies of HLA-B27 and multiple copies of human $\beta_2$m revealed similar features as those seen in AS patients. The unanswered question is, what is the role of ERAP in these rats? Does down regulation of ERAP expression in these rats reduce AS symptoms? To investigate this I would decrease ERAP expression by TALEN (Transcription activator-like effector nucleases) as described (353). The Lewis rats will be examined for AS symptoms following ERAP down-regulation. These results would complement the expected findings described in section 5.2.5 as a result of ERAP overexpression. Based on the results described in Chapter 4, it is expected to see reduced AS symptoms in Lewis rats when ERAP is repressed. These results would complement recent findings by Dr. Matt Brown’s group (personal communication) from the University of Brisbane (Australia) with AS patients indicating that loss of ERAP function is protective.

5.4. Final Conclusion

The experiments proposed here will reveal new insights on the overall role of HLA-B27 and ERAP in relation to influenza infection and AS. Both HLA-B27 and ERAP play important roles in the overall pathogenesis of AS. The results described in the previous chapters could be potentially used as proof of concept to aid in the identification of the arthritogenic epitopes in the future.
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