KIR3DL1 interaction with HLA-B27 is altered by ERAP1 and enhanced by MHC Class I cross-linking

by

Hasan Abdullah

A thesis submitted in conformity with the requirements for the degree of « Master of Science »
« Institute of Medical Science »
University of Toronto

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Abstract

Ankylosing Spondylitis (AS) is an inflammatory arthritis linked to the antigen presenting molecule HLA-B27. The risk of AS is increased in patients possessing endoplasmic reticulum aminopeptidase-1 (ERAP1) polymorphisms, rs30187 and rs27044, encoding amino acid changes K528 and Q730, respectively. Dysfunction of ERAP1 is hypothesized to cause changes in expression of HLA-B27 peptide-HLA trimer (pHLA) and free heavy chain (FHC) ligands, on antigen presenting cells (APCs), which interact with natural killer (NK) cell receptor, KIR3DL1. Dysregulation of this pathway may be pathogenic in AS. APC cell lines expressing HLA-B27 were found to inhibit cytokine production of KIR3DL1+ NK cells due to decreased APC-NK cell adhesion and possibly activating receptor down-regulation. Blocking pHLA and FHC reveals both conformers inhibit cytokine production through KIR3DL1. KIR3DL1 affinity and HLA-B27 surface expression studies suggest ERAP1 R528 and E730 expression protects from AS by generating sub-optimal pHLA, causing reduced KIR3DL1 affinity and weaker cytokine inhibition.
Acknowledgments

This thesis is a comprehensive account of the research I have done during my MSc studies. Independent research is one of the most challenging endeavours of my life and one that must be met with courage, commitment and hard work. As a Lord of the Rings fan, I liken it to the noble quest of going to Mordor to cast the Ring of Power into Mount Doom, but in this particular case my goal was to contribute to our understanding of Ankylosing Spondylitis. Similarly I had to navigate through dark tunnels with occasional shimmering lights here and there to guide me and vast fields with many paths to choose from. Often times the shimmering light is fool’s gold, threatening to lead the wayfarer astray, but the light keeping me on the true path is that of my family members: My mom, who wishes me to be the best in every aspect even more so than I do myself; my dad who always gives me practical advice; my accomplished siblings Shifa, Jamil, and Banna who have great futures ahead of them and motivate me to be a role model. The work published here would not have been possible without their help and guidance as well as that of the individuals mentioned below whom I have had the pleasure of acquaintance and developed friendships with during my studies.

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Contributions

I would like to acknowledge the following people for helping me with technical aspects of my research:

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Dr Debby Burshtyn and Dr Li Fu for contributing the YTS cell lines and technical help with the conjugation assays.

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<td>Ankylosing Spondylitis</td>
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<td>APC</td>
<td>Antigen Presenting Cells</td>
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<tr>
<td>DMARD</td>
<td>Disease modifying antirheumatic drugs</td>
</tr>
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<td>ERAP</td>
<td>Endoplasmic Reticulum Aminopeptidase</td>
</tr>
<tr>
<td>GWAS</td>
<td>Genome Wide Association Study</td>
</tr>
<tr>
<td>FHC</td>
<td>HLA-B27 free heavy chain monomers and dimers</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<tr>
<td>KIR</td>
<td>Killer Immunoglobulin-like receptor</td>
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<td>LILR</td>
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<td>NK</td>
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<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drugs</td>
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<td>pHLA</td>
<td>peptide-HLA trimer</td>
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<td>SNP</td>
<td>Single Nucleotide Polymorphism</td>
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<td>TCR</td>
<td>T-cell Receptor</td>
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Chapter 1 General Introduction

The Role of HLA-B27 and ERAP1 in the pathogenesis of Ankylosing Spondylitis

Ankylosing Spondylitis (AS) is a chronic, inflammatory arthritis that predominantly affects the spinal joints and often times the peripheral joints and entheses as well. Disease progression in AS is variable and is characterized by new bone formation at the vertebral corners that eventually fuse together resulting in a bamboo spine appearance (Schett, Landewe, & van der Heijde, 2007). Pain and restriction of mobility due to inflammation and ankylosis negatively impact physical and social function, as well as mental health, and in general result in a poor quality of life in AS patients (Bodur, Ataman, Rezvani, & Budayci, 2011). There is a gender bias with approximately 2 male patients for every female patient with AS (Weisman, Reveille, & Van Der Heijde, 2006).

The etiology of the disease suggests genetic factors play a prominent role in the pathogenesis of the disease, along with environmental triggers. In particular the antigen presenting molecule HLA-B27 was initially found in much greater frequencies on lymphocytes from individuals in an AS patient cohort compared to healthy controls (Brewerton et al., 1973). Recent Genome Wide Association Studies (GWAS) have revealed that Endoplasmic Reticulum Aminopeptidase 1 (ERAP1) is also strongly associated with AS with the strength of association second only to HLA-B27 (Evans et al., 2011; Haroon & Inman, 2010). The association of ERAP1 with AS is seen only in HLA-B27 positive patients suggesting HLA-B27 and ERAP1 act in concert in the pathogenesis of AS.
1.1 HLA-B27 and ERAP1 in antigen processing and presentation

The purpose of the antigen processing and presentation pathway is to protect the host immune system from harmful pathogens. It consists of two pathways, Major Histocompatibility Class (MHC) I and MHC II pathways that protect from intracellular pathogens and extracellular pathogens respectively. In each case proteolytic pathways process proteins into smaller peptides which are ultimately loaded onto MHC molecules, for example the MHC I molecule HLA-B27, and subsequently recognized by immune cells expressing cognate MHC receptors.

The MHC I pathway starts with the degradation of cytosolic proteins, such as viral or host proteins, by the proteasome. In this pathway proteins present in the cytoplasm are initially targeted to proteasomes which cleave them into smaller peptides (Hammer, Gonzalez, James, Nolla, & Shastri, 2007). The proteasome has a barrel shaped, 20S, core consisting of four stacked rings of seven subunits each, some that are Interferon (IFN)-γ inducible, capped on each end by 19S protein subunits capable of binding ubiquitin-conjugated proteins targeted for degradation(Blum, Wearsch, & Cresswell, 2013). Processed proteins exit the proteasome as peptides ranging 2-25 amino acids in length. Cleaved peptides 8-16 amino acids in length enter the Endoplasmic Reticulum (ER) via the Transporter Associated with Antigen Processing (TAP) (van Endert et al., 1994). TAP forms a peptide loading complex (PLC) in association with proteins such as tapasin, which recruits the MHC I dimer, consisting of the α-heavy chain and β2-microglobulin light chain, as well as the protein folding molecule protein disulfide isomerase (ERp57) and the chaperone calreticulin. The PLC and ER resident chaperones such as calnexin and calreticulin keep the HLA-B27 heavy chain in a stable, β2-microglobulin (β2-m) associated state in the absence of a bound peptide while tapasin brings the unbound complex to the peptide translocated by TAP (Cresswell, Ackerman, Giodini, Peaper, & Wearsch, 2005). Once the proteasome processed peptide enters the ER it is further trimmed to a length of 8-10 amino acids by ER-aminopeptidases such as ERAP1 and loaded onto the HLA-B27 peptide-binding groove (Brouwenstijn, Serwold, & Shastri, 2001). The association of peptide with MHC I creates a MHC I trimer consisting of the α-heavy chain, β2-microglobulin light chain and the aforementioned peptide nestled within the groove formed between the α1 and α2 domains of the
heavy chain (Allen, O'Callaghan, McMichael, & Bowness, 1999; Madden, 1995). Formation of the trimer causes dissociation of the PLC and transport through the Golgi apparatus to the cell surface.

The MHC I molecule HLA-B27 is the earliest known gene associated with AS. It is also the gene with the strongest association with AS with ~90% of patients carrying the gene (Brewerton et al., 1973). The classical role of HLA-B27 is to present cytosolic peptides onto the cell surface as part of the MHC I antigen processing and presenting pathway described above. Fully folded cell surface HLA-B27 can be recognized by cytotoxic CD8+ T-cells with cognate pHLA-B27 specific T-cell Receptors (TCRs) or by Natural Killer (NK) cells with HLA-B27 specific receptors such as Killer Immunoglobulin-like Receptors (KIRs) and Leukocyte Immunoglobulin-like Receptors (LILRs). The interaction of HLA-B27 and cognate receptor bearing immune cells is crucial for inducing an immune response against intracellular pathogens and protecting us from their harmful effects.

The MHC II pathway, unlike MHC I, processes exogenous pathogens into peptides presented by MHC II molecules. MHC II α and β chains associate in the ER with the help of invariant chain (CD74) chaperone protein (Blum et al., 2013). CD74 not only helps folding of MHC II α and β chains but also prevents loading of ER peptides onto folded MHC II molecules. Assembled MHC II is targeted to the endocytic pathway where CD74 is degraded into a 20 amino acid residue class-II-associated invariant chain peptide (CLIP). CLIP is then removed from the MHC II molecule within the endosome by association with a heterodimeric glycoprotein (DM) and is replaced by an antigenic peptide. MHC II bound peptides themselves originate from pathogens internalized by endocytosis, phagocytosis and macropinocytosis. Internalized pathogens are, in general, progressively degraded by proteases within acidic compartments into peptides with high affinity for folded MHC II molecules, therefore replacing CLIP and presenting on the cell surface.
By virtue of the association of HLA-B27 and ERAP1 in AS, the MHC I pathway is also implicated in the pathogenesis of the disease and as such I have focused on it throughout my thesis project.

ERAP1 has been extensively studied in the past and at least two known functions are reported. The first one is to trim cytokine receptors from the cell surface. ERAP1 was initially known as Aminopeptidase Regulating TNFR-I Shedding (ARTS-1). As the name suggests, ERAP1 was found to be involved in the shedding and release of circulating TNFR-I. Subsequently, it was shown that along with TNFR-I it also aids the shedding of IL-6Rα and IL-1R-II. Thus, a defective ERAP1 variant can lead to less shedding and less circulating free receptors. This ultimately leads to an imbalance in the cytokine: receptor ratio and could result in pro-inflammatory effects.

The second function of ERAP1 is in antigen processing. As the name suggest, ERAP1 is an endoplasmic reticulum resident aminopeptidase enzyme that trims the N-terminally extended peptides to be loaded on MHC-I molecules. That it functions as such explains why peptides with proline in P1, P2 or P3 positions can be displayed by MHC I molecules even though they cannot bind TAP (Blanchard & Shastri, 2008). These peptides are initially in an N-terminally extended form and thus able to bind TAP and translocate into the ER. Thus ERAP1 is instrumental in the final shaping of the repertoire of the MHCI-peptide complex. It can be contemplated that abnormal peptides processed by ERAP1 could lead to abnormal antigen presentation by HLA-B27 leading to pathogenic immune response in AS (Figure 1).
Figure 1. ERAP1 affects the generation of stable peptide-HLA-B27 (pHLA) complex and in-turn the formation of FHC. Changes in HLA-B27 expression affect adaptive and innate immune cell response.
1.1.1 ERAP1 structure and function in the context of AS associated polymorphisms

A Genome Wide Association Study (GWAS) conducted in 2007 found the association of ERAP1 with AS from amongst 14,500 non-synonymous Single Nucleotide Polymorphisms (SNPs) analyzed (Burton et al., 2007). The minor allele frequencies at ERAP1 SNPs rs30187 and rs27044 were found to be significantly higher in AS patients compared to controls. A follow up study revealed that ERAP1 is associated with AS only in HLA-B27 positive patients and the association was not found in HLA-B27 negative patients (Evans et al., 2011). Other studies have since identified several ERAP1 SNPs showing genetic background dependent associations with AS but the above mentioned SNPs encoding for the K528R and Q730E amino acid sequence changes respectively are consistently amongst the strongest associations (Bettencourt et al., 2013). Additionally there is evidence from case control studies that certain ERAP1 haplotypes, such as the haplotypes rs27044/10050860/30187-CCT and rs17482078/10050860/30187/2287987-CCTT, confer either protection from or increased risk of AS (Bettencourt et al., 2013; Maksymowych et al., 2009). Our team did a family based genetic study to report an ERAP1-ERAP2 haplotype association with AS (Tsui et al., 2010). This was the first study to implicate ERAP2 in AS.

While the link between HLA-B27 and ERAP1 in AS pathogenesis is well established at the genetic level, there are more diverse theories on the functional association between the two in AS. Functional studies of AS associated ERAP1 mutants have shown that peptide binding affinity and trimming activity is altered depending on the topographical location of the mutations. In particular, the disease-associated K528 and Q730 variants result in highly efficient peptide trimming in comparison with protective 528R and 730E variants (Goto, Hattori, Ishii, & Tsujimoto, 2006). More recently, functional studies of ERAP1 mutants carrying naturally occurring disease associated or protective haplotypes have shown that peptide trimming activity is dependent on the combined mutations rather than mutations at any one site (Evnouchidou et al., 2011). As an example, the relative activity of the R528 variant with the K528 variant is
dependent on the amino acid residue at the 575 position (Evnouchidou et al., 2011; Martin-Esteban, Gomez-Molina, Sanz-Bravo, & Lopez de Castro, 2014). Some mutations resulting in hyperactive ERAP1 function can cause over digestion of the natural HLA-B27 ligand and therefore alter its availability (Evnouchidou et al., 2011; Martin-Esteban et al., 2014). Insight into the structure of ERAP1 and its peptide binding characteristics can offer some explanations on how different ERAP1 polymorphisms affect HLA-B27 expression.

The HLA-B27-dependent association of ERAP1 with AS was recently established by a multi-cohort GWAS study of AS patients and controls (Evans et al., 2011). The study found individuals with the nonsynonymous SNP rs30187 allele T are more likely to develop AS, if they are also positive for HLA-B27. In individuals that are not HLA-B27 positive, the aforementioned ERAP1 SNP does not affect their likelihood of having AS. The HLA-B27 dependent association of ERAP1 with AS is in contrast to the association of genes such as IL-23R, IL12B and KIF21B that are associated with AS regardless of HLA-B27 status. Depending on the patient demographics other ERAP1 SNPs such as rs27044, rs10050860 and rs17482078 are also associated with AS (Burton et al., 2007; Evans et al., 2011; Pazar et al., 2010).

The aforementioned association has led to significant interest in the functional link between ERAP1 and HLA-B27. The ERAP1 gene consists of 20 exons and 19 introns located on chromosome 5 (Hattori, Matsumoto, Mizutani, & Tsujimoto, 2001). Of note exon 2 contains the signal sequence, exons 6 and 7 encode a zinc-binding motif HEXXH(X18)E that is found in many metallopeptidases and is necessary for enzymatic function while the GAMEN motif, also necessary for aminopeptidase function, is encoded in exon 6. The 5’ untranslated region (UTR) flanking the transcription initiation site has transcription factor binding motifs for a variety of proteins, such as MZF-1, IRF1/2, C/EBPα/β, Sp1 and NF-κB, and deletion of these motifs cause loss of transcription. Additionally, it has recently been shown that p53 binding to a response element located in an intron can also regulate ERAP1 mRNA transcription (Wang, Niu, Lai, & Ren, 2013). The mature protein has 941 and 948 amino acid isoforms. Recently the structure of ERAP1 was separately solved using X-ray crystallography by Nguyen et al and Kochan et al (Kochan et al., 2011a; Nguyen et al., 2011). The studies revealed ERAP1 has 4 protein domains where domain I spans amino acids 46-254, domain II 255-527, domain III 528-613 and domain
IV spans 614-941 amino acids. The catalytic site is formed primarily of secondary structures within domain II, but a domain I loop is also shown to bond with substrates within the catalytic site. Within domain II, His353, His357 and Glu376 of the HEXXH(X_{18})E motif coordinate the catalytic Zinc atom. The exo-peptidase function associated GAMEN motif residues are also located in domain II and are shown to interact with substrate peptide. Domains II and IV are oriented relative to each other such that a large cavity is formed, allowing large substrates up to ~16 amino acids, an unusual feature among aminopeptidases, to access the catalytic site in domain II. The S1 site of ERAP1, which accommodates the side chain N-terminal to the peptide bond being cleaved (subsequent sites are referred to as S1’, S2’, etc.), begins near the catalytic Zinc atom site. It is lined with GAMEN (amino acids 317-321) residues that coordinate the N-terminal residue as well as interact with the scissile bond of the peptide substrate, and Gln181 and Glu183 of the D1 loop. This site can comfortably accommodate leucine or methionine as the N-terminal amino acid of the peptide substrate due to the physical and electrostatic characteristics of ERAP1 amino acids mentioned above, providing a physical rationale for ERAP1’s preference for these two amino acids at the N-terminal. Larger amino acids can also be accommodated, but with changes to the orientation of their side chains. The substrate binding cavity, stretching from the Zinc atom into domain IV, is negatively charged but is also lined with shallow pockets, that correspond to sites S1’, S2’, and so on, within the cavity. These sites are presumably responsible for ERAP1 preferring positively charged and hydrophobic peptide residues at specific internal positions (P2, P5, P7 and P8 relative to the N-terminal amino acid because ERAP1 substrate recognition occurs in reference to the N-terminus of the peptide) in the peptide substrate(Evnouchidou et al., 2008). Negatively charged amino acids in any internal position, such as glutamate, reduce ERAP1 trimming. Most notably it may account for ERAP1 strongly preferring positively charged C-terminal residues in bound nonamer (P5: positively charged or aromatic and P7 and P8: positively charged) and decamer peptides. Since the solved x-ray crystallography was of ERAP1 bound to a small substrate, the above comments on the nature of ERAP1 interactions with longer peptides is based on molecular modelling rather than the direct interactions observed with the small substrate.
Figure 2. Schematic of AS associated ERAP1 polymorphisms mapped onto the 3D domain structure of ERAP1 (ref: Structural basis for antigenic peptide precursor processing by the endoplasmic reticulum aminopeptidase ERAP1, Nguyen et al., 2011) (A) ERAP1 consists of 4 domains as indicated in the figure. (B) ERAP1 polymorphisms associated with AS are not found in the catalytic region but rather spread out through different domains affecting enzymatic function by different mechanisms.
ERAP1 exists in a closed conformation that excludes solvent molecules and thus is presumably inaccessible to external peptides as well as an open conformation in which the large cavity formed by domains II and IV is exposed and accessible to substrates. Critical to the transition of ERAP1 from an open to closed structure are the movement of several helices within domain IV. Therefore, in addition to potentially providing C-terminal peptide specificity, domain IV may also be critical for enzyme function by regulating transition from an open to closed conformation and vice versa. The transition between conformations also implicates residues within domain II, in particular Tyr438, which orients towards the active site to participate in amide bond hydrolysis. Experiments studying ERAP1 trimming rates with substrates varying in length show the catalytic site can be allosterically activated by C-terminal peptides. When a fluorogenic dipeptide substrate leucine-7-amido-4-methylcoumarin (L-AMC) is incubated with ERAP1 in vitro, efficient hydrolysis of the dipeptide occurs. However, when increasingly longer peptide substrates up to 8 amino acids in length are added to the reaction, L-AMC hydrolysis is further enhanced. Presumably ERAP1 is able to accommodate both L-AMC and the elongated peptide within the substrate binding cavity and interaction of C-terminal amino acids of the elongated peptide with S’ sites catalyzes amide bond hydrolysis of N-terminal L-AMC. When substrates longer than 8 amino acids are added to the aforementioned reaction L-AMC hydrolysis becomes less efficient because both the peptide substrates can no longer be accommodated within the cavity at the same time and the peptides compete with each other for access to ERAP1. As a result of these experiments, it is proposed that ERAP1 has distinct catalytic and regulatory binding sites in close proximity to each other, presumably on the surface of the substrate binding cavity formed by domains II and IV. In the open conformation, long peptides are able to access the cavity and bind the regulatory site, subsequently causing ERAP1 to change to a closed conformation that allows efficient cleavage at the catalytic site.

Although ERAP1 polymorphisms are associated with AS, it is interesting that the polymorphisms strongly associated with AS, rs30187 and rs27044, which encode amino acids K528 and Q730 respectively, are not key interactors in S1 pocket specificity (Zervoudi et al., 2011). Rather amino acid 528 is at the domain III hinge region between domains II and IV and may be important in ERAP1 conformation change. Amino acid 730 is found in domain IV, which is implicated in ERAP1 preference for peptides with hydrophobic C-terminals and
allosteric activation of the catalytic site. Since SNP rs30187 encodes for an amino acid change in domain III, it is reasonable to expect that changes due to this polymorphism is independent of substrate peptide sequence, whereas rs27044 can be expected to cause peptide sequence dependent changes in ERAP1 activity. In-vitro peptide trimming studies using ERAP1 K528R and Q730E variants have confirmed the variable effect of single amino acid changes at these positions on enzymatic activity (Evnouchidou et al., 2011; Kochan et al., 2011a). Using L-AMC as a substrate, the 528R amino acid change, associated with protection from AS, caused decreased trimming rate compared to the AS associated 528K allele (Evnouchidou et al., 2011). The 730E amino acid change on the other hand caused an increase in L-AMC hydrolysis compared to the AS associate 730Q variant. The variability in association of small peptide trimming rate with AS is not surprising in the context of x-ray crystallography studies showing the 528 amino acid lies in domain III, which is the hinge region, and 730 amino acid lies in the peptide specificity and allosteric activation conferring domain IV. However, in the presence of the activating SIINFEKL peptide, all ERAP1 variants had increased L-AMC hydrolysis. The K528R amino acid change caused the greatest increase in hydrolysis (~4 fold), while the Q730E change caused the least (~1.6 fold). Finally, in a study of various peptides, each variant was shown to have differing activity towards the same nonamer peptide extended by 4 amino acids. In summary, peptide trimming assays have shown AS relevant ERAP1 polymorphisms affect enzymatic function but the mechanism can only be speculated on, such as affinity towards peptide precursors or inherent faults in transitioning between an open and closed state, based on x-ray crystallography structures indicating where the polymorphisms occur.

In order to differentiate between AS-associated and AS-protective ERAP1 polymorphisms, we can utilize a study conducted by Hearn et al. In this study, N-terminally extended precursors to the naturally occurring, HLA-B27 binding SIINFEKL 8-mer was designed and the reactivity of ERAP1 towards the N-terminal amino acid was detected (Hearn, York, & Rock, 2009). As a result they determined the susceptibility of all possible N-terminal amino acids and assigned each a trimming susceptibility score. Using this system, another group has been able to analyze the effect of ERAP1 K528R mutation on endogenous HLA-B*2705 peptide trimming (Sanz-Bravo, Campos, Mazariegos, & LopezdeCastro, 2014). By isolating HLA-B*2705 peptides from two cell lines differing only at ERAP1 528 amino acid, they were able to show that the AS
associated K528 mutation caused the generation of higher molecular weight cell surface peptides, whereas the abundance of such peptides was significantly lower in the cells expressing the AS protective 528R mutation. By scoring the susceptibility of N-terminal amino acid residues, P-2 and P-1 relative to the P1 amino acid (which when cleaved from the precursor generates the HLA-B*2705 binding epitope), as well as the P1 residue to ERAP1 hydrolysis, the study concluded P1 residues in the K528 variant are more resistant to trimming compared to P1 residues in the 528R variant. This is because the K528 variant, already established in previous studies to be more active than 528R, efficiently cleaves susceptible P1 amino acids and as such the resultant epitope is no longer optimal for binding HLA-B*2705. Only those peptide substrates with bulky, ERAP1 resistant P1 peptides are presented by HLA-B*2705 in these cells, in agreement with the theory that peptide presentation is a balance between generation and over-trimming of epitopes by ERAP1 (Garcia-Medel et al., 2012). Conversely, the less active 528R variant is equally inefficient in cleaving susceptible and resistant P1 amino acids, and as such, peptide epitopes presented by HLA-B*2705 in these cells carry both types of P1 residues. This study supports and provides a rationale for the view that ERAP1 polymorphisms at the AS relevant position 528 change the frequency of specific peptides presented by HLA-B*2705 with potential implications for T-cell and NK cell response.

Although the effect of a single polymorphism on ERAP1 function is crucial to our understanding of the enzyme, polymorphisms are also found in the population as “disease-associated” naturally occurring haplotypes (Bettencourt et al., 2013; Kadi et al., 2013). A study was conducted by Reeves et al. investigating the functional effect of naturally occurring ERAP1 haplotypes (Reeves, Edwards, Elliott, & James, 2013). In this study, wild-type, disease-associated ERAP1 haplotype was shown to efficiently trim N-terminally extended precursors of the SIINFEHL antigenic epitope. As a result cells transfected with this wild-type, disease associated, ERAP1 haplotype were able to robustly stimulate cognate TCR expressing cells. In contrast cells expressing AS protective haplotypes were in general unable to generate the SIINFEHL epitope and showed reduced stimulation of cells expressing cognate TCR. In particular, cells expressing only the K528R or the R725Q/Q730E AS protective changes (changes in 725/730 positions are haplotypic) showed decreased TCR stimulation but the K528R change was due to poor trimming of peptide precursors, whereas the R725Q/Q730E change was due to over-trimming of the
peptide precursor leading to antigen destruction. It is important to note, however, that in-vitro studies of 725Q ERAP1 shows reduced trimming of a specific peptide (Evans et al., 2011). The observation that R725Q/Q730E causes over-trimming indicates either the 730 change is dominant to changes in 725 position or that the particular peptide used in the study is conducive to over-trimming. In either case epitope generation is reduced.

Thus far, we have established AS-protective ERAP1 polymorphisms can be differentiated from their wild-type AS associated counterparts based on their activity towards specific peptide precursors. The studies done show this results in reduced HLA-B27 epitope generation in AS protective ERAP1 cells, although the reason for it may be due to inefficient trimming (528R) or due to over-trimming (R725Q/Q730E).

1.1.2 ERAP1 influence on classical HLA-B27 function in AS

The functional changes in ERAP1 due to AS-associated mutations in turn affect TCR-mediated activation, as evidenced in experiments with reporter T-cell lines (Reeves et al., 2013). In an in-vivo study, ERAP1 knockout (ERAP1−/−) and endogenous ERAP1 (ERAP1+/+) mice were immunized with various viruses and the response of CD8+ T-cells isolated from these mice to immunization epitopes was analyzed (York, Brehm, Zendzian, Towne, & Rock, 2006). The study confirmed the frequency of CD8+ T-cell response to the same epitope differs between cells isolated from ERAP1−/− mice and those from ERAP1+/+ mice. Interestingly, cells from ERAP1+/+ mice were less frequently induced by any 8-mer peptide epitopes compared to ERAP1−/−. This observation supports the previously described view that epitope generation is a balance between epitope production and over-trimming. In vitro mass spectrometry studies using mutant ERAP1 cell lines have confirmed that AS relevant ERAP1 mutations indeed change the length and abundance of HLA-B27 ligands, with less active ERAP1 variants generating greater numbers of long peptides (Garcia-Medel et al., 2012). The study by York et al. shows that the lack of ERAP1 changes the HLA-B27 peptide repertoire and elicits a strong CD8+ T-cell response.
Based on the arthritogenic peptide theory of AS pathogenesis, mutations in ERAP1 could generate self-peptides with a particular affinity for HLA-B27, which are subsequently detected by CD8+ T-cell population. Bacterial infection and subsequent presentation of bacterial peptides, which have homology to self-peptides, to HLA-B27 restricted CD8+ T-cells is thought to trigger the expansion of these autoreactive T-cells (Hermann, Yu, Meyer zum Buschenfelde, & Fleischer, 1993). It is possible that mutations in ERAP1 cause altered peptide binding specificity and peptide trimming activity therefore leading to a change in HLA-B27 peptide repertoire, which favors the generation of autoreactive, arthritogenic CD8+ T-cells.

It is important to know if the balance between epitope generation and destruction also causes ERAP1 polymorphism related differences in overall surface HLA-B27 expression as NK cell response is dictated by it. A comparison was done between cells transfected with ERAP1 expressing several AS-associated polymorphisms (ERAP1-high) and cells expressing the opposite AS protective polymorphisms (ERAP1-low) (Seregin et al., 2013). After transfecting these cells with various peptide precursors or mature epitopes, HLA-B27 surface expression was analyzed with a specific antibody (ME1). The study found cells expressing ERAP1-high had reduced HLA-B27 expression, compared to ERAP1-low, regardless of whether the precursor or the epitope peptide had been transfected. The study further established that ERAP1-high destroyed a mature nonamer HLA-B27 binding peptide more efficiently than ERAP1-low and as such reduced surface HLA-B27 expression may be due to over-trimming by ERAP1-high. A different study comparing cell surface H2-Kb expression in mouse spleen cells observed ERAP1−/− mice had reduced H2-Kb compared to mice expressing endogenous ERAP1 (York et al., 2006).

Despite the numerous studies pointing towards the existence of an arthritogenic, B27-binding self-peptide, the peptide itself has not been found. Studies in rat models of spondyloarthritis have also negated the absolute necessity of CD8+ T-cells in arthritis (May et al., 2003). Similarly β2m deficient mice have been shown to develop spontaneous arthritis and thus supporting the view that classical surface HLA-B27 trimers may not be strictly required in spondyloarthritis pathogenesis (Khare et al., 2001).
1.1.3 ERAP1 influence on non-classical HLA-B27 function in AS

Besides its function in the adaptive immune system as a peptide presenting molecule that interacts with TCRs, HLA-B27 could also participate in the innate immune response. HLA-B27 can modulate immune cell effector function by interacting with receptors such as Killer Immunoglobulin-like receptors (KIRs) and Leukocyte Immunoglobulin-like Receptors (LILRs) in a peptide sequence-independent manner. This interaction is particularly important in the missing-self hypothesis of NK cell recognition of aberrant cells. Certain tumor cells and virally infected cells interfere with the antigen presentation pathway, causing down-regulation of MHC I expression and thereby evading TCR mediated recognition (Bubenik, 2005; Petersen, Morris, & Solheim, 2003). However, the missing-self hypothesis postulates that NK cells bearing KIRs and LILRs recognize the absence of surface MHC I and target these aberrant cells for destruction. In the absence of an inhibitory stimulus as a result of MHC I interaction with inhibitory KIRs and LILRs, NK cells are able to carry out cytotoxic effector functions against target cells (Vinay Kumar & Megan E. McNerney, 2005).

An aberrant form of HLA-B27, which consists of two HLA-B27 heavy chains joined together at the Cys67 position by disulfide bonds to form a β2-m free HLA-B27 Free Heavy Chain (FHC) homodimer has also been shown to bind inhibitory KIRs and LILRs in-vitro (Allen, Raine, Haude, Trowsdale, & Wilson, 2001). Elevated levels of FHC have been found in TAP-deficient cell lines which restrict the peptide supply to HLA-B27 molecules and in β2-m knockout mice (Allen et al., 1999). An explanation for this observation is that stable FHC are formed from the dissociation of unstable pHLA-B27 in recycling endosomes and subsequently are transported to the cell surface (Bird et al., 2003). By extension, it can reasonably be expected that any mechanism interfering with the generation of peptides stably associating with HLA-B27 can lead to the formation of elevated levels of surface FHC homodimers. One such molecule which can directly affect the generation of stably binding peptides is ERAP1. If ERAP1 does not trim peptides entering into the ER at an optimal rate, a significant number of ER-resident peptides will not have the ideal length or amino terminal sequence to bind HLA-B27 with high affinity (Haroon, Tsui, Uchanska-Ziegler, Ziegler, & Inman, 2012a). Unstable pHLA-B27 can
subsequently dissociate and form surface FHC. In-vitro peptide trimming assays have shown that ERAP1 variants encoding the K528R and Q730E amino acid changes, which are AS protective, trim peptides at a higher rate than disease-associated ERAP1 variants (Kollnberger et al., 2002). Our studies established the link between ERAP1 peptide trimming and FHC formation in vivo by showing HLA-B27 positive AS patients carrying the ERAP1 Q730 allele have higher FHC on monocytes than their counterparts with the 730E allele (Bowness et al., 2011). The implications of this finding have to be interpreted with caution as it does not support or negate a particular theory over another. If we consider this ERAP1-B27 interaction to be the only pathogenic event and responsible for susceptibility as well, then there are two possibilities. Efficient ERAP1 trimming leads to the generation of more B27-peptide complexes and aberrant immune responses, or lower B27 FHC could lead to higher NK cell activity and pro-inflammatory responses. Another possibility is that the ERAP1-B27 interaction may modify the disease manifestations in a cohort already susceptible to the disease due to HLA-B27. If so, patients with higher FHC could in fact have pro-inflammatory responses due to the triggering of the unfolded protein response (UPR).

Experiments thus far have shown that a majority of inhibitory KIRs and LILRs bind to both stable, peptide associated HLA-B27 and the aberrant FHC, which begs the question: Why would differences in FHC expression be pathogenic in AS? One possibility is that only KIRs and LILRs that only bind to FHC exclusively are pathogenic in AS. In support of this view Kollnberger et al. have shown that a KIR3DL2, which exclusively binds FHC, positive CD4+ T-cell subset which produces IL-17 is stimulated by FHC expressing Antigen Presenting Cells (APCs) to survive and proliferate in spondyloarthritis (SpA) patients (Dangoria et al., 2002; Mear et al., 1999). The above mentioned pathogenesis theory is also consistent with the observation that CD8 + T-cells are not required for arthritis in the rat model.

As described previously, HLA-B27 has free Cys67 residues which can be linked by disulfide bonds to form FHC in the absence of β2m or adequate peptides. Another unusual characteristic of HLA-B27 is its propensity to fold slowly into stable heterotrimeric structures in comparison with other HLA-B alleles (Mear et al., 1999). As such, there accumulates a pool of unfolded or misfolded HLA-B27 heavy chains which can dimerize using the available cysteine residues to
form ER-resident FHC (Dangoria et al., 2002). Accumulation of FHC, and misfolded proteins in general, in the ER activates a three pronged, inter-related ER stress response called the UPR (Todd, Lee, & Glimcher, 2008; Turner, Delay, Bai, Klenk, & Colbert, 2007). ER transmembrane proteins inositol-requiring enzyme 1α (IRE1α), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6) are released from immunoglobulin-heavy-chain-binding protein (BIP) sequestration upon detection of ER stress and activate downstream signaling pathways to scale back the amount of unfolded protein. Of note, IRE1α activation generates X-box binding protein 1 (XBP1) transcription factor, which in turn activates ER-associated protein degradation (ERAD). Independently of XBP1, IRE1α activation also activates autophagy and apoptosis pathways via JUN N-terminal kinase (JNK) activation (Turner et al., 2007). The PERK pathway activates the transcription factor ATF4, which regulates autophagy and apoptosis amongst other functions. Under conditions of ER stress, ATF6 translocates to the Golgi apparatus where it is cleaved into the ATF6f transcription factor responsible for regulating various UPR genes. Another target of UPR is to up regulate BIP chaperone expression in the ER, which improves protein folding capacity. BIP expression, indicating UPR activity, is increased in bone marrow macrophage (BMM)s of HLA-B27 transgenic rats due to the accumulation of intracellular FHC (Turner et al., 2007).

Because ERAP1 activity affects the availability of HLA-B27 ligands it is conceivable that alterations in activity would also affect the levels of misfolded HLA-B27 in the ER. These particular FHC are synthesized by a different mechanism from those observed at the cell surface following endosomal recycling (Bird et al., 2003). In support of the view that ERAP1 activity affects UPR, our in-vitro study has shown suppression of ERAP1 in AS associated HLA-B2704 and HLA-B2705 expressing C1R cell lines results in increased levels of intracellular FHC (Haroon et al., 2012a).
Table I. ERAP1 polymorphisms at amino acids 528 and 730 affect enzymatic function and HLA-B27 epitope generation and in-turn may affect FHC expression.

<table>
<thead>
<tr>
<th>ERAP1 polymorphism</th>
<th>Site of polymorphism</th>
<th>Effect on enzyme function</th>
<th>Effect on B27 epitope</th>
<th>Effect on pHLA</th>
<th>Effect on FHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>R528</td>
<td>Hinge region between DII &amp; DIV</td>
<td>Slow transition between opening and closing</td>
<td>Destruction</td>
<td>Reduce</td>
<td>Increase</td>
</tr>
<tr>
<td>E730</td>
<td>DIV near regulatory site</td>
<td>Over-trimming or under-trimming peptide precursors</td>
<td>Destruction</td>
<td>Reduce</td>
<td>Increase</td>
</tr>
</tbody>
</table>

pHLA: classical peptide bound HLA-B27; FHC: non-classical HLA-B27 heavy chain monomers and dimers.
1.2 KIR3DL1 and AS

The human immune system in essence detects harmful, foreign entities and neutralizes their harmful effects while at the same time allowing host cells to carry out their intended functions. Immune cells of the innate and adaptive (antigen specific) immune system carry out the aforementioned function through various receptor-ligand interactions, and eliminate them in favour of host cells. Cells expressing MHC I ligands interact with innate immune cells such as natural killer (NK) cells through killer immunoglobulin-like receptors (KIR), CD94-NKG2 and leukocyte immunoglobulin-like receptors (LILR), while a specific subset of T-cells expressing T-cell receptors (TCR) and the CD8 co-stimulatory molecule also interact with MHC I molecules. The interaction of MHC I with NK cell receptors differs from that of TCRs in that the former generally induces a bi-modal (on/off) response, dependent on the identity of the interacting MHC I but, with some exceptions, unaffected by the sequence of peptides bound to MHC I, while the latter induces a graded response dependent on the affinity of the TCR for specific MHC I-peptide combinations.

In AS, certain immune cell types have been implicated directly and indirectly compared to healthy subjects. Studies have shown CD4+ Th1 and Th17 subsets of T-cells, which predominantly secrete proinflammatory cytokines IFN-γ and IL-17 respectively, are higher in patient peripheral blood mononuclear cells (PBMC) compared to controls (LIMÓN-CAMACHO et al., 2012). In the same study, cytokines IL-6, IL-17, TNF-α and IL-8 were significantly upregulated in patient serum compared to controls. The implication of CD4+ T-cells in AS is intriguing considering the strong association of HLA-B27, which binds to TCRs on CD8+ T-cells, with AS. A separate study of AS patients and controls revealed CD4+ T-cells and NK cells expressing KIR3DL2 are also upregulated in the peripheral blood and synovial fluid of AS patients (A. T. Chan, Kollnberger, Wedderburn, & Bowness, 2005). Similarly, AS patient PBMCs have increased numbers of CD3- CD56+ NK cells compared to controls (Azuz-Lieberman et al., 2005). However, despite the increased number of NK cells in AS patients cytotoxicity against HLA-B27 expressing target cells did not differ between AS patients and controls due to similar expression levels of KIR3DL1. In a different study, analysis of KIR3DL1
allele expression showed only highly expressed KIR3DL1 variants are decreased in AS patients, while the activating KIR3DL1 allele, KIR3DS1, is increased (Zvyagin et al., 2010).

Given the association of HLA-B27 with AS, the imbalance of CD4+ T-cells and NK cells, but not CD8+ T-cells, in patients suggests NK cells and CD4+ T-cells interacting with HLA-B27 influence the pathogenesis of AS. Furthermore, there are indications that variable expression of NK cell receptors such as KIR3DL1 may be responsible for altered NK cell function and play a role in the pathogenesis of AS.

![Diagram of KIR expressing effector immune cell interaction with pHLA/B27 APCs]

**Figure 3.** KIR expressing effector immune cell interaction with pHLA/B27 APCs can cause inhibition of IFN-γ and pro-inflammatory cytokines and as such is protective in AS (Model 1) or is pathogenic in AS by increasing survival and expansion of effector cells and resultant up-regulation IFN-γ and pro-inflammatory cytokines in the blood stream (Model 2).

Unlike TCRs, multiple KIRs with different MHC I ligand specificities can be expressed in a particular NK cell. The expression or non-expression of a specific KIR, with the exception of
KIR2DL4, which is expressed in all mature NK cells, is a stochastic process during NK cell development and as such mature NK cells can be subdivided into clones expressing different combinations of KIRs. It appears KIRs are sequentially expressed in developing NK cells until they encounter cells expressing cognate, self-reactive, inhibitory MHC I (Li, Pascal, Martin, Carrington, & Anderson, 2008). According to the NK cell licensing-theory, fully mature NK cells also require interaction with cognate MHC I during the development process in order to function properly. Maintenance of KIR expression in mature NK cell clones is regulated by DNA methylation at promoter sites, hypo-methylation inducing KIR expression while heavy methylation silences KIR expression (H. W. Chan et al., 2003; Presnell, Chan, Zhang, & Lutz, 2013). KIR3DL1 has a distal promoter, which synthesizes the sense transcript, as well as a proximal, bi-directional promoter that synthesizes both sense and anti-sense transcripts. The balance between sense and anti-sense transcripts synthesized due to the proximal promoter determines KIR3DL1 expression frequency in NK cells and is affected by SNPs in YY1 and Sp1 transcription factor binding sites within the promoter (Li et al., 2008). Additionally IL-2/IL-15 driven STAT5 expression has been shown to be important in stimulating KIR3DL1 reverse promoter activity and as hypothesized to maintain KIR3DL1 expression in proliferating cells (Presnell et al., 2013). In the context of AS it is important to know the determinants of KIR3DL1 expression as only the highly expressed allele KIR3DL1*001 is associated with AS. As such differences in regulation of KIR3DL1 expression might form an important aspect of the AS pathogenicity model.

**Figure 4. KIR3DL1 expression is primarily controlled by the proximal, bi-directional promoter.** The distal KIR3DL1 promoter activates sense mRNA transcription whereas the proximal promoter also transcribes anti-sense mRNA. Proximal promoter polymorphisms affect the balance between sense and anti-sense transcription and are associated with allelic differences in KIR3DL1. IL-2/IL-15 activation of STAT5 on the other hand is key in maintaining KIR3DL1 expression in proliferating cells.
1.2.1 KIR3DL1 structure and classical HLA-B27 interaction

KIR3DL1 is an inhibitory receptor within the killer cell immunoglobulin-like receptor (KIR) family that binds MHC I proteins with the Bw4 epitope at amino acids 77, 80-83 (NIALR) (Sanjanwala, Draghi, Norman, Guethlein, & Parham, 2008). Its protein structure consists of an extracellular portion with three immunoglobulin domains, D0, D1 and D2 that bind to MHC I molecules, as well as a transmembrane domain and a long intracellular signaling domain (Vivian et al., 2011). X-ray crystallography of KIR3DL1 complexed with peptide-loaded HLA-B*5701 shows all 3 extracellular immunoglobulin domains interact with MHC I. The D0 domain, consisting of amino acids 7-98, binds on top of the HLA-B*5701 antigen binding cleft at a site discontinuous from that of the D1-D2 domains and extends towards the β2m molecule and barely contacting it. On the other hand the D1 and D2 domains, comprising amino acids 99-198 and 203-292 respectively, clamp around the carboxy-terminal of the HLA-B*5701 antigen binding cleft. The interaction between KIR and MHC I molecules is proposed to be based on charge complementarity between D1/D2 and specific MHC I residues. Functional HLA-B interaction with KIR3DL1 is not determined by D0 but is however enhanced by its presence (Khakoo, Geller, Shin, Jenkins, & Parham, 2002). The D1 domain Leucine 166 residue was the only KIR3DL1 amino acid to directly interact with the MHC I bound peptide, doing so at position P8. Despite the view that KIR interaction with HLA is independent of bound peptide sequence, the importance of the identity of amino acid P8 in KIR3DL1 binding to HLA-B*2705 was confirmed using HLA-B*2705-peptide tetramers to stain KIR3DL1 expressing cells. The study found threonine is preferred at the P8 residue and naturally occurring EBV peptides which express P8 glutamate do not bind to KIR3DL1 (Stewart-Jones et al., 2005). A separate study showed KIR3DL1 tolerates histidine, alanine and valine at P8 but does not tolerate negatively charged amino acids at either P8 or P7 of nonamer peptides bound to HLA-B*2705 (Peruzzi, Parker, Long, & Malnati, 1996). Interestingly, it also strongly binds an 11 amino acid long natural HLA-B*2705 peptide. The P2 and P9 anchor residues critical for peptide binding to HLA-B do not affect KIR3DL1 binding.
Figure 5. Model of interactions between KIR3DL1 and HLA-B*5701 residues (ref: Vivian et al. (2011) Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B). (A) KIR3DL1 D0, D1 and D2 domains are shown in yellow, blue and orange colours respectively. HLA-B*5701 (green) with nonamer peptide (purple) and β2m (grey) are shown bound to KIR3DL1. (B) The footprint of KIR3DL1 domains D0 (yellow), D1 (blue), D1-D2 loop (pink) and D2 (orange) and both the loop and D2 (brown) are mapped onto HLA-B*5701 and interacting amino acids indicated. (C) KIR3DL1*001 domains coloured as mentioned above and amino acids interacting with HLA-B*5701 residues are indicated.

Table II. P7 and P8 peptide amino acids affect KIR3DL1 interaction with HLA-B*2705.

<table>
<thead>
<tr>
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<td>High</td>
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<tr>
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</tr>
</tbody>
</table>
1.2.2 KIR3DL1 and non-classical HLA-B27 interaction

KIR3DL1 also binds aberrant HLA-B27 free heavy chain homodimers. Some unique properties of HLA-B27 allow the formation of homodimers. In general the MHC I peptide-binding groove is lined with ‘pockets’ that accommodate and interact with peptide amino acid side chains. In HLA-B27 the P2-binding pocket (pocket B) contains an unpaired cysteine 67 residue that is absent, for example, in HLA-A2 and HLA-B7. The B pocket in HLA-B*2705 was also shown experimentally to reduce peptide loading efficiency compared to the B pocket in HLA-A2 (Mear et al., 1999). Inefficient peptide loading was linked to relatively slow folding of the heavy chain into mature HLA-B*2705 molecules and a tendency to misfold. Later studies showed these free heavy chains can pair via cysteine 67 in the B pocket to form FHC. FHC are found in both the intracellular compartment and cell surface membrane of HLA-B27 expressing cell lines. The FHC molecules in these two cell fractions arise from different sources as experiments show formation of cell surface FHC requires β2-microglobulin and display ER-glycosylation suggesting they originate from mature, peptide-bound cell surface HLA-B27 molecules (Bird et al., 2003). Surface HLA-B27 molecules are endocytosed and subsequently pair via cysteine 67 and recycled to the cell surface as FHC. Exogenous addition of high affinity B27 binding peptides reduces FHC and reconstitutes HLA-B27 heterotrimers. This suggests if HLA-B27 epitopes are destroyed endogenously they may contribute to the formation of FHC (McHugh et al., 2014). KIR3DL1, amongst other NK cell receptors, have been shown to bind cell surface FHC (Allen et al., 2001).
Figure 6. FHC is formed from sub-optimal pHLA complexes. ERAP1 variants can sub-optimally trim N-terminally extended peptide precursors thereby generating unstable pHLA or causing ER accumulation and dimerization of free heavy chains. Unstable cell surface pHLA is endocytosed and recycled back to the cell surface as FHC.
1.2.3 KIR3DL1 influence on NK cell function

KIR3DL1 binding to cognate HLA-B27 ligands initiates a signal cascade that is dependent on the transmembrane and intracellular domain, which influences NK cell function. The KIR3DL1 transmembrane region is a generally conserved stretch of amino acids between 320 to 340 of the mature protein (Campbell & Purdy, 2011). There are some naturally occurring polymorphisms within this region that affect HLA-Bw4 binding. Notably isoleucine at position 320 in 3DL1*002, is substituted for valine in 3DL1*007, and is implicated in HLA-B*2705 binding (Carr, Pando, & Parham, 2005). When isoleucine is substituted for valine in 3DL1*007, inhibitory function in response to HLA-B*2705 is potentiated. Although the mechanism of potentiation is not clear it is hypothesized that this polymorphism may be responsible for homotypic interaction between KIR3DL1 molecules. KIR homotypic interaction and clustering is observed immediately after ligand binding and precedes downstream recruitment of signaling molecules. Ligand binding and receptor clustering is sufficient to induce phosphorylation of KIR independent of cytoplasmic signaling and as such is an early event in KIR function (Faure, Barber, Takahashi, Jin, & Long, 2003).
Figure 7. Schematic of KIR3DL1 inhibiting 2B4 mediated NK cell activation. KIR3DL1 binding to HLA-B27 induces downstream signaling through SHP-1 and prevents recruitment of 2B4 to lipid rafts thereby preventing 2B4 activation functions such as LFA-1 activation and IFN-γ secretion. Additionally, inhibitory signaling can directly inhibit LFA-1 activation to prevent NK cell adhesion.
The cytoplasmic domain of KIR3DL1 consists of 42 amino acids responsible for recruiting signal transduction proteins upon binding of KIR ligands. Inhibitory KIR cytoplasmic domains contain immunoreceptor tyrosine-based inhibition motifs (ITIM), defined as V/I/LxYxxL/V, that contain a single tyrosine residue which is phosphorylated upon receptor-ligand engagement. Most inhibitory KIR receptors, including KIR3DL1, contain two ITIM motifs receptive to phosphorylation although KIR2DL4, which has inhibitory and activating functions, has just one ITIM (Yusa, Catina, & Campbell, 2002). As mentioned previously, receptor-ligand binding is enough to induce phosphorylation of ITIMs. When both ITIMs are phosphorylated Src homology 2 (SH2) domain-containing protein tyrosine phosphatase-1 (SHP-1) is recruited to the cytoplasmic domain whereas SHP-2 can be recruited upon phosphorylation of a single ITIM (MacFarlane & Campbell, 2006). SHP-1 and SHP-2 can subsequently dephosphorylate tyrosines in activation receptors and shut down receptor-mediated NK cell activation.

KIR-mediated inhibition targets early events in NK cell activation. Initially NK cells in contact with susceptible target cells form a contact area termed the NK immune synapse (NKIS). Activating receptors, adhesion and signaling molecules aggregate in the NKIS to form a central supramolecular activation cluster (c-SMAC). Adhesion molecules such as LFA-1, which binds ICAM-1 expressed on the target cell, and receptors inducing high affinity LFA-1 conformation by inside-out signaling such as 2B4, which binds CD48 on the target cell, accumulate in rings around the periphery of the c-SMAC, and upon ligand binding strongly adhere to NK cells (Hoffmann, Cohnen, Ludwig, & Watzl, 2011). NK cell-target cell adhesion is a rapid event with firm adhesion established within 2-5 minutes of initial contact in the absence of inhibitory signaling. 2B4 is shown to enhance actin-dependent and LFA-1-mediated adhesion within 90 seconds of ligand binding.

NK cell signaling molecules such as protein tyrosine kinases (PTK), G-proteins, adaptor proteins and phosphoinositides, along with downstream signaling molecules like SHP-1, Lck, phospholipase C (PLC), Vav, Zap70 and Syk exist in lipid rafts found within the c-SMAC (Lou, Jevremovic, Billadeau, & Leibson, 2000). Clustering of receptors and signaling molecules within
these compartments allows close association with second messengers required to transduce cell surface interactions. Upon NK cell contact with sensitive targets and adhesion, lipid rafts polarize to the contact surface and aggregate as a necessary step preceding activation of signal cascades. KIR inhibition targets both the initial LFA-1 mediated adhesion of NK cells with target cells as well as the redistribution of lipid rafts towards the site of NK cell-target cell contact.

As mentioned previously, KIR-ligand binding induces ITIM phosphorylation and SHP-1 recruitment. Experimental evidence suggests that the aforementioned pathway is important in inhibiting the early NK cell activation event of lipid raft polarization. When KIR3DL1 expressing NK cell clones were co-cultured with MHC expressing sensitive target cells, raft polarization towards the NKIS was inhibited. Furthermore, it was shown using catalytically inactive SHP-1 expressing cells that lipid raft polarization is inhibited only in KIR3DL1 NK cells that express functional SHP-1 (Lou et al., 2000). Presumably, inhibitory KIR recruits SHP-1, which then deactivates signaling proteins required for lipid raft polarization and by extension propagation of activating signals dependent on raft polarization. Despite the necessity of SHP-1 in inhibitory KIR function, only two signal transduction proteins have been shown to directly interact with it, namely: SLP-76 and Vav1 (Binstadt et al., 1998; Stebbins et al., 2003). In the specific case of 2B4 activation in NK cells, lipid raft polarization is essential for 2B4 phosphorylation and downstream activating signaling. Inhibitory KIR not only blocks lipid raft polarization, but also indirectly blocks initial recruitment of 2B4 receptors to the lipid raft by interfering with actin polymerization and as such, prevents 2B4 phosphorylation (Eissmann et al., 2005; Watzl & Long, 2003). In summary, one of the strategies of KIR inhibition is to reduce initial NK cell contact with sensitive targets and prevent contact-dependent NK cell activation.

No known KIR3DL1 polymorphisms that affect HLA-B27 binding have been implicated in AS. However, the inhibitory KIR3DL1*001 allele with highest KIR3DL1 cell surface expression is less frequent in AS patients compared to controls (Zvyagin et al., 2010). The same study found the highest risk of AS is attributed to the activating KIR3DS1 receptor which segregates as a KIR3DL1 allele and is more frequent in AS patients. The role of KIR3DL1 in AS pathogenesis therefore does not seem to be directly due to HLA-B27 binding characteristics, although the
effect of AS-associated HLA-B27 polymorphisms on KIR3DL1 binding has not been studied, but rather due to differences in KIR3DL1 surface expression. It is possible that differences in transcription factor binding, as mentioned in the introduction of this chapter, is responsible for the differences in cell surface expression.

### 1.3 Research aims and hypothesis

As mentioned in the introductory review, there are 3 AS pathogenic mechanisms that tie together ERAP1 function and HLA-B27. The arthritogenic peptide theory hypothesizes there exists a pool of autoreactive T-cells in the body that are specific for self-peptides bound to HLA-B27. When an exogenous pathogen infects the host, the immune system generates pathogenic peptides identical to the host self-peptides. HLA-B27 presentation of these peptides to autoreactive T-cells cause clonal expansion and a strong inflammatory response to both host and pathogenic antigens. It is possible AS-associated ERAP1 polymorphisms aid in the development of autoreactive T-cells as well as the generation of pathogenic peptides that trigger the inflammatory response. For example, an AS-associated ERAP1 polymorphism may under-trim or over-trim a peptide originating from a pathogen. This may change the peptide so that it now resembles a self-peptide and trigger an autoreactive T-cell response. However, studies in rat models of arthritis show that CD8+ T-cells are not necessary for developing arthritis (May et al., 2003) Furthermore, despite comprehensive studies, an arthritogenic peptide as described above has not been found.

The second AS pathogenic mechanism that ties together HLA-B27 and ERAP1 is dependent on the unfolded protein response (UPR). Because HLA-B27 has a propensity to fold slowly it can remain misfolded or dimerize into FHC and accumulate in the ER. If AS-pathogenic ERAP1 generates suboptimal peptides due to under-trimming or over-trimming, there would be a restriction in peptide supply to folding HLA-B27 molecules in the ER. Restriction of peptide supply would hypothetically increase unfolded proteins in the ER. This triggers UPR sensors
such as IRE1, PERK and ATF6. IRE1 activation causes splicing of XBP1, which is required for induction of pro-inflammatory cytokines, and triggers downstream activation of apoptosis signal-regulating kinase 1 (ASK1) and JUN N-terminal kinase (JNK) (Hetz, Chevet, & Harding, 2013). PERK activation causes expression of genes involved in apoptosis, while ATF6 regulates the ER-associated degradation (ERAD) pathway genes. As such, the combination of AS-associated ERAP1 polymorphisms and HLA-B27 can boost the inflammatory response and cause AS.

Alternatively, cell surface FHC can act as ligands for NK cell receptors, such as KIR3DL1 and KIR3DL2, to skew inflammatory responses. In fact, KIR3DL2 is expressed in CD4+ T-cells, so it is not exclusive to NK cells, and is upregulated in AS patient peripheral blood and synovial fluid (Bowness et al., 2011). In addition, these cells have been shown to interact with FHC causing increased survival and proliferation. Similarly, KIR3DL2 expressing NK cells are also increased in number in AS patient peripheral blood and synovial fluid, possibly due to interaction with FHC (A. T. Chan et al., 2005). Other studies have shown KIR3DL1*001 and KIR3DS1 are downregulated and upregulated respectively in AS patients compared to controls (Zvyagin et al., 2010). The question arises: How can ERAP1 and HLA-B27 combine to influence immune function in KIR expressing cells? Answering this question is the objective of this thesis project.

As we know from our background information the impact of ERAP1 polymorphisms on enzyme function is varied. Some polymorphisms such as K528R affect the transition of ERAP1 between close and open states, thereby altering peptide trimming. Others such as Q730E are affected by the amino acids binding to its regulatory site and as such peptide supply to the ER might affect its enzymatic function. In either case, there exists the possibility that polymorphisms affect the generation of naturally found HLA-B27-binding epitopes either by under-trimming or over-trimming peptide precursors. As such, peptide supply to HLA-B27 may be restricted. Research has shown that suboptimal HLA-B27-peptide complexes may be endocytosed and recycled to the cell surface as FHC. As such ERAP1 polymorphisms may directly influence the generation of FHC because of epitope destruction. In fact ERAP1−/− mice have been shown to have decreased
surface HLA-B27. Cells expressing AS-associated ERAP1, with efficient trimming activity, have lower peptide bound HLA-B27 and, although not noted by the authors, higher cell surface FHC, than cells expressing ERAP1 polymorphisms protecting from AS (Seregin et al., 2013). AS patient monocytes expressing the AS-associated ERAP1 Q730 allele have higher FHC than those expressing 730E (Haroon, Tsui, Uchanska-Ziegler, Ziegler, & Inman, 2012b). Altered cell surface expression of FHC, in turn, can alter the function of immune cells expressing KIR receptors.

My hypothesis is that ERAP1 SNPs associated with differential enzymatic activity influence cell surface expression of both peptide-bound HLA-B27 (pHLA) and FHC. The two polymorphisms studied, K528R and Q730E, affect enzymatic activity via different mechanisms, but by changing cell surface expression of HLA-B27 ligands will also affect KIR interaction and function. Hypothetically, AS-associated polymorphisms can be distinguished from AS-protective polymorphisms in how they modulate KIR3DL1 NK cell function, but this hypothesis has not been studied.

In this project, I have:

1.) Investigated the effect of the aforementioned polymorphisms on IFN-γ production by NK cells expressing KIR3DL1, and the individual contributions of pHLA and FHC on KIR3DL1+ NK cell function.

2.) Linked the changes in NK cell function to changes in NK cell – target cell adhesion.

3.) Investigated the effect of ERAP1 polymorphisms on pHLA and FHC expression, and correlated them with changes in NK cell – target cell adhesion and differences in NK cell function.
Figure 8. Hypothetical relationship between ERAP1 activity, HLA-B27 conformer expression and KIR3DL1 function in the pathogenesis of AS. Changes in ERAP1 activity due to protective polymorphisms can change overall pHLA expression or destroy epitopes specific to KIR3DL1. Hypothetically high pHLA/FHC can lead to increased KIR3DL1 interaction but the presentation of sub-optimal KIR3DL1 binding peptides can cause the contrary.
Chapter 2
KIR3DL1 inhibition of IFN-γ production in response to AS protective ERAP1 target cells is significantly reversed by blocking pHLA and FHC

Introduction

NK cells are a vital in innate immune functions as well as modulating adapting immune function. Following exogenous challenge to the immune system due to infection, NK cells can migrate to sites of infection, such as draining lymph nodes, and interact with APCs. APCs such as dendritic cells present antigenic peptides derived from infecting pathogens, complexed with MHC class I molecules, as well as other MHC independent, ligand expression on their cell surface. In particular the KIR and LILR family of receptors interact with peptide bound MHC class I whereas some examples of MHC independent receptors include 2B4 and NKG2D (Lanier, 2008). NK cell contact with APCs triggers both activating and inhibitory receptors on the NK cell surface. The balance between activating and inhibiting stimulus determines the fate of the interacting APC, by triggering or inhibiting NK cell effector function. Some examples of effector functions include pro-inflammatory cytokine secretion, such as IFN-γ, or lysis inducing secretions such as perforin and granzyme release.

MHC class I interaction with KIR receptors is known to modulate NK cell functions such as cytokine secretion and cell survival. In particular, inhibitory KIRs interact with self-MHC class I molecules expressed on APCs to skew the balance between activating and inhibiting signals in NK cells towards inhibition. This mechanism, termed the ‘missing-self hypothesis’, protects host cells expressing self-MHC from autoimmune destruction (Long, Kim, Liu, Peterson, & Rajagopalan, 2013). Some pathogens, such as viruses, have evolved to down-regulate MHC molecules, in order to avoid detection by T-cells expressing cognate TCRs. NK cells can detect the down-regulation as an imbalance between activating and inhibitory signaling, as described above, and destroy these cells, which would otherwise avoid detection by the immune system. As such changes in MHC class I can affect KIR interaction and downstream cytokine secretion.
ERAP1, as described previously, is an ER-resident protein which cleaves peptides prior to loading on to MHC class I molecules. The absence of ERAP1 has been shown to decrease the expression of self-MHC class I molecules on the surface of APCs (York et al., 2006). Because ERAP1 polymorphisms have been shown to affect HLA-B27 peptide epitope generation and cell surface expression, both in its canonical pHLA and non-canonical FHC forms, it is plausible that cells expressing these polymorphisms differentially interact with inhibitory KIR3DL1+ NK cells and affect cytokine production.

The aim of the experiments in this chapter was to identify cytokines inhibited by the interaction of KIR3DL1+ NK cells with cognate, HLA-B27 expressing APCs. Secondarily, the effect of AS associated ERAP1 polymorphisms expressed in HLA-B27+ APCs, in the aforementioned interaction was also assessed. To this end, a cytokine expression screen was performed on supernatants from NK cell and HLA-B2705/ERAP1 target cell co-cultures to identify cytokines affected by KIR3DL1 expression. Further analysis was done using intracellular cytokine staining of NK cells to identify the role of HLA-B27 conformers, pHLA and FHC, in modulating KIR3DL1+ NK cell function, in accordance with aim 1 mentioned in page 32. Hypothetically, both pHLA and FHC should interact with KIR3DL1 to inhibit NK cell cytokine secretion. Also, ERAP1 polymorphisms that increase pHLA and FHC should cause further KIR3DL1 inhibition.

2.1 Methods

2.1.1 Cell lines

C1R-B*2705, a TAP-sufficient human B lymphocyte cell line which expresses endogenous MHC Class I at low levels but HLA-B*2705 at a high level was a kind gift from J López de Castro (Madrid, Spain) C1R-B*2705 cells doubly transfected with GFP-ERAP1 shRNA construct and AS protective ERAP1 constructs were maintained in RPM1640 containing 10% FBS, L-glutamine (2mM), geneticin (0.55 μg/ml), and 5% CO2. The human NK cell lines YTS,
which does not express KIR, and YTS-KIR3DL1, which expresses KIR3DL1*001 were kindly
gifted by Dr. Burshtyn (Edmonton, Canada). YTS was maintained in IMDM containing 15%
HyClone characterized FBS, 50µM 2-mercaptoethanol and L-glutamine (2mM). YTS-KIR3DL1
was maintained in the same media supplemented with puromycin (1µg/ml).

2.1.2 Antibody blocking

For antibody blocking studies, C1R cells were washed with 1% BSA and 1×10^6 cells blocked
with saturating amounts of hybridoma supernatant containing antibody against pHLA (ME1
antibody) and MHC-Class I free heavy chains (FHC/B27; HC10 antibody), or monoclonal
antibody against pan-MHC class I molecules (W6/32, Biolegend) for 1 hour at room temperature
prior to use in conjugation and intracellular cytokine staining assays.

2.1.3 Statistical analysis

Paired comparisons between KIR3DL1 vs non-KIR3DL1, wild-type ERAP1 vs AS protective
ERAP1, ME1 vs HC10 vs W6/32 or vs IgG isotype control conditions, were made to analyze the
effect of KIR3DL1, ERAP1 polymorphisms and specific HLA-B27 conformers. Two-tailed
paired t-tests were performed on data obtained from experiments using the GraphPad Prism 5
software.

2.1.4 NK cell co-culture supernatant assay

1×10^6 ERAP1 cells were resuspended in cell culture medium and either stimulated in 200 U/ml
recombinant human IFN-γ or left un-stimulated overnight at 37°C and 5% CO₂. Cells were
subsequently washed and co-cultured with YTS or YTS-3DL1 NK cells at a 1:1 ratio in 96 well
plates overnight at 37°C and 5% CO₂. Co-culture supernatants were analyzed for cytokine
expression by multiplex capture antibody coupled fluorescent bead assay (Eve Technologies, Calgary, Alberta).

2.1.5 Intracellular cytokine staining

NK cells and C1R target cells were washed in PBS and co-cultured at a 1:5 ratio in 96 well u-bottom plates at a final volume of 200 µl in YTS medium. After 3 hours of co-culture at 37°C and 5% CO₂ cells were treated with GolgiStop (BD Biosciences, Mississauga, Canada) at the recommended concentration for 6 hours to allow intracellular accumulation of cytokines. After co-culture cells were washed in PBS and stained with Zombie NIR fixable viability dye. Subsequently cells were surface stained for the NK cell marker CD56 (PerCP/Cy5.5 anti-human CD56, clone HCD56 from Biolegend) for 20 minutes and then permeabilized and fixed with the Cytofix/Cytoperm kit (BD Biosciences, Mississauga, Canada) in preparation for intracellular staining. Cells were incubated with APC anti-human IFN-γ (clone 4S.B3) and PE anti-human MIP1-α (clone 93342, R &D systems) for 30 minutes followed by washing as per BD instructions and resuspension in FACS buffer. Intracellular cytokine production was assessed using the BD LSR II flowcytometer. Gates were set using FMO controls.

2.2 Results

2.2.1 Screen of NK cell-target cell co-culture supernatants reveal IFN-γ and MIP-1α are significantly inhibited by KIR3DL1

A cytokine secretion screen was performed on supernatants from C1R-B27/NK cell co-cultures in order to select cytokines inhibited by KIR3DL1. Co-cultures were done with un-stimulated and IFN-γ stimulated wild-type ERAP1 cells. 12 cytokines were produced in the 10-100 pg/ml range, 4 in the 101-1000 pg/ml range and 7 were produced in amounts greater than 1000 pg/ml (Table III). For the purposes of this project, we selected those cytokines produced in amounts greater than 1000 pg/ml that exhibited the greatest difference between YTS and KIR3DL1 co-
cultures and which are potentially relevant to the pathogenesis of AS. As a result, IFN-γ and MIP-1α, both important NK cell cytokines, were chosen for further study. Interestingly, IFN-γ dysregulation has been implicated in AS and MIP-1α has been shown to significantly change with TNF inhibitor therapy in AS (Smith et al., 2008) (Akbulut H et al. PMID: 20439531).

Table III. Fold down-regulation of cytokine production in the presence of KIR-3DL1 compared to YTS cells without KIR-3DL1 when co-cultured with C1R-B27 cells.

<table>
<thead>
<tr>
<th>Cytokine produced</th>
<th>10-100 pg/ml cytokine</th>
<th>101-1000 pg/ml cytokine</th>
<th>&gt;1000 pg/ml cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No IFN-γ</td>
<td>IFN-γ</td>
<td>No IFN-γ</td>
</tr>
<tr>
<td>FGF-2</td>
<td>0.72</td>
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<td>0.41</td>
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<td>0.86</td>
<td>0.73</td>
<td>1.04</td>
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<tr>
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2.2.2 Blocking pHLA and FHC significantly reverses IFN-γ inhibition in KIR3DL1+ NK cells

Given the results of the cytokine-screening assay, I decided to analyze IFN-γ and MIP-1α production in greater detail by flow-cytometry. After blocking C1R-B27 target cells with saturating amounts of isotype control, ME1 or HC10 antibodies, I co-cultured them with NK cells for 4 hours and inhibited cytokine secretion for another 6 hours using monensin. Blocking with HLA-B27 conformation specific antibodies ME1 (anti-pHLA) and HC10 (anti-FHC) allows us to measure the individual contributions of pHLA and FHC towards KIR3DL1 inhibition. Cells were subsequently surface stained with a fixable live-dead marker and an NK cell marker followed by permeabilization and intracellular staining with antibodies against IFN-γ and MIP-1α. The flow-cytometry gating strategy is illustrated in Figure 9.

Figure 9. Gating strategy for intracellular cytokine staining assay. (A) Stained NK cell-target cell co-cultures were first gated using FSC-A and SSC-A parameters to exclude debris. (B) Doublet cells were excluded using FSC-A vs FSC-H parameters. (C) Live cells were identified as an APC-Cy7 live/dead marker negative population. (D) NK cells were identified as CD56+PercP-Cy5.5 positive population. (E) IFN-γ positive cells were identified as APC positive events. (F) MIP-1α positive cells were identified as PE positive events.
Intracellular staining of NK cell/wild-type ERAP1 cell co-cultures confirm KIR3DL1 significantly reverses IFN-γ inhibition (Figure 10, comparison between solid circles and open circles). Blocking with ME1 and HC10 antibodies caused a slight up-regulation of IFN-γ production compared to blocking with an isotype control in YTS (non-KIR3DL1) NK cells (Figure 10, solid markers). This may be due to non-specific interaction of the antibody with an NK cell receptor or perhaps due to factors in the hybridoma supernatant. Nevertheless blocking in KIR3DL1 NK cell co-cultures (Figure 10, open markers) showed both ME1 and HC10 antibodies cause up-regulation of IFN-γ compared to isotype control antibody, indicating pHLA and FHC are being blocked from interacting with KIR3DL1 and as such inhibition of cytokine production is being reversed.

Figure 10. KIR3DL1 inhibits IFN-γ production in wild-type ERAP1 co-cultures and antibody blocking causes insignificant specific and non-specific up-regulation. Intracellular staining of wild-type ERAP1 co-cultures show YTS NK cells (filled markers) have greater IFN-γ than KIR3DL1 NK cells (open markers) (p <0.05). KIR3DL1 inhibits IFN-γ (filled circle versus open circles) but blocking with conformation specific antibodies ME1 and HC10 causes non-significant up-regulation in both YTS and 3DL1 cells (further analysis in Figure 11).
To distinguish specific inhibition reversal in KIR3DL1 co-cultures from non-specific reversal seen in YTS co-cultures, further analysis was done as follows. The number of IFN-γ positive cells in isotype control co-cultures was subtracted from ME1 and HC10 blocked co-cultures, in both KIR3DL1 and YTS NK cell-lines (difference(y) = Isotype control – (ME1 or HC10)). The YTS differences (Figure 11, solid black columns) were then plotted against KIR3DL1 differences (Figure 11, open columns) and paired t-test analysis was performed. Analysis shows, although 3DL1 inhibition was indeed reversed to a greater degree by both conformation specific antibodies compared to YTS NK cells, the differences did not reach significance (p>0.05).

**Figure 11.** Blocking HLA-B27 in wild-type ERAP1 cells does not reverse KIR3DL1 specific inhibition. IFN-γ is up-regulated in ME1 and HC10 blocked co-cultures compared to isotype control co-cultures. The difference in up-regulation is higher in 3DL1 co-cultures (clear columns) compared to YTS co-cultures (patterned columns) without reaching statistical significance (p>0.05).
Similarly, we analyzed NK cell co-cultures with ERAP1 knock-down cell lines. Significant inhibition of IFN-γ production was also observed in these co-cultures (Figure 12, compare solid circles with open circles). The IFN-γ production profile was similar to that of wild-type ERAP1 co-cultures with the exception that reversal of inhibition by blocking antibodies seemed lower in KIR3DL1 co-cultures as compared to wild-type ERAP1 cells. As such, further analysis of inhibition reversal was done as described for ERAP1 wild-type co-cultures.

**Figure 12. KIR3DL1 inhibits IFN-γ production in ERAP1 shRNA co-cultures and antibody blocking causes insignificant up-regulation.** Intracellular staining of ERAP1 shRNA co-cultures show YTS NK cells (closed markers) have greater IFN-γ than KIR3DL1 NK cells (open markers) (p<0.05). KIR3DL1 inhibits IFN-γ (closed circle versus open circles) but blocking with conformation specific antibodies ME1 and HC10 causes non-significant up-regulation in both YTS and 3DL1 cells (further analysis in Figure 13).
Differences between non-specific YTS inhibition reversal (Figure 13, solid black columns) and HLA-B27 specific KIR3DL1 inhibition reversal (Figure 13, open columns) were almost identical and not significantly different (p>0.05). Of note, although wild-type ERAP1 co-cultures showed KIR3DL1 specific reversal of inhibition was greater than that of YTS, non-specific reversal, ERAP1 shRNA co-cultures did not exhibit any differences in reversal between YTS and KIR3DL1 co-cultures.

Figure 13. Blocking HLA-B27 in ERAP1 shRNA cells does not reverse KIR3DL1 specific inhibition. IFN-γ is up-regulated in ME1 and HC10 blocked co-cultures compared to isotype control co-cultures. The difference in up-regulation is similar in 3DL1 co-cultures (clear columns) compared to YTS co-cultures (patterned columns) and therefore insignificant (p>0.05)
2.2.3  AS-protective ERAP1 variant cell lines are increasingly dependent on pHLA and FHC contributions to KIR3DL1 inhibition compared to AS-associated ERAP1 or ERAP1 knock-down cell lines

Similar intracellular cytokine staining experiments, as described above, were done on co-cultures with two AS protective ERAP1 cell lines encoding single amino acid changes as follows: ERAP1 K528R and ERAP1 Q730E.

![Graph showing IFN-γ positive cells](image)

**Figure 14.** KIR3DL1 inhibits IFN-γ production in ERAP1 R528 co-cultures and antibody blocking causes both specific and non-specific up-regulation. Intracellular staining of ERAP1 R528 co-cultures show YTS NK cells (closed markers) have greater IFN-γ than KIR3DL1 NK cells (open markers) (p<0.05). KIR3DL1 inhibits IFN-γ (closed circle versus open circles) but blocking with conformation specific antibodies ME1 and HC10 causes up-regulation in both YTS and 3DL1 cells (see Figure 16 for statistics).
Figure 15. KIR3DL1 inhibits IFN-γ production in ERAP1 E730 co-cultures and antibody blocking causes both specific and non-specific up-regulation. Intracellular staining of ERAP1 E730 co-cultures show YTS NK cells (closed markers) have greater IFN-γ than KIR3DL1 NK cells (open markers) (p<0.05). KIR3DL1 inhibits IFN-γ (closed circle versus open circles) but blocking with conformation specific antibodies ME1 and HC10 causes up-regulation in both YTS and 3DL1 cells.
Both variant cell line co-cultures (Figures 14 and 15) had IFN-γ staining profiles similar to ERAP1 wild-type cells (Figure 10) rather than ERAP1 shRNA cells (Figure 12). In both cases 3DL1 inhibited IFN-γ production as expected (Figures 14 and 15, compare solid circles with open circles). Non-specific inhibition reversal was observed as in other co-cultures and as such detailed analysis of non-specific versus specific inhibition reversal was performed as described previously.

Figure 16. Blocking FHC in ERAP1 R528 cells significantly reverses KIR3DL1 specific inhibition. IFN-γ is up-regulated in ME1 and HC10 blocked co-cultures compared to isotype control co-cultures. The difference in up-regulation is higher in 3DL1 co-cultures (clear columns) compared to YTS co-cultures (patterned columns). Blocking FHC with HC10 antibody causes a significant reversal in KIR3DL1 inhibition (p<0.05).
Both AS-protective variants R528 and E730 had increased KIR3DL1 specific inhibition reversal (Figures 16 and 17, open columns) compared to non-specific inhibition reversal (Figures 16 and 17, solid columns). The inhibition reversal due to both pHLA and FHC blocking were statistically significant (p<0.05) in ERAP1 E730 co-cultures, whereas only FHC blocking reached statistical significance in ERAP1 R528 co-cultures. ERAP1 R528 assay had n = 5 to 3 whereas ERAP1 E730 had n = 5 and as such may have contributed to the strength of association. Notably, the strength of association was greater with FHC than pHLA in both cases. Across all ERAP1 cell lines, the strength of KIR3DL1 inhibitory dependency on pHLA and FHC can be described as follows: ERAP1 shRNA << wild-type ERAP1 < ERAP1 R528 < ERAP1 E730.

Figure 17. Blocking pHLA and FHC in ERAP1 R528 cells significantly reverses KIR3DL1 specific inhibition. IFN-γ is up-regulated in ME1 and HC10 blocked co-cultures compared to isotype control co-cultures. The difference in up-regulation is higher in 3DL1 co-cultures (clear columns) compared to YTS co-cultures (patterned columns). Blocking pHLA and FHC with ME1 and HC10 antibodies cause a significant reversal (p<0.05 and p<0.005 respectively) in KIR3DL1 inhibition.
2.2.4 MIP-1α production is highly sensitive to KIR3DL1 and is not sufficiently reversed by blocking pHLA or FHC

Intracellular MIP1-α production was also analyzed in all NK cell-target cell co-cultures in concert with IFN-γ. In all co-cultures, KIR3DL1 inhibited MIP1-α to a basal level of almost 0% (Figure 18 A,B,C and D, compare solid circles with open circles). Once more, slight but reproducible KIR3DL1 non-specific up-regulation of MIP1-α was seen in all co-cultures (Figure 18 A,B,C and D, solid markers). Upon blocking with ME1 and HC10 antibodies, there was no noticeable reversal of MIP1-α inhibition, possibly because the unblocked conformer was sufficient to inhibit MIP1-α to a basal level (Figure 18 A,B,C and D, open markers). This result is in accordance with the view that MIP1-α is a rapidly inducible cytokine, compared to IFN-γ, and as such is highly sensitive to inhibitory receptor ligation.
Figure 18. KIR3DL1 inhibition of MIP-1α production occurs in all ERAP1 co-cultures and is not reversed by HLA-B27 antibody blocking. (A, B, C and D) Comparison of YTS co-cultures (black markers) with 3DL1 co-cultures (open markers) show KIR3DL1 inhibits MIP1-α to a basal level in all co-cultures (p<0.05). Blocking pHLA (square markers) and FHC (triangle markers) does not reverse MIP1-α inhibition.
In an effort to analyze whether MIP1-α inhibition can be reversed if cytokine levels are upregulated, NK cells were stimulated with PMA and ionomycin prior to co-culture with target cells. While MIP1-α was in fact up-regulated by PMA/ionomycin treatment, it seemed to abrogate inhibition by KIR3DL1 to statistical insignificance (Figure 19). As was the case with unstimulated NK cell co-cultures, KIR3DL1 specific reversal of inhibition due to blocking antibodies was absent confirming the sensitivity of MIP1-α to KIR3DL1 interaction with the unblocked HLA-B27 conformer (Figure 19 A, B, C and D, compare open markers).

**Figure 19.** KIR3DL1 inhibition of MIP-1α production is abrogated by PMA/ionomycin stimulation of NK cells. (A, B, C and D) Comparison of YTS co-cultures (black markers) with 3DL1 co-cultures (open markers) show KIR3DL1 inhibition of MIP1-α is not significantly inhibited in all co-cultures. Blocking pHLA (square markers) and FHC (triangle markers) does not reverse MIP1-α inhibition.
2.2.5 MHC class I conformation specific antibody W6-32 is insufficient to reverse KIR3DL1 inhibition of IFN-γ and MIP-1α

To strengthen observations seen using anti-HLA-B27 antibody ME1, intracellular staining experiments were also done after blocking with pan-MHC class I (pHLA) antibody W6/32. Surprisingly W6/32 blocking did not reproduce results seen with ME1 blocking. Neither IFN-γ nor MIP-1α inhibition was reversed in any co-culture by blocking MHC class I with W6/32 (Figure 20).
Figure 20. Blocking MHC class I molecules with pan-MHC class I antibody does not reverse IFN-γ or MIP-1α inhibition. (A-D) All target cells were blocked with pan-MHC class I antibody W6/32 prior to co-culture did not exhibit reversal of IFN-γ inhibition (compare circle markers with square markers) by KIR3DL1. (E-H) Similarly MIP-1α inhibition was not reversed after blocking with W6/32.
2.3 Discussion

To evaluate NK cell effector function, HLA-B*2705 expressing target cells were co-cultured with non-KIR3DL1 (YTS) and KIR3DL1 NK cells. Supernatants of these co-cultures were analyzed for cytokine expression and revealed several candidate cytokines regulated by KIR3DL1. Of 64 cytokines analyzed, 12 were expressed in low levels (10-100 pg/ml), 4 in moderate levels (101-1000 pg/ml) and 7 at high levels (>1000 pg/ml), the rest being undetectable in the supernatant (Table III). A majority of cytokines regulated by KIR3DL1 had chemoattractant functions although many of them are not known to be produced by NK cells and may have been produced by the B lymphoblastoid target cell line (C1R). It is known KIR3DL1 regulates NK cell activation by targeting early NK cell events such as cell-cell adhesion but conversely it could possibly indirectly influence cytokine production by the target cell line, by regulating the amount of time NK cells are interacting with the target cell line.

In this study, I chose to analyze IFN-γ for its important role in modulating innate and adaptive immune responses. In humans, NK cells are a primary producer of IFN-γ. They are known to up-regulate the antigen processing and presentation pathway, leading to increased MHC Class I and Class II presentation (Schoenborn & Wilson, 2007). IFN-γ produced by NK cells is also known to affect CD4 T-cell development, and is important in regulating the adaptive immune response. With respect to AS, IFN-γ treatment of macrophages from AS patients induces a substantially different gene expression profile compared to controls (Smith et al., 2008). The cytokine secretion screen revealed MIP-1α is substantially regulated by KIR3DL1, and was the primary basis for further analysis. Additionally MIP-1α plays an important role in recruiting mononuclear cells to sites of inflammation and may play a role in inflammatory diseases such as AS (O'Grady et al., 1999).

When HLA-B*2705 expressing C1R cells were co-cultured with NK cells, there was noticeably lower levels of intracellular IFN-γ and MIP-1α in co-cultures with KIR3DL1+ NK cells.
indicating the latter is responsible for inhibiting these two cytokines (Figure 10). Previous research had shown both these cytokines are induced in response to 2B4 activation and therefore KIR3DL1 is likely inhibiting 2B4-mediated NK cell activation (Figure 6). The question arises ‘Which HLA-B*2705 conformer, pHLA or FHC, is responsible for inducing inhibition?’ Each conformer was blocked with conformation-specific antibodies, ME1 (which recognizes the conformation of residues 67-71 on the HLA-B27 α1 helix that contact connecting loop residues 41 and 43), HC10 (which recognizes a linear epitope in the α1 domain that becomes exposed in β2m- and peptide-free MHC class I molecules) and W6/32 (which recognizes a conformation dependent on β2m and all 3 alpha helices in MHC class I molecules) (Arosa, Santos, & Powis, 2007; Martayan et al., 2009; McCutcheon, Smith, Valenzuela, Aalbers, & Lutz, 1993). None of these antibodies are absolutely HLA-B27 specific, but the relative absence or low abundance of MHC-I molecules other than HLA-B27 on the C1R-B27 cells, likely reflect HLA-B27 mediated changes.

IFN-γ production from NK cells was inhibited by KIR3DL1 and this was reversed by both ME1 and HC10 antibody blocking. The first surprising result in this chapter is that W6/32 blocking of pHLA does not reverse IFN-γ inhibition in KIR3DL1+ NK cells.

MIP-1α inhibition, on the other hand, could not be reversed by blocking any HLA-B27 conformer. MIP-1α regulation in NK cells is sensitive to low levels of MHC I and is rapidly regulated compared to IFN-γ and this could be responsible for the lack of reversal with MHC-I antibodies (Bryceson et al., 2011). Even after enhancing MIP-1α production by PMA/ionomycin stimulation, no changes in inhibition could be observed. Another possibility is the relative lack of effect of KIR3DL1-B27 interaction on MIP1α production pathways.

The variant of ERAP1 present in the target cell appeared to influence NK cell IFN-γ production. Blocking either pHLA or FHC caused significant reversal of IFN-γ inhibition in the AS protective ERAP1 co-cultures and to a lesser extent in wild-type ERAP1 co-cultures, but could
not be observed when targets cells had ERAP1 levels suppressed by shRNA treatment. The results confirm both pHLA and FHC functionally interact with KIR3DL1+ NK cells, but ERAP1 status affects how significant this functional interaction is. Reflecting on our hypothesis that ERAP1 polymorphisms can affect cause differences in pHLA and FHC expression followed by changes in KIR3DL1 NK cell inhibition; across all ERAP1 cell lines, the strength of KIR3DL1 inhibitory dependency on pHLA and FHC can be described as follows: ERAP1 shRNA << wild-type ERAP1 < ERAP1 R528 < ERAP1 E730.
Chapter 3
ERAP1 expression affects KIR3DL1 binding and is potentiated by MHC I cross-linking

Introduction

The previous chapter revealed the roles of pHLA and FHC in inhibiting IFN-γ production by interacting with KIR3DL1 on NK cells, as well as the changes in this interaction with ERAP1 variants present in the target cell. Consensus suggests these changes may be because KIR3DL1 interaction also varies with ERAP1 polymorphism expression.

NK cell contact and subsequent firm adhesion with target cells is a proximal step in NK cell activation. Contact with susceptible target cells causes formation of the NK immune synapse (NKIS). Within the NKIS activating receptors, adhesion and signaling molecules aggregate to form a central supramolecular activation cluster (c-SMAC). Some examples of proteins found in the c-SMAC include the adhesion molecule LFA-1, which binds ICAM-1 on target cells, and receptors known to induce the high affinity LFA-1 conformation such as 2B4, which binds CD48 on the target cell. Within minutes of initial contact and in the absence of inhibitory signaling, activation of LFA-1 causes strong adherence to NK cells (Hoffmann et al., 2011).

Formation of the c-SMAC allows NK cell signaling molecules such as protein tyrosine kinases (PTK), G-proteins, adaptor proteins and phosphoinositides, along with downstream signaling molecules like SHP-1, Lck, phospholipase C (PLC), Vav, Zap70 and Syk found within lipid rafts in the c-SMAC allows close association of receptors with their down-stream signaling molecules (Lou et al., 2000). Upon NK cell contact with sensitive targets and establishment of firm adhesion, lipid rafts polarize to the contact surface and aggregate as a necessary step preceding activation of signal cascades.

Inhibitory KIR receptors target proximal steps of NK cell activation described above, such as LFA-1 mediated adhesion and actin dependent recruitment of activating receptors. This
mechanism ensures inhibitory KIR signals are dominant over activating signals (Long et al., 2013).

If in fact ERAP1 causes changes in KIR3DL1 ligand (HLA-B27) expression as hypothesized, it is highly likely that the inhibitory effect of KIR3DL1 on NK cell-target cell adhesion will also affected, given the information above. Changes in NK cell-target cell adhesion may precede changes in downstream NK cell function, such as cytokine secretion, and as such explain observations found in the previous chapter.

In this chapter, KIR3DL1 affinity for the various ERAP1 expressing cell lines was analyzed at the molecular and whole cell levels with the aim, as described in page 32, of correlating proximal events in NK cell activation, namely NK cell target-cell adhesion, with downstream effector functions, assessed previously in terms of IFN-γ production. Hypothetically, stronger KIR3DL1 inhibition, in terms of cytokine production, should correlate with stronger inhibition of cell-cell interaction. Cell lines used, antibody blocking experiments and statistical analysis were performed as described in section 2.1.1-2.1.3. KIR3DL1 affinity was determined using soluble KIR3DL1-Fc fusion proteins and KIR3DL1+ NK cells, as described in the following materials and methods section.

3.1 Materials and Methods

3.1.1 KIR3DL1-Fc binding assay

ERAP1 cell lines were washed in FACS buffer blocked with either 0.5 µg IgG isotype control antibody (Biolegend), 50 µl ME1 hybridoma supernatant diluted in 100 µl FACS buffer, 100 µl HC10 hybridoma supernatant or 0.5 µg W6/32 antibody (Biolegend) for 1 hour at room temperature in 96 well u-bottom plates. Cells were subsequently washed in FACS buffer and resuspended in 40 µl FACS buffer. An equal volume of 100 µg/ml KIR3DL1-Fc (R & D) was added and cells were incubated at 4°C for 1 hour. Cells were subsequently washed and stained with 1:50 dilutions of F(ab’)2 fragments of APC goat anti-human IgG-Fc (Jackson
ImmunoResearch) at 4°C for 30 minutes. Cells were washed once more and fixed in 1% paraformaldehyde. KIR3DL1-Fc binding was analyzed using the BD LSR II flowcytometer.

3.1.2 NK cell-target cell conjugation assay

Cell-cell conjugation studies were performed as described (Burshtyn & Davidson, 2010). Briefly, NK cells were labeled with PKH26 red fluorescent cell membrane labeling dye (Sigma, Oakville, Canada) and added to C1R cells at a 1:5 ratio and a final volume of 200µl in 5 mL FACS tubes. Cells were immediately centrifuged at 300×g for 1 minute and incubated in a 37°C water bath for various time periods followed by fixation with 0.5% formaldehyde.

3.2 Results

3.2.1 KIR3DL1-Fc weakly binds AS-protective ERAP1 cells

The affinity of KIR3DL1 to target cells with different ERAP1 variants was tested using recombinant KIR3DL1-Fc chimeras. KIR3DL1-Fc consists of disulfide-bonded monomers of the extracellular portion of KIR3DL1 ligated to the Fc portion of human IgG. Surface staining of the various target cell lines with KIR3DL1-Fc revealed differences in KIR3DL1 binding depending on the ERAP1 variant present. Protective ERAP1 variants led to less affinity for KIR3DL1 than either shRNA or wild-type ERAP1 in target cells, with differences reaching statistical significance in ERAP1 E730 cell lines (Figure 21).
Figure 21. Protective ERAP1 variants have lower affinity for KIR3DL1 than wild-type and ERAP1 shRNA expressing cell lines. Both ERAP1 variants have lower affinity for KIR3DL1-Fc than wild-type or shRNA expressing cells (p<0.05).
3.2.2 Blocking pHLA and MHC class I increases binding while blocking FHC decreases KIR3DL1-Fc binding to ERAP1 expressing cells

![Figure 22](image)

**Figure 22. Blocking pHLA and MHC class I increases KIR3DL1 binding whereas blocking FHC decreases it.** Blocking pHLA and MHC class I prior to staining with KIR3DL1-Fc causes increased KIR3DL1 affinity (A and C: compare open and closed markers). Blocking FHC decreases KIR3DL1-Fc binding (C: compare open and closed markers).

The effect of blocking pHLA, MHC class I, and FHC on KIR3DL1 binding was investigated. Surprisingly blocking both pHLA and MHC class I with ME1 and W6/32 respectively increased KIR3DL1 binding (Figure 22 A and C) across all cell lines. Blocking FHC, however, decreased KIR3DL1 binding (Figure 22 B). Plotting changes in KIRDL1 binding after ME1, HC10 and W6/32 blocking revealed ERAP1 shRNA cell lines had the greatest change due to ME1 and W6/32 blocking followed by wild-type ERAP1 and ERAP1 R528 and E730 cell lines (Figure 23 A and C).
Figure 23. Increase in KIR3DL1-Fc binding is greatest in ME1 and HC10 blocked ERAP1 shRNA cells. KIR3DL1-Fc binding is enhanced after ME1 (A) and W6/32 (C) blocking but reduced by HC10 (B) blocking (* = p ≤ 0.05; ** = p < 0.005; *** = p < 0.001).

3.2.3 Blocking pHLA potentiates KIR3DL1 interaction with wild-type ERAP1 and ERAP1 shRNA cells

The results of the previous assay revealed both a difference in KIR3DL1 binding depending on ERAP1 expressed in target cell lines, as well as surprising increases in binding after ME1 and W6/32 blocking. Whether this phenomenon occurs at the cellular level was investigated in the conjugation assay.

In a flow-cytometry, based NK cell adhesion assay, NK cells were labeled with red fluorescent dye and co-cultured with target cells that express GFP. After co-culture for 0, 5 and 10 minutes cells were fixed with PFA and analyzed by flow-cytometry. The gating strategy is shown in Figure 24. Unconjugated NK cells are represented by a PE+/GFP- population in the upper left quadrant of the FACS plot, whereas target cells are in a PE-/GFP+ population at the bottom right hand quadrant. NK cells adhered to target cells are in a PE+/GFP+ population in the top right quadrant.
Figure 24. NK cell-target cell conjugation assay gating strategy. (A) YTS/wild-type ERAP1 co-cultures were first gated by size in the left-hand plot (SSC-A vs FSC-A) to exclude debris and dead cells. Cells co-cultured for 0 minutes and 10 minutes were segregated based on PKH26 and GFP fluorescence in the middle panel and right-hand panel respectively. (B) 3DL1/wild-type ERAP1 co-cultures were similarly gated on and exhibit lower double positive events at the 10 minute time point compared to YTS/wild-type ERAP1 co-cultures (compare top and bottom right-hand panels).

Results show KIR3DL1 expressing NK cells form less conjugates compared to non-KIR3DL1 YTS cells after both 5 minutes and 10 minutes of co-culture (Figure 25). Between 5 minutes and 10 minutes of co-culture, NK cell conjugation reaches a steady state and therefore conjugation percentage remains almost the same at these time points.
Figure 25. NK cell conjugation with target cells is inhibited by KIR3DL1. YTS/wild-type ERAP1 co-cultures have increased NK cell-target cell conjugates (circle marker) after 5 minutes and 10 minutes of co-culture compared to 3DL1/wild-type ERAP1 co-cultures (square marker).

Next the individual contribution of pHLA and FHC towards KIR3DL1 specific conjugation inhibition was assessed. Target cells were blocked with isotype control, ME1 and HC10 antibodies prior to co-culture for 10 minutes. KIR3DL1 inhibited conjugation as expected in wild-type ERAP1 co-cultures (Figure 26 A, compare solid circles with open circles). Blocking with ME1, HC10 and W6/32 prior to co-culture caused KIR3DL1 specific reversal of NK cell conjugation inhibition (Figure 26 A, compare open markers). Further analysis shows this reversal is statistically significant only in W6/32 blocked co-cultures (Figure 26 B). Whereas KIR3DL1-Fc studies indicated both ME1 and W6/32 significantly increase KIR3DL1 affinity for wild-type ERAP1 shRNA cell lines, at the cellular level only W6/32 significantly increased affinity. HC10 blocking did not reduce affinity but rather slightly up-regulated cellular adhesion, although statistically insignificant.
Figure 26. W6/32 antibodies reverse KIR3DL1 inhibition of NK cell adhesion with wild-type ERAP1 target cells. (A) YTS co-culture (solid markers) with isotype control antibody (circle marker) had significantly higher conjugates than 3DL1 co-cultures (open markers). (B) Blocking with W6/32 significantly reverses KIR3DL1 inhibition of NK cell adhesion (p<0.05).

Figure 27. NK cell conjugation with target cells is inhibited by KIR3DL1. YTS/ERAP1 shRNA co-cultures have increased NK cell-target cell conjugates (circle marker) after 5 minutes and 10 minutes of co-culture compared to 3DL1/ERAP1 shRNA co-cultures (square marker).
Similarly, KIR3DL1 inhibition of NK cell adhesion was observed in the time course experiment (Figure 27). Blocking with ME1 and W6/32 reversed increased cellular adhesion although once more it was statistically significant in W6/32 blocked co-cultures (Figure 28 B). HC10 blocking did not cause any change in cellular adhesion.

These findings indicate at the cellular level, KIR3DL1 inhibits adhesion, but ME1, and more significantly W6/32 blocking up-regulates adhesion. Thus, the phenomenon seen at the molecular level in the KIR3DL1-Fc studies also occurs at the cellular level, albeit this assay is not as sensitive in detecting the changes in KIR3DL1 affinity.

Figure 28. Blocking pHLA reverses KIR3DL1 inhibition of NK cell adhesion with ERAP1 shRNA target cells. (A) Profiles of YTS co-cultures (solid markers) and 3DL1 co-cultures (open markers) with ERAP1 shRNA cells after blocking are represented graphically. (B) Blocking with W6/32 significantly reverses KIR3DL1 inhibition of NK cell adhesion (p<0.05).
3.2.4 Blocking pHLA potentiates KIR3DL1+ NK cell affinity for AS protective ERAP1 expressing cells

Conjugation assays were performed, as described above, with ERAP1 R528 and ERAP1 E730 expressing cell lines. The time course assay shows once more that KIR3DL1 drastically inhibits NK cell conjugation with either target cell (Figure 29 A and B).

![Figure 29. NK cell conjugation with AS protective ERAP1 target cells is inhibited by KIR3DL1. (A and B) YTS/ERAP1 variant co-cultures have increased NK cell-target cell conjugates (circle marker) after 5 minutes and 10 minutes of co-culture compared to 3DL1/ERAP1 variant co-cultures (square marker).](image)

When pHLA and MHC class I was blocked with ME1 and W6/32 antibodies respectively, affinity for KIR3DL1 NK cells was increased significantly, whereas HC10 blocking did not change adhesion compared to isotype blocked co-cultures (Figure 30 A and B). No non-specific differences in conjugation were seen after antibody blocking in YTS co-cultures.

Further analysis of the reversal of inhibition reveals the differences due to ME1 and W6/32 blocking are statistically significant (p<0.05) (Figure 31 A and B).
Figure 30. Blocking pHLA and MHC class I upregulates KIR3DL1 NK cell adhesion with AS protective ERAP1 target cells. Profiles of YTS co-cultures (solid markers) and 3DL1 co-cultures (open markers) with ERAP1 R528 cells (A) and ERAP1 E730 cells (B) after blocking show only ME1 and W6/32 (compare open circles with open squares) significantly reverses KIR3DL1 inhibition of NK cell adhesion.

Figure 31. Blocking pHLA and MHC class I significantly increases KIR3DL1 NK cell adhesion with AS protective ERAP1 target cells. The difference in up-regulation of NK cell adhesion in 3DL1 co-cultures after blocking with ME1 and W6/32 is significantly higher (p<0.05) compared to isotype blocked (A) ERAP1 R528 (B) ERAP1 E730 expressing target cells (* = p<0.05; ** = p<0.005).
3.3 Discussion

The previous finding showing antibody blocking does not reverse KIR3DL1 inhibition of NK cell cytokine production, when co-cultured with target cells having ERAP1 shRNA and only weakly with cells having wild-type ERAP1, was a surprising finding since HLA-B27 is an established ligand for KIR3DL1. Staining the various ERAP1 cell lines with recombinant human KIR3DL1-Fc offered some insight into why this may be happening. In the experiments described in this chapter KIR3DL1 affinity for the different ERAP1 cell lines was analyzed at both the molecular and cellular levels. Additionally, the roles of pHLA and FHC in KIR3DL1 were assessed. The results show that cells containing AS-protective ERAP1 can be distinguished from those with the AS-associated wild-type ERAP1 on the basis of KIR3DL1-Fc binding. AS-protective ERAP1-cells have significantly lower affinity than the AS-associated ERAP1-cells. It is possible that because KIR3DL1 has higher affinity for ERAP1 shRNA and wild-type ERAP1 cell lines, blocking is insufficient to reverse IFN-γ inhibition in these two cell lines, but is sufficient for the AS-protective ERAP1 cell lines. The KIR3DL1-Fc assay therefore reveals affinity for KIR3DL1 at the molecular level in the various cell lines is as follows: ERAP1 shRNA = wild-type ERAP1 >> ERAP1 R528 > ERAP1 E730.

After blocking MHC class I with W6/32, KIR3DL1-Fc staining dramatically increases in all cell lines compared to those blocked with an isotype control antibody (Figure 23 C), mirroring results seen with ME1 blocking. This phenomenon is also seen in cells expressing HLA-C that are blocked with pan MHC class I antibodies W6/32 and G46-46 antibodies prior to staining with KIR2DL1 and KIR2DS1 tetramers (Stewart et al., 2005). The fact that MHC I blocking with ME1 and W6/32 increase KIR3DL1 binding, especially in the ERAP1 shRNA cell line, also contributes to the lack of inhibition reversal (Figure 23 A and C). The protective ERAP1 variants exhibit the least increase in KIR3DL1-Fc binding in response to MHC I cross-linking. HC10 blocking significantly reduced KIR3DL1-Fc binding although the magnitude of change was minute compared to that seen with MHC I blocking (Figure 23 B). This result suggests FHC play
a small role in modulating KIR3DL1 affinity compared to pHLA, although both are important for inhibiting IFN-γ.

Early NK cell adhesion events are known to be regulated by inhibitory KIR receptors in order to inhibit NK cell function. As such, adhesion between the various ERAP1 target cell lines and NK cells lacking or expressing KIR3DL1 was investigated. Additionally the role of pHLA and FHC in regulating NK cell adhesion was also investigated. The results of the experiments show KIR3DL1 does indeed inhibit NK cell adhesion to ligand expressing targets (Figure 25). Inhibition starts early after co-culture and stabilizes around 10 minutes after co-culture. The conjugation assay confirms MHC I cross-linking increasing KIR3DL1 affinity also occurs at the cellular level. Cross-linking MHC I with W6/32 significantly increased KIR3DL1+ NK cell conjugation with all ERAP1 target cells (Figures 26B, 28B and 31).

Blocking with HC10 however, does not cause any changes in KIR3DL1+ NK cell conjugation, suggesting once more that it plays a small role in mediating KIR3DL1+ NK cell adhesion compared to pHLA. That HC10 blocking does not significantly change NK cell adhesion at the cellular level is surprising since it causes significant reversal of IFN-γ inhibition in both AS-protective ERAP1 cell lines (Figures 16 and 17) and slight decrease in KIR3DL1 affinity at the molecular level. That FHC is a ligand for KIR3DL1 and binding is inhibited by HC10 has been confirmed independently by Hatano et al. (Hatano et al., 2015). In the same study the authors could not find a functional role for FHC in inhibiting IFN-γ production although this study confirms it plays a significant role in ERAP1 cells expressing AS-protective ERAP1, perhaps because the 721.221 cells used in the assay express AS-associated ERAP1, and as such, do not play a significant role in KIR3DL1 IFN-γ inhibition.

It is difficult to compare across the different cell lines the contribution of this phenomenon, as was done in the KIR3DL1-Fc assay, since each cell line was tested separately due to the challenging timing requirements of the assay. The sensitivity of the conjugation assay is
decreased in comparison, as significant inhibition due to HC10 blocking could not be replicated. Nevertheless, the results of this assay confirm increased KIR3DL1 affinity after ME1 and W6/32 blocking is not only seen at the molecular level as determined by KIR3DL1-Fc staining, but also in KIR3DL1 expressing NK cells.

Interestingly, the results indicating blocking with W6/32 causes increased KIR3DL1+ NK cell affinity for target cells, and yet blocks IFN-γ inhibition suggests KIR3DL1 can cause cytokine inhibition without inhibiting early NK cell activation events such as cell-cell adhesion. Whereas ME1 blocking causes inhibition reversal, even though it increases KIR3DL1 affinity by MHC I cross-linking. In combination, these observations suggest first, that W6/32 does not productively inhibit KIR3DL1 interaction with HLA-B*2705 and secondly, indirectly suggests productive KIR3DL1 interaction with HLA-B*2705 inhibits recruitment of activating receptors such as 2B4 to the cell surface as a means of inhibiting cytokine secretion without affecting NK cell-target cell adhesion (Figure 7 and Table III). HC10 blocking of IFN-γ inhibition may also work in a similar fashion, although it does not cause cross-linking and only marginally reduces affinity for KIR3DL1.

Returning to our hypothesis, KIR3DL1 does inhibit NK cell-target cell adhesion but the effect of ERAP1 polymorphisms on this function could not be evaluated with the current methods. The role of pHLA and FHC was evaluated using antibodies, as described, but this method in fact amplified KIR3DL1 interaction, instead of blocking it. As such the individual roles of pHLA and FHC cannot be evaluated with antibody blocking studies.
Table IV. KIR3DL1-Fc binding characteristic after blocking with various MHC I antibodies predicts KIR3DL1 inhibition can occur in the absence of NK cell-target cell adhesion down-regulation

<table>
<thead>
<tr>
<th>Cell line</th>
<th>KIR3DL1-Fc binding</th>
<th>Effect of ME1 blocking</th>
<th>Effect of HC10 blocking</th>
<th>Effect of W6/32 blocking</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>KIR binding</td>
<td>IFN-γ inhibition</td>
<td>KIR binding</td>
<td>IFN-γ inhibition</td>
</tr>
<tr>
<td>ERAP1 shRNA</td>
<td>High</td>
<td>Increase</td>
<td>Reversed</td>
<td>Slight decrease</td>
</tr>
<tr>
<td>Wild type ERAP1</td>
<td>High</td>
<td>Increase</td>
<td>Reversed</td>
<td>Slight decrease</td>
</tr>
<tr>
<td>ERAP1 R528</td>
<td>Low</td>
<td>Increase</td>
<td>Reversed</td>
<td>Slight decrease</td>
</tr>
<tr>
<td>ERAP1 E730</td>
<td>Low</td>
<td>Increase</td>
<td>Reversed</td>
<td>Slight decrease</td>
</tr>
<tr>
<td>Hypothetical blocking mechanism</td>
<td>-</td>
<td>Down-regulates activating receptors</td>
<td>Inconclusive</td>
<td>Does not block NK IFN-γ inhibition</td>
</tr>
</tbody>
</table>
Introduction

As described in the introduction, ERAP1 and MHC class I molecules are intimately tied together as components of the antigen processing and presentation pathway. Intracellular proteins, targeted for degradation by ubiquitination, are first cleaved by the proteasome into 2-25 amino acid peptides (Hammer et al., 2007). These peptides subsequently enter the ER via TAP and subsequently N-terminally cleaved by ERAP1 into 8-9 amino acid peptides. It must be noted that not all precursor peptides are trimmed equally as certain N-terminal amino acid residues are more susceptible to cleavage compared to others and as such are more likely to be trimmed by ERAP1. Another level of complexity is added to ERAP1 trimming function due to the effect of SNPs on aminopeptidase activity. Some ERAP1 polymorphisms, such as K528R, affect the transition of enzyme between open and closed conformations whereas others, such as Q730E, affect binding of the peptide precursor to the enzyme itself (Kochan et al., 2011b; Nguyen et al., 2011). In general, the combination of precursor peptide sequence and ERAP1 polymorphism decide the susceptibility of precursor peptide to enzymatic degradation. Because ERAP1 trimmed peptides are subsequently loaded onto MHC class I molecules, exit the ER and proceed through the Golgi apparatus before finally being presented on the cell surface, polymorphisms that effect epitope generation have an effect on MHC class I cell surface expression. Polymorphisms that generate weakly binding MHC class I epitopes may not be stably expressed on the cell surface, and thus cause low surface MHC class I expression. Weakly bound pHLA complexes may dissociate once they reach the cell surface and become endocytosed only to reform at the cell surface as stable, FHC dimers or monomers (Bird et al., 2003). These ERAP1 polymorphisms may cause functional changes that manifest themselves in the amount of cell surface pHLA and FHC expression. This phenomenon is supported by studies described in the introduction, where mouse and human studies have shown ERAP1 status affects cell surface expression of pHLA and FHC.
Since both HLA-B27 conformers are responsible for interacting with KIR3DL1, changes in expression may be correlated with changes in NK cell functions such as cytokine production and target cell adhesion. The results presented thus far show ERAP1 status does in fact cause changes in NK cell cytokine production and target cell adhesion. By staining target cells with ME1, HC10 and W6/32 antibodies, a correlation between these changes and cell surface expression of KIR3DL1 ligands can be made. The aim of this study, in accordance with aim 3 in page 32, is to combine the results of studies in previous chapters to show whether there exists a chain of events linking ERAP1 polymorphisms K528R and Q730E and MHC class I expression on APCs, interaction of KIR3DL1 with MHC class I ligands expressed on the APCs as well as resultant modulation of NK cell cytokine secretion. Hypothetically ERAP1 polymorphisms correlated with high KIR3DL1 inhibition should also increase expression of pHLA or FHC. Cell lines, antibody staining and statistical analysis was performed as described in sections 2.1.1-2.1.3.

4.1 Methods

ERAP1 cells were washed in FACS buffer and 200,000 cells resuspended in either 0.5 μg IgG isotype control antibody (Biolegend), 50 μl ME1 hybridoma supernatant diluted in 100 μl FACS buffer, 100 μl HC10 hybridoma supernatant or 0.5 μg W6/32 antibody (Biolegend). Cells were incubated at room temperature for 1 hour and then washed in FACS buffer and resuspended in 0.2 μg APC goat anti-mouse IgG for 30 minutes at room temperature. After staining cells were washed in FACS buffer and fixed in 1% paraformaldehyde. Analysis of antibody binding was done using the BD LSR II flowcytometer.
4.2 Results

The results of the cell surface staining assay indicate the differences in expression are statistically significant when comparing the protective ERAP1 variants (Figure 32 A, B and C). It appears ERAP1 R528 has lower pHLA expression than ERAP1 E730 cell lines (Figure 32 A) and it is confirmed by W6/32 staining (Figure 32 C). FHC expression is opposite that of pHLA expression in both ERAP1 variants (Figure 32 B). FHC expression varies widely among the different cell lines, unlike pHLA expression. In addition to the differences mentioned above, ERAP1 R528 has higher FHC than wild-type ERAP1 and ERAP1 E730 has lower FHC than ERAP1 shRNA cell lines. Statistically significant differences in pHLA and FHC expression could not be found between the shRNA and wild-type cell lines. W6/32 staining also indicates the presence of non-HLA-B27 MHC class I molecules, since the MFI of W6/32 staining is drastically higher than that of ME1 staining (Figure 32 A and C, compare MFI). This observation is not surprising since C1R cells are known to express low levels of HLA-Cw4 and HLA-B35.

Figure 32. pHLA, FHC and MHC class I expression are similar across all ERAP1 cell lines. (A) pHLA (B) FHC and (C) MHC class I expression across all ERAP1 cell lines are similar although slight reproducible changes in pHLA, FHC and MHC class I are seen in ERAP1 shRNA cells (square markers) compared to other cell lines. Additionally pHLA and MHC class I expression is significantly higher than FHC expression in all cell lines (* = p<0.05, ** = p<0.005).
4.3 Discussion

ERAP1 expression has been previously shown to affect HLA-B27 cell surface expression. As a result it is thought that the differences in expression may cause differential regulation of cognate KIR expressing cells. In order to investigate this hypothesis and correlate KIR3DL1 affinity with cell surface expression of HLA-B27, the various ERAP1 cell lines were surface stained with ME1, HC10 and W6/32 antibodies.

The results reveal that there are no differences in MHC class I expression between ERAP1 shRNA and wild-type ERAP1 cells. This finding is surprising given the vast difference in ERAP1 expression between the two cell lines. However, a recent study by Cifaldi et al. revealed ERAP1 knock-down does not necessarily correlate with changed MHC I expression in human cell lines. In the study HeLa, Raji, SK-N-SH, SK-MEL-93, MNT-1 and DAOY cell lines were transfected with lentiviral shRNA constructs and MHC I expression determined by W6/32 staining. None of the cell lines revealed significant differences in expression compared to the parental cell lines, although ERAP1 expression was significantly different (Cifaldi et al., 2015).

It is possible that ERAP1 knock-down causes the expression of MHC I peptides that do not require N-terminal processing by ERAP1 and as such overall expression levels are unchanged. The protective ERAP1 polymorphisms, however, can be distinguished on the basis of MHC I expression. The R528 polymorphism does not change pHLA expression, in accordance with a recently published study that detected MHC I expression on monocyte derived dendritic cells (mDC) from individuals expressing the K528R mutation (Costantino et al., 2015). Rather this mutation, which is inherited in a haplotype also encoding for changes in the 575 and 725 amino acids, affects ERAP1 mRNA and protein expression levels without causing changes in MHC I expression. The E730 mutation, on the other hand, significantly up-regulates MHC I expression, while FHC expression is unchanged. The R528 mutation does, however, cause significantly increased FHC expression.
Comparing KIR3DL1-Fc study results with surface staining results reveals correlations between pHLA or FHC expression and KIR3DL1 affinity. The results of KIR3DL1-Fc staining indicate protective ERAP1 expressing cell lines have the least affinity for KIR3DL1, whereas wild-type and ERAP1 shRNA cell lines have the greatest affinity (Figure 21). The major differences in HLA-B27 expression between the AS-protective variants and wild-type AS-associated ERAP1 are increased pHLA (E730) and FHC (R528), suggesting increased expression of conformer is associated with less KIR3DL1 affinity. Although this hypothesis is initially counterintuitive, it is possible given the fact that KIR3DL1 binding is dependent on the P7 and P8 residues of peptides presented by HLA-B27 and the observation that FHC plays only a small role, compared to pHLA, in adhesion to KIR3DL1 (Figure 23 A and B). Assuming the protective ERAP1 variants display sub-optimal peptides, their affinity to KIR3DL1 would be reduced compared to the wild-type ERAP1 variant. Accordingly studies using ERAP1 knock-down cell lines that have been pulsed with high affinity peptides and subsequently co-cultured with freshly isolated NK cells have shown high affinity peptides inhibit NK cell degranulation (Cifaldi et al., 2015). Crucially, pulsing with high affinity peptides did not affect the overall MHC class I expression, and as such the inhibitory effect is not due to increased MHC class I expression but due to higher affinity of these peptides for cognate KIR. This hypothesis can be further probed by analyzing peptides displayed by each ERAP1 variant and comparing the affinity of pHLA and FHC for KIR3DL1. The results also suggest FHC expression does not significantly affect KIR3DL1 binding, as FHC over-expression in the E730 mutant does not increase KIR3DL1 affinity. That blocking FHC with HC10 causes only a minor, but significant, change in KIR3DL1 affinity supports the view that FHCs have lower affinity for KIR3DL1 than pHLA, although both are important in regulating IFN-γ production.

The results do suggest the two AS-protective ERAP1 polymorphisms affect enzymatic function in different ways. As detailed in Table II, the poorly trimming ERAP1 R528 polymorphism occurs in the hinge region between domains II and IV, whereas the similarly poor trimming ERAP1 E730 occurs near the peptide binding regulatory region in domain IV. The former polymorphism affects transition between open and closed states and the latter affects peptide
binding. Recently, it has been shown AS-associated ERAP1 variants are deficient in trimming compared to AS protective variants, and as such, have lower MHC class I expression (Reeves, Colebatch-Bourn, Elliott, Edwards, & James, 2014). The same study showed individual ERAP1 polymorphisms have lesser effect on enzymatic function than combinations of polymorphisms, which are found in naturally occurring haplotypes. Nonetheless, in this study we can conclude a single nucleotide change in ERAP1 does affect cell surface HLA-B27 expression and that these differences are HLA-B27 conformation specific.
5.1 Protective ERAP1 polymorphisms decrease KIR3DL1 interaction through different mechanisms

In this study, I investigated the effect of single nucleotide polymorphisms in ERAP1 on intact HLA-B*2705 and FHC HLA-B*2705 cell surface expression and the resultant effects on KIR3DL1 interaction and function at the molecular and cellular level. All three molecules are implicated in the pathogenesis of the inflammatory arthritis Ankylosing Spondylitis, and as such investigating them, as has been done in this project, could directly benefit those affected by AS, and additionally reveal mechanisms and observations applicable to other diseases.

The predominant hypothesis connecting these three molecules suggests that ERAP1 function affects cell surface expression of HLA-B27 pHLA and FHC, which in turn affects the ability of these cells to functionally interact with cognate KIR-expressing immune cells such as NK cells and CD4+ T-cells (Figure 8). In this study, I have found KIR3DL1 inhibits IFN-γ production in NK cells and that both pHLA and FHC HLA-B27 expressed on APCs functionally interact with KIR3DL1 in imparting this effect. The studies also suggest KIR3DL1 cytokine inhibition is due to reduction in NK cell-target cell adhesion and possibly activating receptor down-regulation. Additionally, I found single nucleotide changes in ERAP1 that are associated with protection from AS do in fact affect cell surface expression of pHLA and FHC and can be distinguished from one another on this basis (Figure 33). Depending on the polymorphism either one of the two conformers is up-regulated compared to the AS-associated variant. The effect of up-regulated HLA-B27 expression is, surprisingly, that it decreases KIR3DL1 interaction at the molecular level, possibly because the up-regulated pHLA does not display peptides optimal for KIR3DL1 binding and that FHC does not play a significant role in modulating KIR3DL1 affinity. In support of the finding that AS-protective ERAP1 polymorphisms reduce KIR3DL1 affinity, I found IFN-γ inhibition in KIR3DL1+ NK cells responding to AS-protective ERAP1
cells can be more easily reversed by blocking pHLA and FHC compared to AS associated ERAP1 cells.

These findings suggest that low KIR3DL1 affinity is protective in AS and that ERAP1 confers this property by up-regulating suboptimal pHLA. In line with my results, I would expect to find in AS patients with these SNPs would have higher pHLA or FHC on antigen presenting cells. Previous research with KIR3DL2+ NK and CD4+ T-cells shows increased interaction of KIR3DL2 with HLA-B27 causes increased survival and expansion of these effector cells in HLA-B27+ AS patients (A. T. Chan et al., 2005). In HLA-B27- AS patients, the number of KIR3DL2+ NK and CD4+ T cells are not significantly increased compared to controls. Additionally KIR3DL2+ NK cells exhibited an activated phenotype characterized by perforin, β7 integrin and CD38 expression, whereas KIR3DL2- NK cells expressed lower levels of these markers. The activating phenotype is possibly a product of NK cell licensing, which allows NK cells expressing cognate inhibitory KIRs to robustly activate in response to activating signals (Cruz-Munoz & Veillette, 2010). As such, I would expect patients expressing AS-protective ERAP1 polymorphisms that cause reduced KIR3DL1 interaction to have less KIR3DL1+ NK and CD4+ T-cells than patients expressing AS-associated ERAP1 polymorphisms. Whether these cells are activated may depend on environmental triggers, but because ERAP1 polymorphisms affect KIR3DL1 affinity it may also affect NK cell licensing and as such affect NK cell activation.

It would be important to examine this hypothesis by quantifying the percentage of KIR3DL1+ NK cells in HLA-B27+ AS patients with the aforementioned ERAP1 mutations. Additionally, the KIR3DL1 affinity for pHLA expressed by the patients could be assessed using recombinant KIR3DL1-Fc staining, as described in this thesis. This thesis provides the necessary mechanistic background to interpret results of the future study. However, the study could be complicated by the fact that NK cells generally express more than one type of KIR that binds to MHC class I molecules and as such it would be difficult to separate the effect of KIR3DL1 from that of other cognate KIRs.
Figure 33. AS protective ERAP1 polymorphisms both decrease KIR3DL1 interaction but do so by up-regulating either pHLA or FHC.

If in fact there is a direct relationship between protective ERAP1 polymorphisms leading to reduced KIR interaction with HLA-B27, the interaction between KIR and HLA-B27 could be targeted in AS patients as a therapeutic intervention. Optimal doses of KIR blocking antibodies could be administered to patients as a means of blocking KIR-HLA interaction and may lead to reduced activated NK cell/CD4 T-cell proliferation and as a result reduced pro-inflammatory cytokine secretion.
5.2 MHC Class I cross-linking enhances KIR3DL1 interaction

A particularly exciting finding was that antibody binding to MHC class I increased KIR3DL1+ NK cell affinity for target cells. The implication of this finding is that, if MHC class I antibodies are found in human serum, they could bind to antigen presenting cells and potentiate KIR3DL1 function in NK and CD4+ T-cells as mentioned in the previous paragraph. Interestingly, AS-associated ERAP1 cells have increased affinity for KIR3DL1 after blocking with anti-HLA-B27 antibodies, compared to protective ERAP1 expressing cells, and therefore the effects on KIR3DL1 expressing cells mentioned in the previous paragraph would be amplified in patients expressing AS-associated ERAP1 (Figure 23). This finding also brings back into question the molecular mimicry hypothesis of AS pathogenesis. The hypothesis postulates bacterial pathogens such as *Klebsiella* share homology with HLA-B27 epitopes and thus when these pathogens infect HLA-B27+ individuals they trigger an antibody response, which not only acts against the infecting pathogen, but also cross-reacts with HLA-B27 (Rashid & Ebringer, 2012).

A few studies have shown the presence of HLA-B27 autoantibodies in the serum of AS patients and that they cross react with *Klebsiella pneumonia* peptides and *Proteus mirabilis*, to name a few (Ogasawara, Kono, & Yu, 1986; Schwimmbeck, Yu, & Oldstone, 1987; Tani et al., 1997). The antibodies implicated in *Klebsiella pneumonia* studies are reported to be IgG or IgA antibodies (Stone et al., 2004; Tani et al., 1997). Several studies have shown monoclonal HLA-B27 antibodies cross react with bacterial pathogens such as *Yersinia enterocolitica, Klebsiella pneumonia* and *Shigella flexineri* (van Bohemen, Grumet, & Zanen, 1984). An intriguing aspect of this hypothesis is that it provides a simple connection between pathogen infection and AS onset which could explain why only ~10% of HLA-B27 positive individuals develop AS (Braun et al., 1998). The hypothesis is however very controversial and there are conflicting reports on the importance of serum antibodies to pathogens implicated in AS (Stone et al., 2004).

Nonetheless, the results of this research implicate cross-linking of MHC class I molecules in potentiating KIR3DL1 interaction, but whether it is occurring in patients due to anti-MHC class I antibodies or due to some other factors that cause MHC class I cross linking is worth investigating. To this end, it would be interesting to see if AS patients in fact up-regulate serum
antibodies that cross-link MHC class I. The HLA-B27 target cells utilized in this study could be incubated with patient and control serum and KIR3DL1-Fc affinity analyzed. If in fact there is an up-regulation in KIR3DL1-Fc binding, my findings provide a mechanistic basis for such a phenomenon.

5.3 Clinical applications

Although the relationship between AS and HLA-B27 is clear cut, with some studies showing up to 90% of AS patients having HLA-B27, this relationship is complicated by the fact that only ~10% of HLA-B27 positive individuals go on to develop AS (Braun et al., 1998; Brewerton et al., 1973). Therefore, simply targeting HLA-B27 in therapeutic interventions, and especially in light of its important function in the immune system, is not a prudent choice. In addition GWAS studies have since shown several other genes are also associated with AS, though not as strongly as HLA-B27. Studies have also shown patients with SpA have significantly increased signs of bowel inflammation, suggesting an imbalance in gut microbiota may also contribute to the disease (Asquith, Elewaut, Lin, & Rosenbaum, 2014). However, despite indications of imbalance in microbiota and the possibility of a pathogen trigger, antibiotics are not commonly used in treating AS, with the exception that some studies show sulfasalazine improves clinical symptoms and only one study showing the efficacy of moxifloxacin, an antibiotic against Gram negative and positive bacteria but the mode of action of these agents may be due to anti-inflammatory functions as opposed to targeting specific bacteria (Dougados et al., 2002; Ogrendik, 2007) As such, both genetic and exogenous factors likely combine to contribute to AS pathogenesis and complicate the search for appropriate therapeutic interventions.

Commonly used interventions against AS include non-steroidal anti-inflammatory drugs (NSAIDS), disease modifying anti-rheumatic drugs (DMARDS), corticosteroids and biologic drugs targeting specific proteins involved in inflammation. All these interventions generally target inflammatory pathways to relieve symptoms of AS. Standard NSAIDs such as ibuprofen are cyclooxygenase enzyme inhibitors have been used to relieve inflammatory back pain (Sieper, Braun, Rudwaleit, Boonen, & Zink, 2002). The efficacy of NSAIDs in relieving core AS symptomatic outcomes (e.g., physical function, pain, spinal mobility, spinal stiffness/inflammation and patient’s global assessment) have shown to improve these outcomes (Dougados et al., 2002). However, NSAIDs are considered short-term relief from symptoms and
in general symptoms reappear soon after withdrawal of treatment and a significant number of AS patients do not achieve a satisfactory clinical response with NSAIDs. In some cases NSAID treatment coincides with adverse gastrointestinal effects (Song, Poddubnyy, Rudwaleit, & Sieper, 2008).

DMARDs are used as a second line of treatment when patients become refractory to NSAIDs. Some examples of DMARDS include the aforementioned sulfasalazine as well as methotrexate, anti-malarial drugs, gold and azathioprine. The mechanisms by which DMARDs exert their effects are varied and often poorly understood, as in the case of sulfasalazine, but include reduction in circulating CD8+ T-cells (azathioprine), transcription factor binding inhibition (gold) and decrease in chemotaxis of polymorphonuclear cells (methotrexate) (Munster & Furst, 1999). Studies show that in general DMARDs have been ineffective in relieving AS clinical symptoms (Sieper et al., 2002).

Corticosteroids may also be used as treatment for patients refractory to NSAID treatment. Corticosteroids diffuse through the cellular membrane and bind to glucocorticoid receptors to form a complex that subsequently localizes to the nucleus. The complex binds response elements in DNA and recruits coactivator molecules that induce histone acetylation and subsequent DNA transcription (Barnes, 2006). It is presumed the primary mechanism by which corticosteroids reduce inflammation is to induce the expression of anti-inflammatory genes through the aforementioned mechanism. However, within the context of inflammatory diseases, it is hypothesized corticosteroids exert their anti-inflammatory functions by repressing expression of inflammatory genes, contrary to its well-known mechanism of transcription activation, although the mechanism is as of yet unclear. It is hypothesized that the mechanism may involve histone deacetylation or repression of activating transcription factor binding to repress the genes in question. Corticosteroid administration has been shown to relieve clinical measurements such as stiffness, pain and mobility (Dougados et al., 2002). Despite its beneficial short-term anti-inflammatory effects, corticosteroids have a wide range of adverse side effects that affect normal skin, endocrine, metabolic and immune function (Stanbury & Graham, 1998).

The latest treatments of AS involve the administration of biological agents targeting TNF-α. Because of the importance of TNF-α in inflammation, interventions targeting TNF-α have been successful in improving disease activity and function indices (Goh & Samanta, 2012).
Commercially available TNF-α inhibitors target production in subtly different ways. Etanercept is a soluble TNF-receptor construct that exerts its action by binding available TNF-α and therefore preventing binding to immune cell subsets expressing TNF-receptor. Infliximab is a chimeric monoclonal anti-TNF antibody that similarly binds available TNF-α to inhibit its downstream effects. Adalimumab is a fully human anti-TNF monoclonal antibody that works in a similar fashion as infliximab (Keyser, 2011). For patients refractory to TNF inhibitors other biologics such as rituximab (B-cell depletion) and various non-TNF cytokine-inhibiting monoclonal antibodies are currently under investigation (Goh & Samanta, 2012).

Although several current therapies have been useful for improving AS symptoms, they have significant drawbacks. Patients may become refractory to specific interventions and may be more susceptible to infections due to the general immunosuppression functions of therapies. Future interventions may need to target specific immune cell subsets, rather than target proteins such as TNF that exert their effects on a wide range of immune cells. In order to target specific immune cells subsets, interventions must recognize, in concert, several markers that distinguish them from other cells. Current monoclonal antibody therapies are unable to do that. If for example, certain KIR-expressing CD4+ T-cells are expanded in AS patients, as mentioned previously, it would be more effective to target those specific subsets at sites where they are overexpressed. Therapies may include genetically modified cells, which express receptors for the targeted immune cell markers, and at the same time deliver proteins that destroy these cells upon contact. An example of such a cell could be genetically modified NK cells or macrophages that have innate cytolytic function.

The focus of this thesis was to determine molecular mechanisms that may be pathogenic in AS. The goals and findings in this thesis are relevant to early stages of developing clinical applications, given the fact that it is based on basic cell culture models. Findings must be extrapolated to animal models and relevance in AS patients must be determined before developing clinical applications. Nonetheless, we can predict potential future applications based on the therapy model outlined in the previous paragraph. This study shows that KIR3DL1-positive cells may be important in AS patients expressing specific ERAP1 polymorphisms and thus, perhaps KIR3DL1-expressing NK or T-cell subsets may be potential targets for therapy. Specifically, it appears low KIR3DL1 interaction with HLA-B27/ERAP1-expressing target cells.
may be protective in AS. Therefore, one application of this finding may be to deplete KIR3DL1+ immune cells, in a similar manner to that of the B-cell depleting biologic, rituximab. However, further research needs to be done to identify KIR3DL1+ immune cell subsets that can be targeted in AS patients, before genetically modified cells, or perhaps even bioengineered vesicles with similar function, can be created and used in therapeutic interventions. The second part of this thesis shows anti-HLA-B27 antibodies may also be pathogenic in AS. However, we must first determine the presence of elevated amounts of anti-HLA-B27 antibodies before concluding their significance in AS. If in fact patients have elevated amounts of anti-HLA-B27 antibodies, they could also be targeted with recombinant proteins to neutralize these antibodies. The possible applications are numerous but there exists the need to further investigate the significance of these findings in patients, as outlined, before we can develop applications.


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in ankylosing spondylitis implicates peptide handling in the mechanism for HLA-B27 in disease susceptibility. Nature Genetics, 43(8), 761-767. doi:10.1038/ng.873; 10.1038/ng.873


Hammer, G. E., Gonzalez, F., James, E., Nolla, H., & Shastri, N. (2007). In the absence of aminopeptidase ERAAP, MHC class I molecules present many unstable and highly immunogenic peptides. *Nature Immunology, 8*(1), 101-108. doi:http://dx.doi.org.myaccess.library.utoronto.ca/10.1038/ni1409


of Sciences of the United States of America, 108(19), 7745-7750.
doi:10.1073/pnas.1101262108; 10.1073/pnas.1101262108

Kollnberger, S., Bird, L., Sun, M. Y., Retiere, C., Braud, V. M., McMichael, A., & Bowness, P.
Arthritis and Rheumatism, 46(11), 2972-2982. doi:10.1002/art.10605

Immunology, 9(5), 495-502. doi:10.1038/ni1581; 10.1038/ni1581

variegated KIR gene expression: Polymorphisms of the bi-directional KIR3DL1 promoter
are associated with distinct frequencies of gene expression. PLoS Genetics, 4(11),
e1000254. doi:10.1371/journal.pgen.1000254; 10.1371/journal.pgen.1000254

Limón-Camacho, L., Vargas-Rojas, M. I., Vázquez-Mellado, J., Casasola-Vargas, J.,
Moctezuma, J. F., Burgos-Vargas, R., & Llorente, L. (2012). In vivo peripheral blood
proinflammatory T cells in patients with ankylosing spondylitis. The Journal of
Rheumatology, 39(4), 830-835. doi:10.3899/jrheum.110862

killer cell responses: Integration of signals for activation and inhibition. Annual Review of
Immunology, 31, 227-258. doi:10.1146/annurev-immunol-020711-075005;
10.1146/annurev-immunol-020711-075005

and negative signals in cytotoxic lymphocytes regulates the polarization of lipid rafts during


May, E., Dorris, M. L., Satumtira, N., Iqbal, I., Rehman, M. I., Lightfoot, E., & Taurog, J. D. (2003). CD8 alpha beta T cells are not essential to the pathogenesis of arthritis or colitis in
HLA-B27 transgenic rats. *Journal of Immunology (Baltimore, Md.: 1950)*, 170(2), 1099-1105.


doi:10.1038/nri2359; 10.1038/nri2359


doi:10.1002/art.22295


leukocyte antigen B. *Nature, 479*(7373), 401-405. doi:10.1038/nature10517; 10.1038/nature10517


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