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Roles of ERAP1 and PPARγ in the development of arthritis

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A importância de ERAP1 e PPARγ para o desenvolvimento de artrite

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor David Moulin, Investigador Sénior CR1 do Centre National de la Recherche Scientifique e da Doutora Luísa Helguero, Investigadora Auxiliar do Departamento de Química da Universidade de Aveiro.

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palavras-chave

PPARγ, ERAP1, Artrite, Artrite Reumatóide, Espondilite Anquilosante, transferência de sérum K/BxN.

resumo

A inflamação das articulações, conhecida por artrite, é uma doença que afecta mais de 175 milhões de pessoas no mundo e, além de ter um grande impacto na qualidade de vida dos doentes, constitui uma carga monetária elevada. Mutações em ERAP1 – uma aminopeptidase comummente encontrada no RE e responsável pela apresentação de péptidos ao sistema imunitário – e PPARγ – um receptor nuclear mais frequentemente associado com o processamento de ácidos gordos e glucose – foram previamente associadas com um maior risco de desenvolvimento da doença. Com este trabalho, estudaram-se as funções de ERAP1 no desenvolvimento de artrite experimental induzida por sérum K/BxN e de PPARγ num modelo generalista de inflamação, tanto em mastócitos como em macrófagos. Em mastócitos, PPARγ aparenta ter um papel pró-inflamatório, visto que células KO libertam significativamente menos IL-6. Já em macrófagos, a ausência do receptor faz com que as quantidades da citocina encontradas no sobrenandante sejam consideravelmente maiores, apontanto para uma acção anti-inflamatória de PPARγ neste tipo celular. Quando à acção de ERAP1, esta enzima mostrou-se crucial para o desenvolvimento de artrite induzida por sérum K/BxN, visto que ratinhos KO não apresentaram sintomas artríticos, ao contrário dos WT. Finalmente, animais ERAP1$^{-/-}$ envelhecidos podem ser um modelo espontâneo de espondilite anquilosante.

keywords

PPARγ, ERAP1, Arthritis, Rheumatoid Arthritis, Ankylosing Spondylitis, K/BxN serum transfer.

abstract

Arthritis affects over 175 million people in the world, heavily decreasing the patients' quality of life and representing a considerable financial weight. Mutations in ERAP1, an ER aminopeptidase involved in peptide presentation to the immune system, and PPARγ, a nuclear receptor most associated with fatty acid and glucose metabolism, have previously been associated with a higher risk for arthritic development. In this work, ERAP1's activity in arthritis was assessed using K/BxN serum transfer, an experimental model of arthritis. PPARγ's role in general inflammation was also examined in both mast cells and macrophages. It appears that, in mast cells, PPARγ has a pro-inflammatory effect, resulting in a lower release of IL-6 by KO cells. On the contrary, larger amounts of the cytokine were found in supernatants of PPARγ$^{-/-}$ macrophages, suggesting an anti-inflammatory effect of the receptor in these cells. Regarding ERAP1, the enzyme's presence proved to be crucial for the development of K/BxN-induced arthritis, since KO mice were not affected by the exposure to the serum. Finally, ERAP1$^{-/-}$ mice might be a spontaneous model of ankylosing spondylitis if these are heavily aged, around 40-weeks-old.

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Abbreviations and Acronyms

AS – ankylosing spondylitis

ER – Endoplasmic reticulum

ERAP1 – Endoplasmic Reticulum Aminopeptidase 1

HLA-B27 – Human Leukocyte Antigen B27

IFN-γ – Interferon-γ

IL-1RII – Interleukin-1 Receptor type II

IL23R – Interleukin-12 Receptor

IL-6R – Interleukin-6 Receptor

LAMC - L-leucine-7-amido-4-methylcoumarin

LPS – Lipopolysaccharide

MEF – Mice Embryonic Fibroblasts

MHC – Major Histocompatibility Complex

TAP - Transporter associated with antigen processing

TNFR – Tumour Necrosis Factor Receptor

RA – Rheumatoid Arthritis

COX – Cyclooxygenase

BMMC – Bone Marrow-derived Mast Cell

BMM – Bone Marrow-derived Macrophage

I. Introdution

Arthritis is a group of conditions that includes more than one hundred different diseases[1], all characterized by the impairment of the joints and surrounding tissue, but different in their causes, mechanisms of action and molecules involved. It is estimated to affect over 175 million people in the world[2], with 10% of the world population above 60 years old presenting symptoms of arthritis[2]. It costs governments and people about 1500 euros per person and per year[3], which totalises almost 250 billion euros every year. Osteoarthritis, rheumatoid arthritis, gout, ankylosing spondylitis and juvenile arthritis represent some of the most common forms of this pathology[1]. For the purpose of this report, emphasis will be given to rheumatoid arthritis and ankylosing spondylitis.

1. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory auto-immune disease and its most striking features are joint swelling followed by a destruction of the synovial joints, an outcome that heavily affect patients' lives[4,5]. These complain of pain, stiffness and swelling of the articulations. It is estimated that RA might have an incidence of about 25 cases in each 100.000 Europeans[6] and 75 in every 100.000 Americans[7]. Women are particularly affected, with an incidence of approximately double of that found in men[6,7]. Whereas the latter are unlikely to present symptoms of RA before the age of 45, after which the probability of developing the disease rises abruptly[6], women usually present symptoms earlier in life and rarely develop the disease after the age of 80 years-old[6,7].

The accepted criteria for RA diagnosis were updated in 2010[8], substituting the previous 1987 classification[9]. Currently, the disease is diagnosed if the patient presents with symptoms that include synovitis of at least one joint, serological presence of rheumatoid factor (RF) or anti–citrullinated protein antibody (ACPA) and abnormal levels of an acute-phase response, it being erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP)[8]. To allow for a more accurate diagnosis, symptoms should also be present for more than six weeks[8]. Among other molecules, tumour necrosis factor α (TNFα)[10], interleukins (ILs)-1[11] and -6[12,13] and cyclooxygenases (COXs)-1[14,15] and -2[14,15] were found to be either over-expressed at a mRNA level or present in quantities higher than normal. The auto-immune aspect of RA comes from the presence of auto-antibodies, such as RF and ACPA,

which are often detected in the patients' sera years before other clinical manifestations[16-18]. Despite the large knowledge about RA mediators, the cause for this disease has been unknown. Recently, however, some authors have proposed that a Gram-negative bacterium most often associated with oral pathologies, *Porphyromonas gingivalis*, could influence the development and the severity of RA[19,20]. They defend that the pathogen citrullinates terminal residues or arginine[21], thus exacerbating the immune response. This association explains the fact that smoking individuals are more likely to be positive for ACPA[22] and to be diagnosed with RA[23], since several studies have connected the practice of smoking with the presence of periodontal pathogens[24,25]. Nonetheless, the hypothesis fails to explain the origin of RF and it seems more likely that *P. gingivalis* would only increase the severity of the disease rather than cause it on its own.

RA is currently managed with disease-modifying anti-rheumatic drugs (DMARDs)[8], a group that is mainly composed of TNF inhibitors but also contains molecules that suppress the action of IL-1. An early attack on the disease can potentially be beneficial[26], but currently changing diagnostic criteria and ambiguity of some of the parameters make it difficult to detect initial stages of this pathology. More research on the origin of RA, stable diagnostics criteria and large screenings of RF and ACPA in asymptomatic individuals could potentially help unveil new targets for the treatment and management of this disease.

2. Ankylosing Spondylitis

Ankylosing spondylitis (AS) is an inflammatory disease belonging to the group of spondyloarthropathies, in which, traditionally, four other diseases – reactive arthritis, psoriatic arthritis, inflammatory bowel disease-associated spondyloarthropathy and undifferentiated spondyloarthropathy – are also included. According to the new diagnostics criteria, AS is now the prototype disease for axial spondyloarthritis[27]. It is characterized by a chronic inflammation of the axial skeleton, pelvis and possibly peripheral joints. It is thought to start by an inflammation of the enthuses – the points at which the ligaments, tendons or muscles insert into the bones – which leads to a slight erosion of the surrounding osseous tissue. The latter calcification to correct such erosion is too accentuated and the joints eventually fuse with the bone. Aside from pain, this causes difficulty of movement that might include expansion of the chest, due to the compromising

of the joints between the ribs. The onset of the symptoms is usually between late teens and early twenty's but it may also be before 10 or after 30[28].

Spondyloarthropathies affect 1,7 to 2,7 millions of caucasian adults in the USA[29] and the latest study on AS' prevalence estimates that 5 in each 1000 Europeans were diagnosed with this disease[30]. Aside from the incidence and prevalence of the disease, the quality of life of each patient is also heavily altered as it progresses. There is an association between AS and sick leaves, both paid and unpaid work productivity, retirement, depression and social isolation. In the UK, 40% of the patients with working age are unemployed[31] and, in Central America[32] and Canada[33], respectively 39% and 46% of the patients are classified as unable to work. Overall, patients go on permanent work disability three decades before the retirement age[32]. A Dutch study reported a loss of about 5000€ per patient and per year only due to work instability[34]. Work productivity decreases in about 21% for patients with AS[33] and 42% of patients in the Netherlands reported the need of help in basic daily responsibilities[34]. Similar conclusions arised from a French report[35].

Since AS is a genetic disease[36], most of the research has revolved around candidate genes that were possibly deregulating normal mechanisms. The strongest association with AS and other spondyloarthropathies is Human Leukocyte Antigen B27 (HLA-B27)[37-39]. About 90% to 95% of the patients with AS express this gene[40,41] and it represents 37% to 50% of the risk for developing the illness[42,43]. However, it is now known that other factors must be involved, given that only 1% to 6% of the individuals that tested positive for HLA-B27 develop AS[37,44,45]. Two other genes are also thought to have a role in the development of this pathology: interleukin-23 receptor (IL23R) – whose SNPs, namely rs11209026[43], have been associated with AS[43,46] – and Endoplasmic Reticulum Aminopeptidase 1 (ERAP1)[39,47]. After HLA-B27, ERAP1 is the second most significant gene, as mutations in the protein's sequence account for 26% of the risk of developing AS[43].

3. The Major Histocompatibility Complex (MHC) pathway

An essential part of immunity is the presentation of specific antigenic peptides to T lymphocytes. This allows the immune system to recognize and eliminate cancerous, virus infected or damaged cells and it is intrinsically dependent on the proper functioning of

peptide presenting machinery. There are two possible and distinct pathways used by MHC class I and class II molecules in order to exhibit peptides to CD8+ and CD4+ T cells, respectively. The MHC class I pathway is present in all nucleated cells and its chief purpose is to expose their internal proteome, while MHC class II molecules react mainly with extracellular pathogens, like bacteria. In both, the presented peptide is stably integrated as part of the fully folded protein.

In mammals, nearly all proteins are degraded by the proteasomes[48,49] and this proteolysis is the biggest source of ligands for MHC class I molecules[48,50]. In fact, this is a key step in the peptide presentation pathway, since inhibition of the proteasome also inhibits the referred process[50]. There are three types of proteasomes. The most ubiquitous form is commonly known as the 26S proteasome and comprises the catalytic 20S core and two 19S regulatory subunits. This form can also be referred to as the constitutive proteasome. In response to particular stimuli, like interferon-γ (IFNγ)[51], the core assembly is made differently and the complex is then called immunoproteasome due to its role in inflammation, as the name suggests. Another form is known as thymus-specific proteasome and expressed in thymic epithelial cells (TECs)[52]. The alternative proteasomes have different patterns of peptide degradation[53,54], as expected considering the distinct role they play in the organism. The peptides generated through this degradative process often have the correct carboxylic terminal (C-terminal) to bind to MHC class I molecules, however the amino terminal (N-terminal) is rarely ideal after this step[55-58]. Aside from this complex, there is little or no carboxypeptidase activity in the cytoplasm[56] and N-terminal trimming has to be conducted by aminopeptidases, given that blocking the N-terminal also blocks the digestion process[55,58]. These N-extended peptides are generated preferentially by both proteasome[58] and immunoproteasome[58-61], instead of the mature epitope, which provides evidence that these might be important intermediates in antigen presentation. Indeed, some authors postulate that such peptides have a higher survival rate in the cytoplasm and are thus more likely to be presented in the surface of the cell[58,59,62]. Despite the equal digestion rate of proteasomes and immunoproteasomes, the former have considerably lower efficiency – 60% to 100% less – in generating antigenic precursors and that supports their primary part of degrading proteins to amino acids[58]. Only occasionally these peptides are able to escape proteasomal degradation and be displayed as antigens.

95% of the peptides produced in this first step have 2 to 25 residues in length[62,63] and although this is a critical stage in MHC class I peptide presentation, proteasomes and immunoproteasomes (from now on to be indistinctly designated as proteasomes) destroy more epitopes than they create. As described below, MHC class I molecules preferably bind to peptides between 8 and 10 amino acids in length and only 15% of the peptides generated by the proteasome have this size, while 20% are longer and 60% to 70% are too short[58,62,63] (Figure 1).

As mentioned, proteasomal degradation is capable of generating some antigenic peptides but most of them still need additional cleavages by aminopeptidases[48,55,64]. It is in the lumen of the endoplasmic reticulum (ER) that this next step takes place and thus an intermediate phase is required in which the peptides are transported to this organelle. Transporter associated with antigen processing 1 and 2 (TAP1 and 2) dimerize, forming the TAP complex that selects peptides of about 7 to 20 amino acids long[65-67], although it binds with higher affinity to those between 9 and 16[65,68,69], which comprise mainly precursors and not mature epitopes[67,69]. Peptides with 20 amino acids have about 50% less affinity to TAP than those with 11 amino acids[65]. The fact that longer peptides have lower affinity for the enzyme makes them more susceptible to cytosolic degradation[65] and this in turn affects the MHC class I pathway.

Some MHC class I molecules bind to 8 residue long peptides while others prefer substrates that have 9 or 10 amino acids[65,70,71]. Given that the proteasome generates peptides between 2 and 25 residues long, TAP transports those that are 7 to 20 and MHC class I molecules need 8 to 10 residue epitopes, there must be a cleavage in the ER prior to the final binding. In fact, authors have supported this hypothesis by showing that peptides that are not efficiently transported to the ER still appear on the surface of the cell[67]. More specifically, MHC class I molecules repeatedly present epitopes that contain proline in one of the first three positions, even though TAP is not capable of transporting peptides with such characteristics[69,72]. These evidences led to the search of ER aminopeptidases and ERAP1[73] and, later, ERAP2[74] were described. Humans have both these enzymes, while mice do not possess ERAP2[74,75], though there are some evidences that the latter might have an ER aminopeptidase other than ERAP1[75]. The genes for these two proteins are localized head to head in chromosome 5[76] and their role is to trim peptides in the N-terminal, therefore contributing to the antigenic repertoire of the cell[73,74]. It appears that ERAP2's

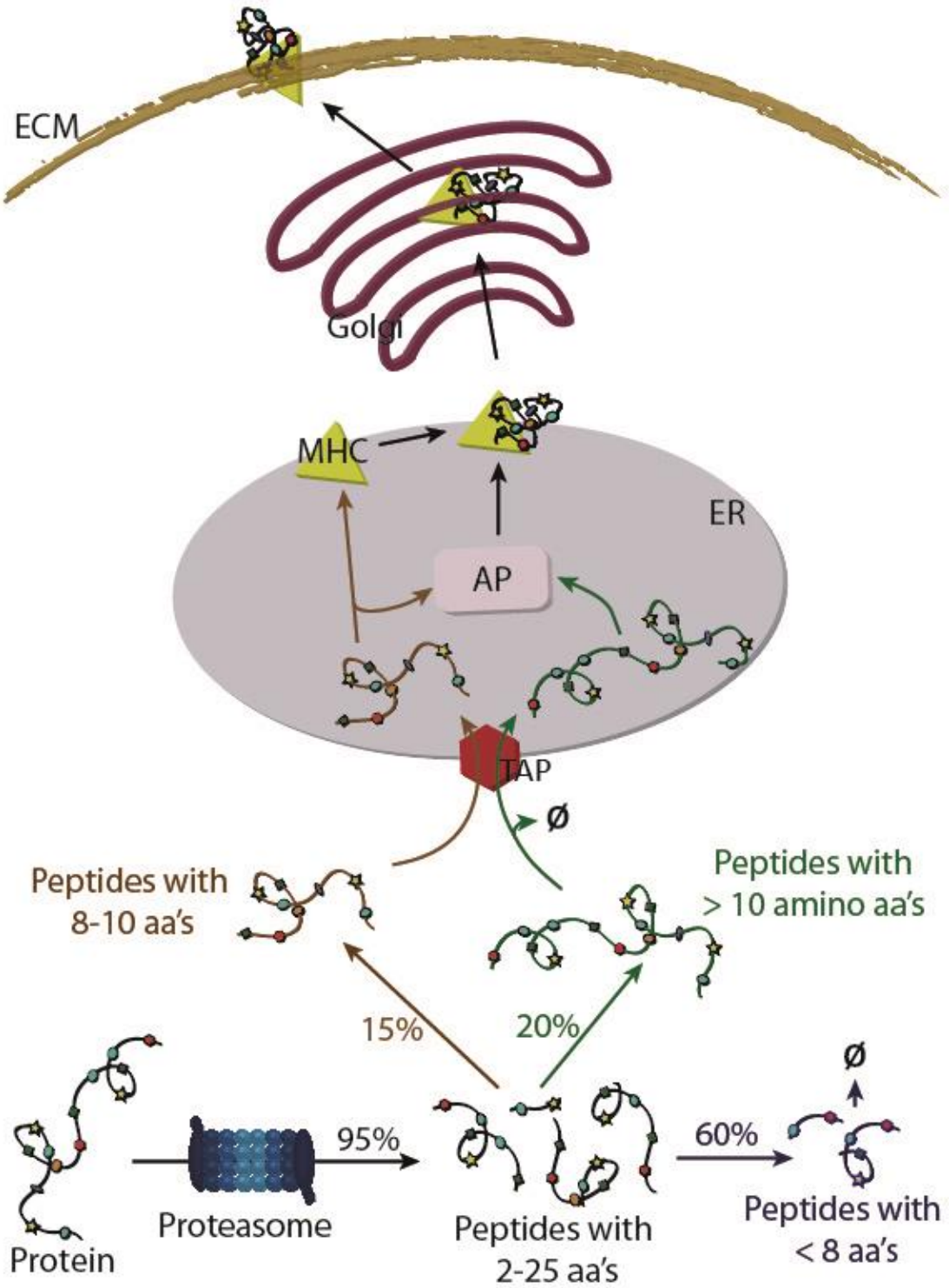


Figure 1: MHC class I peptide presentation. After proteasomal degradation, peptides between 7 and 20 amino acids can bind to TAP and be transported into the ER, where they bind to MHC class I molecules with or without suffering and intermediate aminopeptidasic step. These are then presented to T lymphocytes, on the surface of the cell. ECM, extracellular matrix; MHC, Major Histocompatibility Complex class I molecule; ER, Endoplasmic Reticulum; AP, aminopeptidases; TAP, Transporter Associated with Antigen Processing; Ø, cannot bind to TAP.

main function is to decrease the presentation of epitopes by cleaving them to peptides that are too small for MHC class I binding (S.-C. Chang, N. Bhutani and A.L.G., unpublished results, reviewed in [77]). On the other hand, ERAP1 has a unique mechanism, discussed below, that makes it essential to the generation of epitopes of the proper length. If incorrectly cleaved, these epitopes will not form stable complexes with MHC class I proteins[64]. When bound to these[67] or glycosylated[65], antigenic molecules remain stable, whereas free epitopes are rapidly exported from the ER[65]. Therefore, in order to be exposed on the surface of the cell, peptides must survive proteolytic destruction – from the proteasome, cytosolic peptidases and ERAP2 – and compete for TAP binding, ERAP1 trimming and MHC class I incorporation.

Considering all that has been said, it seems that these ER aminopeptidases, particularly ERAP1, together with other peptide processing machinery have a critical role in maintaining the balance between protection against infectious diseases and immunological tolerance to somatic peptides. Variants of both ERAP1 and ERAP2 have been associated with ankylosing spondylitis[43,46,78].

4. ERAP1

Endoplasmic Reticulum Aminopeptidase 1 (ERAP1)[73], also known as ER Aminopeptidase Associated with Antigen Processing (ERAAP)[79], Aminopeptidase Regulator of Tumour Necrosis Factor Receptor type I shedding (ARTS-1)[80] and Puromycin Insensitive Leucine Specific Aminopeptidase (PILS-AP)[81], was first described in 1999 as Adipocyte-derived Leucine Aminopeptidase (A-LAP)[82]. Given that it is a key enzyme in MHC class I peptide presentation – a process that occurs in all nucleated cells –, its expression was found in most cells types[81,82], with higher levels in lung, liver, thymus and spleen – tissues that also express greater concentrations of MHC class I molecules[79].

4.1. Structure, the “molecular ruler” model and size-dependent cleavage

ERAP1 belongs to the M1 family of metalloproteases[82,83] which are characterized, among other features, by the presence of GAMEN and HEXXH$(X)_{18}$E motifs[a] and a

[a] GAMEN: Gly-Ala-Met-Glu-Asn; HExxHx18E: His-Glu-Xaa-Xaa-His-$(Xaa)_{18}$-Glu

catalytic zinc atom[83]. It comprises four protein domains – I, II, III and IV – with a large cavity between domains II and IV. This is the largest cavity known for a protein so far, suggesting easy access for longer substrates[84]. Similarly to other members of the M1 family, the zinc atom, the catalytic centre and the GAMEN and HEXXH$(X)_{18}$E motifs are found in domain II, in a thermolysin-like conformation[84]. It is in this catalytic region that may be found one of the differences between the two human ER aminopeptidases. The amino acid in the position 181 is glycine (Gln) for ERAP1 and aspartate (Asp) for ERAP2. Because this residue is located in the top of the catalytic pocket, a conversion of such type will alter its specificity by making it longer and more acidic[84,85]. Domain I is made entirely of β-barrels that fit on top of domain II. This "tunnel" allows the interaction with terminal amino acids of the substrate peptide, thus completing the catalytic pocket[84]. Domain III is the smallest domain of the four, also rich in β-barrels[84,86] and located in the middle of subunits II and IV[84]. The last domain, IV, is the most variable one of the M1 group, with 10 to 17 α-helices[84]. In ERAP1, it has 16 helices of variable size arranged side by side in a way that creates a concave structure facing the catalytic centre[84,86]. Unlike other sister proteins, ERAP1 has its fourth domain positioned rather far from the active centre[84], resulting in the previously mentioned uncommonly large cavity.

There are at least two possible conformations for this protein, one less active than the other[84,86]. It is hypothesized that the conversion between these two forms is regulated by a centre distinct from the catalytic pocket but physically close to it[84]. The open state of the enzyme, although possible, is not catalytically favourable. Instead, by moving particular domains, ERAP1 will acquire a more closed conformation that locks the substrate in, facilitating cleavage[84]. Even in this closed form, the concave shape of the C-terminal domain assures that the enzyme is able to accommodate large peptides. It is fairly established that the conformational change of the enzyme is responsible for its activation, by positioning the highly conserved tyrosine residue located upstream from the GAMEN motif (Tyr438) towards the catalytic centre[84,87]. The substitution of this residue by phenylalanine (Phe) decreases ERAP1's activity by 190 times[84]. Similarly, mutations in the equivalent conserved residue in *S. cerevisiae*'s leukotriene A4 hydrolase lead to a significant loss of the enzyme's activity, without affecting its affinity to the substrate, due to a drop in the stabilisation of the intermediate product[88]. So, why is it an advantage to have both open and closed conformations? The open form facilitates the capture of the

substrate. After binding, ERAP1 will switch to the closed conformation, clasping the peptide and rotating the Tyr residue, all of which favour catalysis. As mentioned, the interactions that prompt conformational conversion are not yet clear but, as it will be explained ahead, this is probably due to the occupation of a regulatory domain, critical for the protein's function in the MHC class I pathway. The region of this regulatory centre is not yet known but it is thought to be in the C-terminal domain[86], specifically near helices 20 and 22[84], 20 Å away from the catalytic zinc atom, a distance that is consistent with its "molecular ruler" function that is yet to be discussed in this paper. This distance will allow the enzyme to select peptides 9 to 11 residues long, but longer peptides may also bind to ERAP1 in a non-stretched position, *i.e.*, "wrinkling" central portions of the substrate that still fit into the large enzyme's cavity[86]. Comparing with other members of the metallopeptidases' M1 family, homology happens only in the catalytic centre located in the N-terminal domain[86,89], but though the architecture may be unequal, the binding strategies are alike, namely through the enzyme's interaction with both terminals of the substrate, allowing flexibility in the size and sequence by wrinkling[70,71]. Hence, both the protein's structure and the fact that it is activated by longer precursors allow ERAP1 to trim longer peptides while sparing shorter ones that might have the correct length to bind to MHC class I molecules.

This distinctive catalytic process that is allosterically activated and identifies both the peptides' size and the amino acid content of the C-terminal is titled as the "molecular ruler" mechanism[90] and, to the present knowledge, no other aminopeptidase has the ability to generate peptides of specific length. The first indication that ERAP1 had a functioning of such sort was the fact that it showed a preference for longer peptides[73] between 9 and 16 amino acids[73,74,90,91]. In fact, the enzyme hydrolyses precursors until they are left with 8 or 9 residues, losing its activity for peptides below that size[90,91]. Regardless of the N- and C-terminal sequence, approximately 50% of the precursors are cleaved to 9-residue-long epitopes, while the other 50% have a final length of 8 residues[90]. Unlike other aminopeptidases, that prefer substrates shorter than 4 amino acids, ERAP1 shows maximum activity for substrates with 9 to 12 amino acids, even though 16-residue-long peptides are still efficiently processed[90]. However, precursors with 18 amino acids already have a low rate of cleavage and 20- and 30-residue-long substrates are barely cleaved[90]. Unexpectedly, a different set of substrates pushes ERAP1's maximum activity towards

peptides with 10 to 14 amino acids[90]. In an experiment aimed to clarify the enzyme's peculiar mechanism, the amount of peptides with 10, 11 and 12 residues was measured in a time-dependent manner. The results revealed an increase in these peptides' amounts followed by a decrease, emphasizing their intermediary role in the trimming reaction[90]. Subsequent research that measured the concentrations of epitopes or epitopes' precursors with 8 to 11 amino acids concluded that peptides with 11 residues are the most rapidly trimmed, followed by 10- and 9-residue-long substrates, respectively. During this process, the percentage of epitopes with a length of 8 amino acids increased and the discontinued trimming was not determined by a permanent loss of enzyme's activity, since the supplementation with more suitable peptides yielded again 8-residue-long products[73]. It appears that ERAP1's apparatus evolved to assist in immune surveillance, fitting into the specificities of both TAP and MHC class I molecules. As mentioned before, researchers believe that a regulatory centre not far from the catalytic domain allosterically activates the enzyme's trimming activity. To be efficiently cleaved, the peptide needs to be able to position itself both in the catalytic pocket and the regulatory domain, so that the C-terminal induces the change in conformation that will in turn accelerate the cleavage of the N-terminal placed near the zinc atom[84,86,90]. Accordingly, smaller peptides will not be able to stabilize the more active closed conformation, making its processing much slower. However, small molecules have been shown to activate the catalysis of other short peptides by binding to the regulatory centre while the catalytic pocket is occupied by one of them[84,86,90] (Figure 2). The ovalbumin epitope SIINFEKL, a non-ideal substrate for ERAP1, induces hydrolysis of L-leucine-7-amido-4-methylcoumarin (LAMC)[92]. Even though smaller peptides might facilitate the trimming of others, this is not the case with large size precursors, given that the occupation of the regulatory centre will prevent binding of the substrate's C-terminal, inhibiting its hydrolysis[84]. This provides further evidence for the proximity between regulatory and catalytic cores. Despite the fact that it all points to a very well adapted mechanism adopted by ERAP1, this enzyme might also destroy epitopes by degrading them below the minimum length required for MHC class I binding[91]. However, given its inability to trim peptides shorter than 8 amino acids *in vitro*, it seems unlikely that *in vivo* this will happen. Still, about 90% of the molecules presented by MHC class I proteins are 9 or 10 amino acids[93], which means that in some cases ERAP1 might cleave and destroy epitopes. Length preferences could potentially attenuate this

effect, *i.e*, the fact that ERAP1 trims 11-residue-long peptides faster than those with 10 or 9 amino acids could direct its binding towards precursors instead of mature epitopes. Nonetheless, proximity to the enzyme due to recent catalysis also has to be taken in consideration. So, on a basal level, there is a high chance that ERAP1 will destroy MHC class I epitopes with 9 or 10 residues[90], but, when overexpressed, the total amount of these epitopes was only slightly diminished[90]. Presumably, binding to MHC class I proteins protected them from further trimming[91]. Even though it may seem contradictory to eliminate epitopes on a basal level, these events most likely play a crucial part in avoiding exacerbated inflammatory reactions against somatic molecules - autoimmunity. Ultimately, "the concept of a molecular ruler is that ERAP1 trims down to a final core size (…) [that] may or may not result in a mature epitope"[93].

4.2. Substrate specificity

ERAP1 was first described as a leucine aminopeptidase, due to its preference for substrates with this N-terminal residue[73,82]. Despite these initial findings, the enzyme is capable of trimming a large scope of residues[73], displaying however a higher affinity for hydrophobic amino acids instead of charged or hydrophilic ones[94], which may be explained by the presence of a hydrophobic region near the terminal amino group[82]. Remarkably, proline is the only amino acid that ERAP1 is incapable of removing[64,95,96]. It quickly trims leucine and methionine, while glutamate, aspartate, tryptophan, arginine, cysteine and glycine are harder to remove[74,90,93,94,96], possibly because aside from being hydrophobic, the catalytic pocket is shallow and occupied by the lateral chains of Ser316, Met319, Gln181 e Gln183[84]. Thus, leucine or methionine can easily fit into the core, while larger amino acids, like tryptophan, have to position themselves in a very specific manner, even though they are able to form the same kind of interactions. On the other hand, cysteine and glycine are too small to stably fit into the catalytic pocket[84]. The C-terminal content also plays a role in ERAP1 specificity, as this enzyme selects peptides that have hydrophobic residues in this terminal as well[90]. The cavity that is thought to be regulatory has a strong electronegative potential that can account for the fact that negatively charged peptides are poor substrates of ERAP1[94]. Amino acids with positive charges however are similarly slowly trimmed[90]. Specifically, peptides with lysine, arginine and aspartate on C-terminal have a much smaller cleaving rate than when alanine, tyrosine or leucine are in the same position[90]. It could be that the regulatory centre is mainly electronegative but with

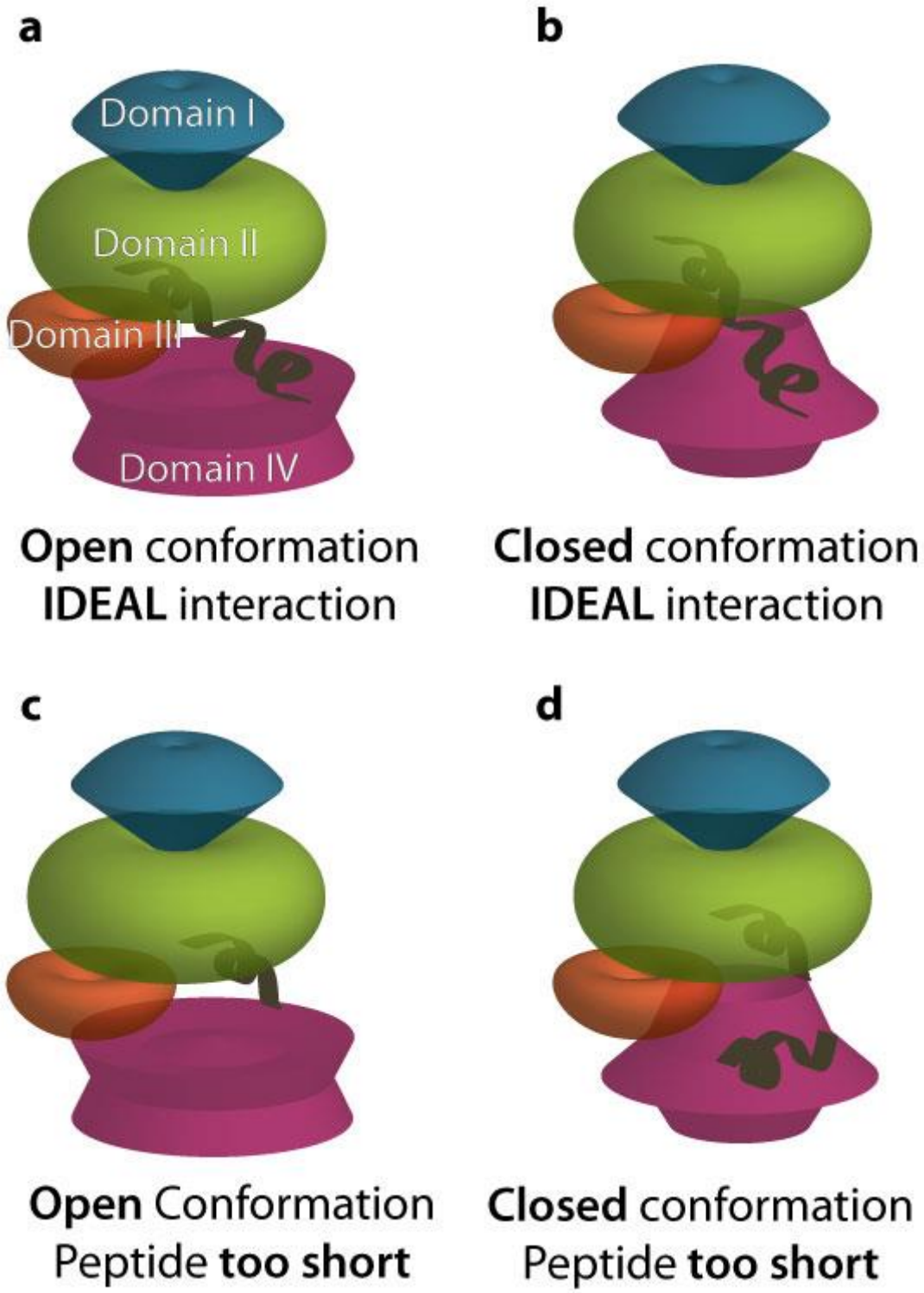


Figure 2: ERAP1's molecular ruler mechanism. ERAP1's structure is optimized to trim longer peptides that can bind to both to the catalytic pocket and the regulatory center (a), thus activating the protein (b). Since shorter peptides cannot simultaneously bind to domains II and IV, these are slowly trimmed due to the inability to convert the enzyme into its more active closed form (c). However, if another short peptide binds to the regulatory center, the enzyme changes its conformation, closing the opening between domains II and IV and rotating an important tyrosine residue (not shown), therefore facilitating catalysis (d). Figure based on data from [75,84,86,90,94].

a few hydrophobic residues that reduce the stability of this core, causing it to seek other hydrophobic-residue-containing peptides that can therefore reduce the potential energy of the complex. Some authors hypothesize that the presence of small depressions near the catalytic centre, thought to be responsible for anchoring amino acids' side chains, can also account for C-terminal preferences[84]. The presence of a free carboxylic group, however, does not appear to play any role in the enzyme's specificity, since the substitution of leucine for leucine-amide did not alter the velocity at which the peptide was hydrolysed[90]. A free N-group, on the other hand, is vital to the enzyme's function. Acetylation of this group completely arrests ERAP1 trimming activity, as it happens with other aminopeptidases[90]. Aside from N- and C-terminal selectivity, residues in internal positions also affect specificity[94,97]. It was already said that proline was not tolerable if located in the first position of the N-terminal but this is also true if this residue appears in P2[93], because its side chain cannot accommodate in the catalytic pocket without disrupting the highly conserved GAMEN motif[84]. It is not surprising that immunoproteasomes preferentially generate peptides that have a hydrophobic or positively charged C-terminal or, in other words, acidic residues rarely appear on the C-terminal of precursors after immunoproteasome cleavage, unlike basic and hydrophobic ones[58,98]. Indeed, these peptides are favoured by TAP[67,69], ERAP1[90,94] and MHC class I molecules[93], demonstrating once again the synchronism present in MHC class I pathway machinery and exalting the role of each of these complementary steps.

5. ERAP1's polymorphisms and disease

Often there is a correlation between gene polymorphisms and disease. Because ankylosing spondylitis is a genetic disorder, it is fair to assume that the cause can perhaps lie on a mutation of such sort. Six ERAP1 polymorphisms were found to be associated with ankylosing spondylitis: rs27044[43,47,92,99-101], rs30187[39,43,46,47,92,99,101-103], rs10050860[43,47,99,101,104], rs2287987[43,47,99,101,104], rs17482078[43,101,104] and rs27037[101]. Out of all of them, rs30187, a missense mutation that alters the lysine528 for arginine[99,100], appears to be the most significative one[39,47,92] followed by rs27044[47,92]. The rs30187 variant of ERAP1 affects the trimming of the epitopes' precursors, thus disturbing MHC class I antigen presentation[39,92]. This SNP is located in a coding region[46] and causes a 4-

fold decrease in the catalytic activity of the enzyme, when compared to the native form[92,102]. Rs27044 allele in turn consists of a switch of glutamine by glutamate in the residue 730[99,100]. Both these polymorphisms and the native form are able to generate antigenic peptides *in vitro*, though the cleaving activity varies from one to the other[92]. The enzyme with the rs30187 SNP is the least active of the three against LAMC, followed by the native form and the one carrying the rs27044 allele, this last one representing the most active one of the three enzymes. However, when a small activating peptide was added to the reaction mixture, rs30187-containing enzyme suffered the biggest increase in activation while the one with the rs27044 SNP was the least activated of them all, most probably because its architecture was already in a favourable state for catalysis. In the presence of this small peptide, the native form is the most active one with small difference from the form carrying rs27044[90]. So, extrapolating, rs27044-containing ERAP1 is active even when there is no sufficient activation, possibly destroying epitopes due to a failure of the "molecular ruler" mechanism. On the other hand, as described[92,102], the enzyme that incorporates the rs30187 mutation has lower activity than the native form, diminishing the presentation of some peptides while others are increased, something that will be further discussed in the topic *Modulation of ERAP1 and its Consequences*. In Caucasian ankylosing spondylitis patients from the United Kingdom, alleles rs30187 and rs27044 are present with a significatively higher frequency than in control patients, while rs10050860, rs17482078 and rs2287987 are more likely to be found on the control group than on the one diagnosed with ankylosing spondylitis[43]. Rs10050860 allele possesses an asparagine instead of aspartate in residue 575, rs17482078 switches arginine725 for glutamine725 and rs2287987 changes the amino acid 349 from methionine to valine[99,100]. Lastly, all of the polymorphisms that are associated with ankylosing spondylitis are found either on the catalytic centre, C-terminal regulatory pocket – responsible for the enzyme's specificity –, domain junctions – potentially and indirectly altering the enzyme's activity or specificity by altering its conformation or its ability to change between the open and closed form –, gene promoter or boundaries concerning introns and exons[47,84], these last two affecting splicing.

6. Modulation of ERAP1 and its consequences

Due to its critical role in the MHC class I pathway, ERAP1's existence is key for the correct cleavage of peptides, as demonstrated by an experience performed with mice embryonic fibroblasts (MEFs)[75]. Nonetheless, ERAP1 knock out (KO) mice grow normally and present no apparent signs of disease, demonstrating that this is not a vital gene[75]. Also, excluding ER cleavage, MHC class I pathway is not altered in ERAP1-deficient mice[75], which again proves that this is the most important enzyme in one of the last steps of the peptide-presenting machinery. Given the association of ERAP1 mutations with AS and other diseases, ERAP1$^{-/-}$ models are highly used to study potential target processes. In general, mice KO for this enzyme have diminished MHC class I peptide presentation on the surface of the cell[105] by about 20% when compared with controls[77,91]. However, as explained before, ERAP1 deficiency inhibits the presentation of some peptides while enabling that of others (*see Structure, the "molecular ruler" model and size-dependent cleavage*). When it comes to the ovalbumin epitope SIINFEKL, precursors extended by 1 and 3 amino acids have 75% and 54% less breakdown, respectively, and the 12-residue precursor HGEFAPGNYPAL is 40% less trimmed, in ER fractions immunologically depleted of ERAP1[73]. Reduction of the gene's expression by interference RNA also hinders showing of SIINFEKL by MHC class I molecules, for HeLa cells[91]. A contradictory study reported no difference in this epitope's presentation for wild-type and ERAP1-deficient MEF cells[75]. Differences in the cell type can account for this inconsistency, but it seems unlikely that, after all the experiences proving ERAP1's crucial role, this would not be the case in MEFs. More studies with a wide range of cell varieties and epitopes are indispensible. This is in fact a lack in the research on this topic, given that most studies focus solely on ovalbumin precursors and not enough on perhaps more significant ones as well as on live animal models that can experience the under- or overexpression of the enzyme for longer periods of time. For KO thymocytes and B and T cells, MHC class I presentation is reduced[75]. New peptides can be detected on ERAP1$^{-/-}$ mice cells that were not on the WT ones[105]. As said, some epitopes are presented in higher amounts whereas others are less or not at all shown, both for pathogenic proteolytic fragments and endogenous peptides[59,75]. So, changes on the enzyme's activity greatly modify the peptide repertoire of a cell and, consequently, of an organism. Expectedly, the

immune response varies from WT to ERAP1 KO mice. After infection with Lymphocytic Choriomeningitis Virus (LCMV), the ERAP1$^{-/-}$ group grossly exhibited a reduced immune response against some model antigens, compared with the WT subjects[59,106], and when transplanted into control individuals, these cells exert an immunogenic role[105].

Given that ERAP1 modulation conditions the immune response, it is only appropriate to determine if and how the latter influences the former. IFNγ, an inflammatory cytokine, induces MHC class I peptide presentation[73] through ERAP1 overexpression[73,79,91]. This result is valid for the tumour model cells HeLa, U937 and SW620, without disturbing the abundance of mRNA belonging to other aminopeptidases[73]. When wild-type HeLa cells are treated with IFNγ, the conversion from 9- to 8-residue peptides is accentuated[73]. Immunodepletion of ERAP1 completely stops this phenomenon[73] and diminishes antigenic presentation[91]. Other factors like conversion of proteasome into immunoproteasome – which generates preferentially longer peptides[93,107] – and over-expression of TAP[80,91] are also IFNγ dependent and complement the enzyme's role. Because generation of smaller antigens from longer ones is dependent on ERAP1's presence, its loss leads to reduced and increased inflammatory action, respectively if referring to peptides with 8 or 9 to 10 amino acids[75]. So, the enzyme is an important regulator of antigenic peptide presentation, enhancing or reducing inflammatory response according to the epitopes.

Both ERAP1[108] and ERAP2[109] are secreted when over-expressed in human cells, presumably due to saturation of proteins responsible for the aminopeptidases' retention[110]. In normal circumstances, ERAPs bind to other molecules that prevent their exit from the ER. However, once inflammatory molecules like IFNγ are released, these aminopeptidases are over-expressed, thus overloading their retaining molecules. In a free state, they can leave the ER[109]. Through production of chimeric proteins, Hattori *et al.* concluded that the exon 10-containing sequence between 485 and 615 was responsible for ERAP1's confinement to this compartment[111]. The highest levels of secretion happen when cells are exposed to both IFNγ and lipopolysaccharide (LPS)[108]. Research shows that monofactorial treatments cannot fully stimulate this process[108]. IFNγ induces the over-expression of ERAP1 but not its release from the ER[84,92,108]. On the other hand, LPS does not enhance the levels of the enzyme but seems to be responsible for its presence in the extracellular medium[108]. So, in order for the enzyme to be secreted, low concentrations of LPS and IFNγ

are required, causing a 1.5-fold increase in the aminopeptidasic activity of the extracellular medium[108]. These results suggest that IFNγ is mainly responsible for ERAP1's over-expression while LPS induces its secretion. Brefeldin A (BFA), a molecule that prevents protein transport from the ER to the Golgi complex, highly decreases the aminopeptidase's secretion, exposing the importance of these cell structures in the transport of ERAP1 and, consequently, in the processes it is involved in. Different forms of the protein exist in the ER and extracellular medium, with 105 kDa and 115 kDa respectively[108]. Altered sugar chains account for the observed weight disparity[108]. After 48h, both forms are found in the extracellular matrix most probably due to release of the lower molecular weight form from dying cells[108].

Summarizing, moulding of ERAP1's properties further demonstrates its importance for MHC class I presentation, enhancing or reducing inflammatory response by shifting immunodominance patterns.

7. Alternative localizations and functions of ERAP1

Subcellular localization of ERAP1 has been a target for debate over the past years. Some authors refer to it as an ER protein[82,84,89,92], while others place it on the cell membrane[55,94] or claim it is secreted[112]. In mice, however, it appears that the enzyme is present only in the ER. Co-localization with KDEL sequence proves ERAP1's presence in the ER, both for mice[108] and human cell lines[73]. In bronchial cells, ERAP1 was found to be a type II transmembrane protein that co-immunoprecipitates with Tumour Necrosis Factor Receptor I (TNFRI)[80]. Although it is found mainly in the ER, the alternative locations of the enzyme suggest involvement in different processes, according to the situation.

Indeed, aside from functioning in peptide presentation, ERAP1 is associated with the shedding of TNFRI, Interleukin-1 Receptor type II (IL-1RII) and Interleukin-6 Receptor (IL-6R). The enzyme binds to the extracellular domain of TNFRI, promoting its cleavage, *in vitro*[80]. Despite its aminopeptidasic role, ERAP1 is not responsible for the cleavage of this receptor[80]. Instead, it complexes with nucleobindin 2 and RNA-binding motif protein X chromosome (RBMX)[113,114], supposedly helping other metalloproteinases in their shedding function[80]. It was proposed that abnormal activity of ERAP1 could result in a decrease in the levels of soluble cytokines' receptors, therefore increasing the

biological activity of these inflammatory molecules[110]. However, no relationship exists between serum cytokine receptors' levels and three of the most significant ERAP1 polymorphisms – rs27044, rs10050860 and rs30187 – in patients with AS[115]. This is probably due to the fact that ERAP1 is not the enzyme that actually cleaves the receptors and therefore its activity might not be crucial. The same paper concluded that TNFRI is cleaved as a response to inflammatory molecules[115], generating decoy receptors that neutralize TNF. As mentioned, ERAP1 is also involved in the shedding of IL-6R[116] and IL-1RII[117]. Similarly to what happens with TNFRI, the enzyme interacts with the extracellular domain of IL-1RII[117]. In this case, however, it is more likely that ERAP1 directly cleaves the receptor, given that cells KO for ERAP1 present no basal shedding of IL-1RII, while those that overexpress the peptidase have increase shedding, less membrane-associated receptor and decreased IL-1β biologic activity[117]. Cleaving the extracellular domain of IL-1RII generates soluble proteins capable of binding to IL-1, thus preventing the cytokine's excessive pathway stimulation[117]. Type I receptor's levels (IL-1RI), on the contrary, are not affected by the enzyme[117]. So, ERAP1 is associated with the shedding of some receptors either by directly cutting them or indirectly promoting their cleavage. More information is needed on how the receptor is cleaved when ERAP1 is not the main player.

As aforesaid, the enzyme's location is arguable and some authors defend its presence in the extracellular matrix as a secreted protein[108,112] (see *Modulation of ERAP1 and its Consequences*, third paragraph). There certainly seem to be evidences for that but ERAP1's role in those cases is not entirely known. It could merely be a side-effect of inflammation due to saturation of the retaining proteins but could also have an impact in cancer metastasization, through the degradation of the extracellular matrix. A proven effect of ERAP1 in the extracellular medium is the activation of macrophages. Goto *et al.* have found that, after an inflammatory stimulus, ERAP1 is excreted from the macrophages' ER and this process allows the cells a higher phagocytic activity[108]. To date, nothing else was found on this topic, so more research will be needed in order to clarify ERAP1's potential functions.

8. Hypotheses on why ERAP1 is related to AS

As seen, polymorphisms both on ERAP1 and ERAP2 are correlated with AS. Abnormal peptide processing can lead to unstable peptide-MHC complexes, susceptible to misfolding[118]. In fact, improperly folded HLA-B27 molecules tend to accumulate in the ER triggering stress in this compartment, which can culminate in a proinflammatory response termed Unfolded Protein Response (UPR)[119-121]. The activation of this pathway first arrests protein translation and increases the production of molecular chaperones. If this approach is not successful, UPR will direct the cell towards apoptosis. Aberrant peptide processing, due to ERAP1's mutations, is very likely to alter peptide repertoire, as described above, and thus represent a disequilibrium in somatic arthritogenic molecules, *i.e.*, the presence of abnormal peptides on the surface of cells can potentially lead to an auto-immune response. All of the above mentioned mechanisms somehow affect inflammatory processes. An inflammation of the bone ligaments prompts bone erosion and the repairing process induces calcification that, if too accentuated, translates in AS. It is plausible that the presence of soluble cytokine receptors prevents, to some extent, an uncontrolled inflammatory response. Therefore, non-shedding of receptors, likely to be associated with ERAP1's function, could be a mechanism by which inflammation is increased (Figure 3). Aberrant inflammation has innumerous side-effects, one of which is presumably the secretion of ER proteins, mentioned earlier. This increased extracellular aminopeptidasic activity could further enhance the damage done to the bone. Furthermore, extracellular ERAP1 has been said to activate macrophages' phagocytic activity[108]. Although further studies examining this mechanism should be conducted, this is yet another possibility to how ERAP1 contributes to AS.

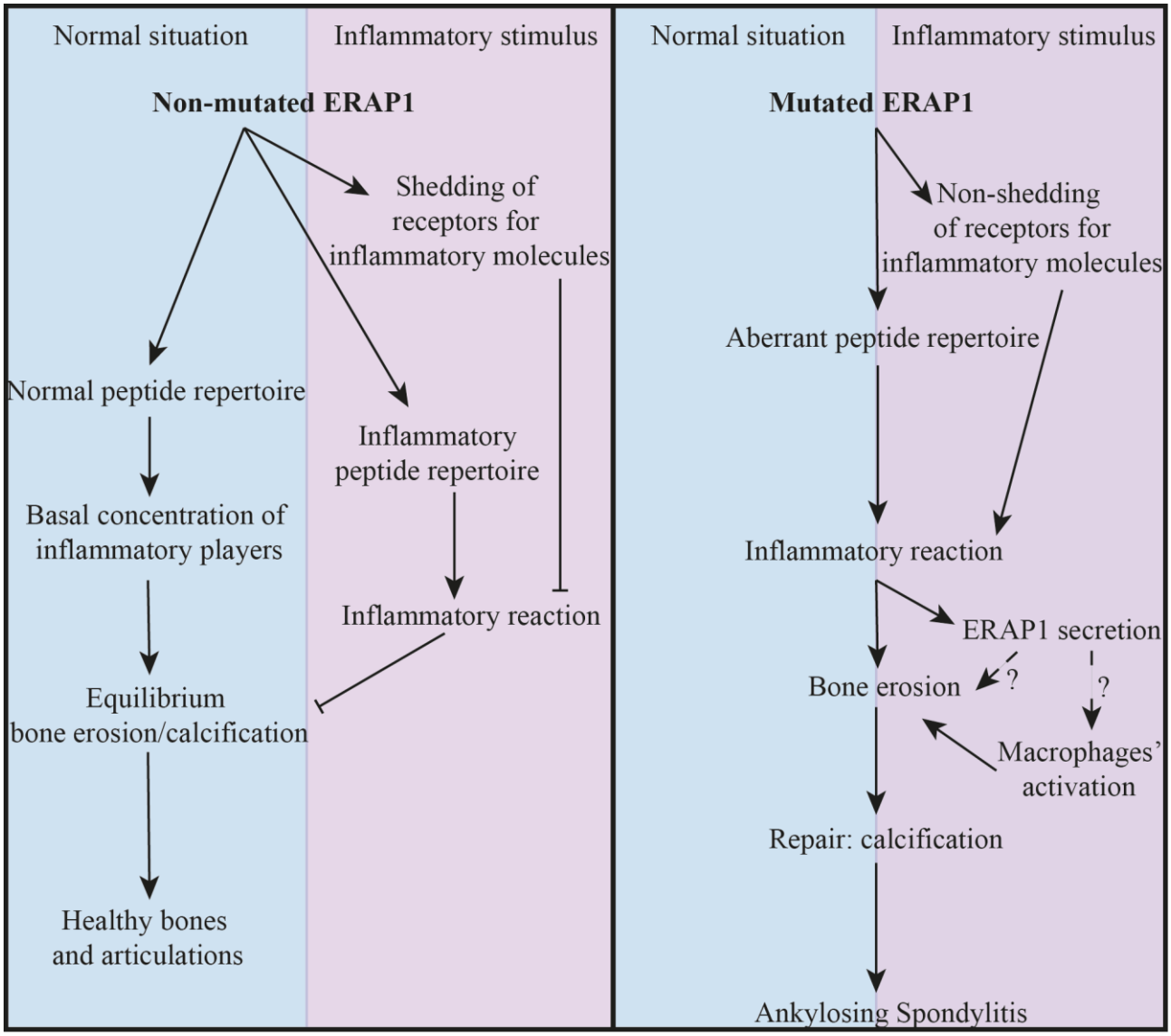


Figure 3: Possible ERAP1 involvement in ankylosing spondylitis. With a non-mutated ERAP1, the cells' internal peptides are correctly cleaved and presented to lymphocytes in patterns that the body recognizes as normal. Therefore, no inflammatory signals are generated and a balance between bone anabolism and catabolism is maintained. When there is an inflammatory stimulus in a healthy organism, the ensemble of peptides presented by the cells change and an inflammatory reaction begins. In order to negatively regulate this phenomenon, ERAP1 cleaves some of the inflammatory receptors, thus reducing the amount of inflammatory signalling. However, when ERAP1 has its functioning compromised, the peptide repertoire is aberrant whether there is a stimulus or not, turning inflammation into a constant rather than an exceptional situation. Additionally, the fact that the receptors are not cleaved further enhances the extent of the inflammation, leading to a destruction of the surrounding bone that can later lead to ankylosing spondylitis. Finally, because inflammation is so accentuated, the ER retaining molecules become saturated and ERAP1 is released into the extracellular medium, where it might imaginably play a role in bone erosion either directly or by inducing macrophages' phagocytic activity.

9. ERAP1 and other diseases

Alternative forms of ERAP1 have been correlated with several diseases other than AS. An impaired ability to present self- and pathogen-derived peptides can lead to inadequate immune responses. Multiple sclerosiss[103], arterial hypertension[122], diabetes *mellitus*[123], hemolytic uremic syndrome[124], pre-eclampsia (non-direct relashionship)[125], certain forms of cervical cancer[126-128] and psoriasis[97,129] are some of the diseases that showed an association with the enzyme. While its role in Crohn's Disease (CD) remains uncertain, the allele rs30187 has been significatively correlated with multiple sclerosis (MS)[103], hemolytic syndrome[124] and hypertension[122]. The exchange of a lysine for arginine in position 528 decreases ERAP1's activity[102] (see *ERAP1's polymorphisms and disease*). Because the enzyme is responsible for angiotensin II inactivation and conversion of kallidin to bradykinin[112,122], this reduction in activity leads to vasodilation and, consequently, hypertension. The same variant of the protein seems to induce pre-menopausal osteoporosis in Japanese women, which suggests that this enzyme may be of importance for the bone's catabolic/anabolic metabolism[130]. ERAP1-defficient mice are unable to correctly process the peptides produced by the protozoa *Toxoplasma gondii* and die from infection when exposed to this pathogen[131]. A similar mechanism is conceivable for cancer spreading, if a certain polymorphism diminishes MHC class I presentation and is therefore responsible for a reduced immune surveillance. The numerous and diverse diseases that ERAP1 is associated with sustain the assumption that the enzyme is involved in multiple pathways and that it may play different and perhaps complementary roles according to its location and environment.

10. PPARγ

Peroxisome proliferator-activated receptors (PPARs) are nuclear proteins capable of regulating the expression of genes. These were initially described as targets for molecules that induce the proliferation of peroxisomes[132], thus their name. To date, three types of PPARs have been identified: PPAR-α, -β/-δ and -γ[132]. Of the three, PPAR-γ is the most widely studied one, essentially for its role in glucose metabolism[133], since peroxisomes are mainly responsible for fatty acid β-oxidation. Due to alternative splicing,

three isoforms of this receptor can be produced: -γ1, -γ2 and γ3. Whereas PPAR-γ1 has a diffused expression – skeletal and cardiac muscles, liver, pancreas, kidney and fat[134] – PPAR-γ2, with 30 more amino-acids at the *N*-terminus, is mainly found in fatty tissue[134] and PPAR-γ3 in fat[135], colon epithelium[135] and peritoneal macrophages[136]. Upon activation, PPAR-γ binds to peroxisome proliferator response elements (PPREs) by dimerization with retinoid X receptor (RXR)[137]. These genomic regulatory sequences are mainly located upstream from genes related with metabolism, but can also be found near those controlling stress response, immunity, development and cell cycle control, among others[138]. This highlights the roll of biological functions that PPARγ is able to control or, at least, have an influence upon. The regulatory action of this receptor is not only attributed to PPRE binding but also to interference with activator protein-1 (AP-1), signal transducer and activator of transcription-1 (STAT-1) and nuclear factor-κB (NF-κB) pathways[139,140]. In the latter scenario, PPARγ is SUMOylated and directed towards nuclear repressor complexes (NCoR) that prevent gene transcription. This interaction inhibits NCoR removal via the ubiquitin/proteasome pathway, subsequently preventing the transcription factor's access to the promoter[140]. A continuous state of repression is therefore maintained. The exposure of human chondrocytes to 15d-PGJ2, a natural agonist of PPARγ, reduced the IL-1β-induced mRNA expression of COX-2 and the release of NO, probably by restricting NF-κB and AP-1 DNA binding[141]. Similarly, in synovial cells from RA patients, this agonist inhibits TNF-α and IL-1β expression and NF-κB activity[142]. So, targeting PPARγ appears as a promising anti-inflammatory therapy. Further proof includes the facts that non-stimulated human monocytes[143] and murine macrophages[139], typical inflammation-associated cells, express low levels of PPARγ mRNA whereas activated ones have high levels of mRNA for this receptor. 15d-PGJ2 treatment of monocytes decreases the release of IL-1β, IL-6 and TNF-α[143,144], while increasing IL-1 receptor antagonist (IL-1Ra) production[143]. When exposed to 15d-PGJ2, IFNγ-activated macrophages have the morphological characteristics of rest cells[139], which means that this receptor may be able to reverse or prevent macrophage activation. In fact, 15d-PGJ2 supresses genes that become over-regulated upon macrophage activation, such as iNOS[139]. However, when an inflammatory stimulus is not provided, agonists have no effect on cytokine release[143]. This could perhaps be due to the receptor's agonist-dependent conformational change and/or dislocation towards an inflammation-related pathway that upon activation by an

inflammatory stimulus will be blocked, at least partially, from transmitting a signal. For example, agonist-induced PPARγ activation could leave the receptor in a favourable state to bind to NCoR, therefore inhibiting inflammatory signalling. Protein-protein interaction studies would help clarify this potential mechanism.

Aside from its localization in the above-mentioned tissues, PPARγ can also be found in cartilage, both at the mRNA and protein levels[145]. In fact, its expression in osteoarthritic (OA) cartilage is 2.4-fold lower than in a normal one[146]. However, in RA synovial lining, macrophages, fibroblasts and endothelial cells, PPARγ is over-expressed[147]. These facts demonstrate that arthritis cannot be treated as a homogeneous group and that each disease has its own mechanisms, possibly independent from one another. On the other hand, some common ground can be found. PPARγ's under-expression in OA is attributed to IL-1 and blocking NF-κB signalling inhibits IL-1 suppression of the receptor's expression[146]. Intraperitoneal administration of 15d-PGJ2 improves experimental RA in rats[147] and an association was found between a PPARγ polymorphism and psoriatic arthritis (PsA)[148]. So, even if the mechanisms are not the same, this receptor seems to be involved in arthritis in general. A therapy with a synthetic PPARγ agonist has been tried in PsA individuals in 2005[149]. Despite the promising results, the study population was small and the drug exerted some side effects, like weight gain and fluid retention. Coupling of a PPARγ agonist with a diuretic has been previously done[150] and a different agonist could resolve the weight gain issues, since PPARγ activation is more often associate with increased fat processing[151,152]. Nonetheless, some authors postulate that the agonists' effects may not always be PPARγ-dependent[153,154] and thus care should be exerted when working with these molecules.

11. Purpose of the work

The present work aimed at elucidating the role of ERAP1 in an experimental model of arthritis. PPARγ's function in inflammation was also examined to a lesser extent.

12. Models and Techniques

12.1. Cell culture and animal colonies

In order to perform scientific studies and to understand a particular process, it is essential to be able to control variables, using models that respect the research ethics. Cell and animal models are thus the best approach known so far, but, as the name implies, these are models and might not always overlap with the real phenomena intended to be studied. For this reason, it is vital to permanently keep in mind the gap that may – and does – exist between these two worlds.

12.1.1. Mast cells and macrophages growth and differentiation

Mast cells are present in several types of tissues and some of their biological roles include phagocytosis, antigenic processing, cytokine production and release of vasoactive peptides. The cells' vasomodulatory properties are mainly due to their histamine-rich granules, a very well-known amine with vasodilatory properties, but can be attributed to heparin as well[155], a glycosaminoglycan also abundant in mast cells' granules. Researchers frequently use this cell type to study immunity[156] and host-defense mechanisms[157], among other processes, given the diversity of adhesion molecules and immune-response receptors that these cells carry, along with the cytokines that they release. Furthermore, mast cells are especially known for their determining part in allergy development[158,159]. Although less frequently, they have also been associated with arthritis[160], owing to the cytokines and other inflammatory mediators that they release and that can lead to chronic inflammation.

Mast cells derive from pluripotential hematopoietic cells in the bone marrow[161,162] and have specific growth conditions. In order to differentiate, they require stem cell factor (SCF)[163,164], a molecule specific for mastocytes because, despite the fact that other cells also respond to it in the beginning of their differentiation process, they need additional lineage-specific growth factors as they mature[165]. This factor induces the proliferation of murine mast cells both *in vitro*[163,166] and *in vivo*[167]. Granulocyte-macrophage colony-stimulating factor (GM-CSF)[168,169], macrophage-colony-stimulating factor (M-CSF)[169] and IFNγ[169] inhibit mast cells' differentiation and transforming growth factor-β1 (TGF-β1) conditions their proliferation[170]. For murine mast cells, IL3[171], IL4[169], IL9[169,172], IL-l0[169,173] and nerve growth factor (NGF)[174] have been shown to promote proliferation. However, in

the absence of IL-3, neither IL-4[169] nor IL-9[172] or NGF[169,174] alone have the ability to promote the proliferation of these cells. Similarly, IL-10 requires both IL-3 and IL-4 for the effective stimulation of the cells' development[173]. So, IL-3 is crucial and sufficient for the growth of mast cells, at least *in vitro*, but the maturation process requires additional factors. A combination of IL-3 and SCF generates populations of cells that most resemble the mast cell phenotype found in connective tissue[167]. This approach was therefore used when obtaining cells for the work described ahead.

Macrophages, like mast cells, are present in several types of tissues. Their main roles are to phagocyte and present pathogens to T-cells during an inflammatory stimulus. Due to the fact that macrophages result from the differentiation of monocytes when these leave the blood, this cell type can directly be obtained from an abundant tissue[175] or from differentiating either bone marrow monocyte precursors[175,176] or blood monocytes[177]. Bone marrow monocyte differentiation into macrophages simply requires the use of a macrophage-specific factor, M-CSF[178]. Macrophage isolation from blood, on the other hand, is a long and complicated procedure and, although in humans it is most likely the best approach due to the facility and little invasiveness of blood collection, with animals alternative options are preferable. Tissue collection is a viable and easy option in animals, particularly peritoneum due to the large dimensions of the tissue and its accessibility. However, macrophage yield is usually of $0{,}5\text{-}1 \times 10^6$ macrophages per mouse[175], a low amount. In order to increase this number, molecules that stimulate the differentiation of monocytes into macrophages, causing the tissue to become rich in these cells, can be injected a few days prior to cell gathering. This modification allows for a simple and productive method, but the physiological characteristics of the macrophages will be different from those observed in cells collected without the stimulation of monocyte differentiation[175]. Therefore, and because bone marrow is also extracted for mast cells' isolation, obtaining macrophages from this tissue is the most appropriate method for the work in question. Moreover, it allows for the generation of uniform cell populations[175], an important characteristic for further biochemical analysis.

12.1.2. Inflammatory stimuli and targeted molecules

Inflammation induction in cellular and animal models is a widely used technique when studying inflammatory processes. LPS is probably the most commonly used inflammatory stimulus. It activates the NFκB pathway culminating in the transcription of an array of inflammatory molecules, among them IL-1β, IL-6 and TNFα. Although not so well known and more controversial[179], IL-33, a member of the IL-1 family, can also induce the release of inflammatory cytokines, including IL-1β, IL-6 and TNFα[180].

12.1.3. The K/BxN mouse model

The K/BxN mouse model resulted from crossing KBN mice, a strain that is transgenic for a T-cell receptor, with a non-obese diabetic (NOD) murine line[181]. Within 25 to 35 days, the resulting offspring presented with swelling and redness of the distal joints, hyperextension of the ankle, valgus deviation of the knee, hyperpronation of the toes and compromised mobility, among other symptoms[181]. After further study, it was noted that these animals also showed oedema under the synovial lining, neovascularization, synovitis, pannus, fibrosis, pyknosis of the chondrocytes and extensive infiltration of inflammatory cells, mainly neutrophils. After several months of disease, there was a large decrease in the inflammation, but the joints had suffered remodelling of their architecture, with the appearance of irregular bone structures – due to bone erosion and uncontrolled restoration –, substantial fibrosis and severe loss of cartilage[181]. The mechanism by which these animals develop spontaneous arthritis depends on the recognition of glucose-6-phosphate isomerase (GPI)[182] and bovine pancreatic ribonuclease (RNase 42–56)[181], the later presented by the mouse's MHC class II molecule H2-IA^k. Since specific haplotypes are responsible for the specificity of the autoimmune reaction[183], the presence of this MHC class II molecule is most likely the factor that directs inflammation towards the joints. The basis of the anti-GPI-induced arthritis, however, has been a cause for research, since this protein is ubiquitously expressed[184]. It appears that, despite the non-specificity of the intracellular location, extracellular and membrane-bound expression of this enzyme is preferentially located at the synovial fluid and endothelial surface of the synovium[182]. Different placement of innate control mechanisms[185] and antibody-antigen complex precipitation in the joints may also explain why these areas are particularly targeted in this model, although this question remains unanswered.

The theory of arthritis induction through antibody action is further sustained in the K/BxN serum transfer model, where serum from K/BxN mice is transferred into animals with a different genetic background[186]. In this model, the recipients develop a transient state of disease that presents itself with the same symptoms as those perceived in mice with spontaneous arthritis. TNF-α and IL-6 were detected in the synovial fluid of K/BxN mice, although the former was only present at a messenger state rather than a protein one. However, later studies, performed by inducing the disease through K/BxN serum transfer, have found that IL-6 has no role in the development of arthritis in this model[187] and that TNF-α, although important, is not essential[187,188], since IL-6$^{-/-}$, TNF-α$^{-/-}$, TNFR1$^{-/-}$, TNFR2$^{-/-}$ and TNFR1/TNFR2-double deficient mice developed arthritis similarly to control ones. IL-1, on the other hand, has proved crucial for the development of K/BxN-induced arthritis. Mice KO for IL-1R did not develop arthritis[187]. They presented with no clinical signs of disease, no histological manifestations of joint inflammation and absence of cartilage destruction and bone erosion[187]. FcγRII/III[189], mast cells[190] and neutrophils[191] have also been reported as central for this model of serum transferred arthritis.

The features presented by K/BxN mice point towards a diagnostics of RA. However, some important hallmarks of the disease in humans differ from the mouse model, notably the presence of rheumatoid factor[181]. So, although this is a valid model for the study of arthritis and its mediators, caution should be exerted when extrapolating the results onto humans, such as with any disease model.

12.1.3.1. The relevance of the IL-1 pathway

The purpose of this work is to evaluate the role of ERAP1 in arthritis development. As previously mentioned, this enzyme has been associated with the shedding of IL-6R, TNFRI and IL-1RII. However, due to the fact that the K/BxN model is independent of IL-6 and only partially dependent of TNF-α, the results of this work will more likely be explained by the cleavage of IL-1RII.

There are three types of IL-1 receptors – IL-1RI, IL-1RII and IL-1RAcP (accessory protein) or IL-1RIII – that can exist either on the soluble or membrane-bound forms. In order for a signal to be transduced through the IL-1 pathway, either IL-1α or IL-1β is required to bind to the membrane-bound form of IL-1RI. The IL-1RAcP is then recruited to dimerize with IL-1R1 and a cascade of events if triggered, activating the NF-κB and AP-

1 pathways. IL-1Ra, an antagonist for the IL-1 receptors, can also bind to IL-1RI, preventing the recruitment of IL-1RAcP and, therefore, the signal transduction[192]. Cleaving of either one of these receptors into their soluble form will also affect transduction, since they lack the intracellular domain essential for response initiation. Type II receptor for IL-1 acts as a decoy receptor, since it has affinity for IL-1 but lacks the referred intracellular domain. Thus, binding to this receptor results in an absence of response. The presence of IL-1Ra, IL-1RII and the soluble forms of the receptors is anything but purposeless, since it helps regulate inflammation, preventing an exaggerated response. In fact, signaling of the IL-1 pathway might naturally activate genes that will up-regulate these molecules, through a negative feedback mechanism[193,194]. IL-1α has a relatively similar affinity to all of these receptors, however, IL-1β and IL-1Ra bind preferentially and almost irreversibly to IL-1RII and IL-1RI, respectively[195]. So, on a normal situation, basal levels of IL-1β will almost completely not bind to the type I receptor, not only because this molecule has a much higher affinity towards the decoy receptor but also because IL-1RI is practically saturated with IL-1 antagonist, all of this thus preventing a signaling cascade (Figure 4). When it comes to the soluble forms of these receptors, IL-1β has an even lower affinity towards soluble IL-1RI (sIL-1RI) than it does for the membrane-bound one (mIL-1RI). Similarly, this cytokine binds with lower affinity for sIL-1RII than for mIL-1RII, although it is still greater comparatively with type I receptor. The difference between soluble and membrane-bound forms of IL-1RII lies with the recruitment of the accessory protein. mIL-1RII, unlike mIL-1RI, does not require dimerization with mIL-1RAcP for effective IL-1α/β binding. sIL-1RII, on the other hand, does require the soluble form of the accessory protein for adequate cytokines' sequestration, much like sIL-1RI[195]. Despite not necessarily dependent of IL-1RAcP recruiting, IL-1RII can engage this receptor, thus seizing not only IL-1β but the accessory receptor as well, both essential for IL-1 signalling. Accordingly, the presence of the decoy receptor in its membrane-bound form plays an important role in the control of the inflammatory response. Thus, IL-1RII shedding by ERAP1 can be predicted to have an impact on arthritis development. However, due to the many players involved, the direction of this effect cannot be anticipated beforehand.

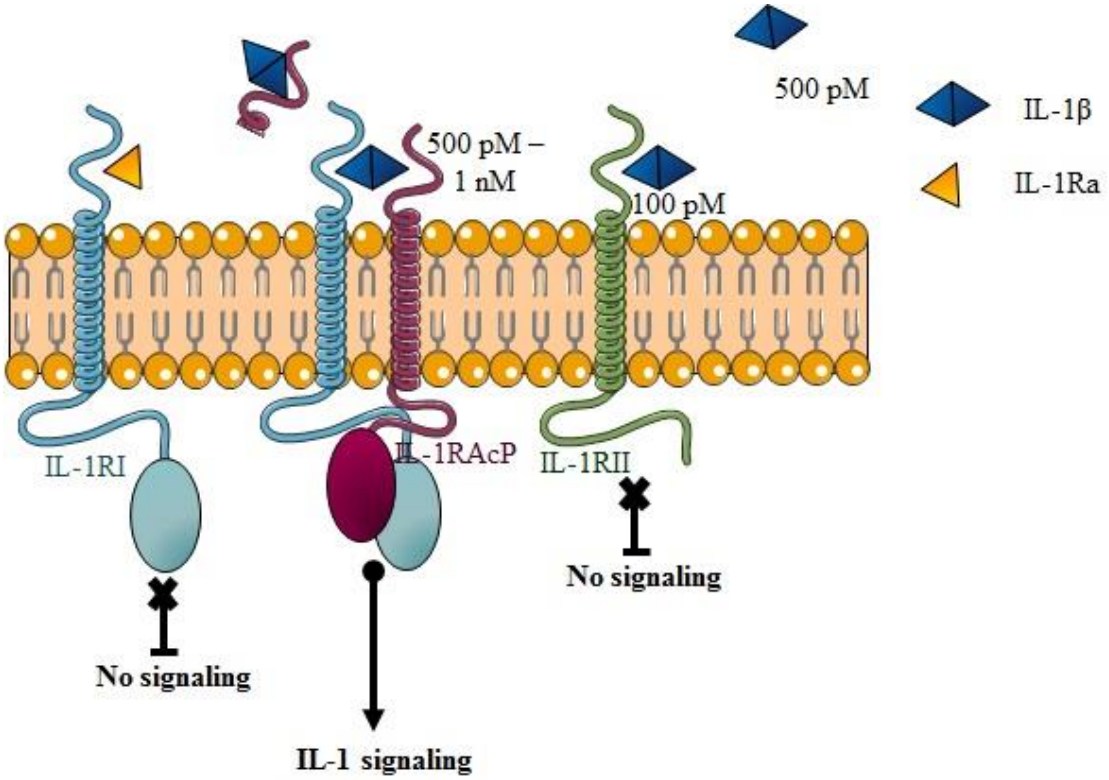


Figure 4: IL-1 signaling pathway. In order for there to be an IL-1 signal, IL-1α/β (IL-1α not depicted) has to bind to IL-1RI, which recruits IL-1RAcP, forming a dimer. Both these receptors possess an intracellular domain that allows signal initiation. IL-1RII, on the other hand, does not have a Toll-Interleukin receptor (TIR) domain and thus cannot initiate any signalling. When bound to this receptor, IL-1α/β is captured and cannot bind to IL-1RI. Furthermore, there is a recruitment of IL-1AcP, crucial for IL-1 signalling, that also stays trapped by binding with IL-1RII. This molecule is therefore a decoy receptor, same as the soluble forms of both IL-1RI and IL-1RII. Aside from decoy receptors, the extracellular medium also contains IL-1Ra, an antagonist of IL-1 that can bind the same molecules as IL-1α/β, although with different affinities, but prevents the recruitment of the accessory protein, and thus, the signal transduction. It has a particularly high affinity for IL-1RI. IL-1β affinities towards each of the receptors are shown, as the amount of cytokine necessary for half-maximum (50%) receptor saturation 153.

12.2. Molecular biology

The study of biological processes, including diseases, very often requires the use of basic molecular biology techniques. In this work, produced and/or released molecules were studied both in their messenger and mediator forms, respectively through real-time polymerase chain reaction (RT-PCR) and western blot (WB) and enzyme-linked immunosorbent assay (ELISA). Due to the characteristics of the K/BxN model, it is also important to study joint features for an accurate disease classification. This can easily be achieved through histological staining of tissue sections. In this section, these techniques will be briefly explained.

12.2.1. Real Time PCR

By targeting a specific DNA region with primers and incubating these molecules with a DNA polymerase, genetic amplification is possible, in a reaction termed PCR. When to the reaction mixture are added either fluorescent dyes that intercalate with the DNA or DNA probes labelled with a fluorophore, the product amplification can be followed in real time. With information regarding the number of cycles used and the concentration of a DNA standard, absolute quantification of the targeted gene can be achieved. Alternatively, relative quantification is obtained by using internal reference genes.

12.2.2. Western Blot and ELISA

When an experience requires the detection of a particular protein, incubating the sample with specific antibodies coupled with either fluorescent or luminescent molecules is an easy and affordable approach. If the antibody has a high specificity with little cross-reaction or if the targeted molecule exists in high amounts in the sample, the substrate medium can be incubated as a whole, using either slot-blot or ELISA. The latter is a very specific method and it requires less antibody than a regular membrane incubation. However, when antibody specificity or protein concentration are low, or when different proteins or different forms of the same protein are the target, it is necessary to perform a separation step prior to antibody incubation. In a western blot, the samples are first separated by size with a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and only after incubated with an antibody directed towards the targeted molecule. Because the polyacrylamide gel is a sensitive material that is hard to manoeuvre, the proteins are transferred into a membrane that has a high protein affinity. The two most common membrane materials are nitrocellulose and polyvinylidene difluoride (PVDF) and although both have approximately equal binding properties for different protein types, PVDF membranes are preferable when working with hydrophobic proteins. They are also more resistant than nitrocellulose though being costlier.

12.2.3. Histology

Histology is the microscopic study of tissues and cells. It can be aided by the use of colorants that stain specific structures. Nonetheless, it is a technique that requires previous

training to be able to identify the tissue or cell morphology and their characteristics. If the sample is in a solution, a simple montage between a microscope slide and a cover slip is sufficient, however, when working with tissues it is necessary to either dissolve them into a cell suspension or section them in thin layers, using paraffin impregnation to allow for the cuts.

II. Materials and Methods

1. PPARγ studies

1.1. Cell Culture

Bone marrow-derived mast cells (BMMC) and macrophages (BMM) were obtained from KO mice by Doctor David Moulin, according to the following protocols. BMMC[196] and BMM[176] were generated from bone marrow of 8-week-old C57BL/6 male mice as previously described. Briefly, mice were sacrificed and intact femurs and tibias were removed. Sterile endotoxin-free RPMI-1640 medium (Gibco®, Life Technologies™) was repeatedly flushed through the bone shaft using a syringe with a 25-G needle. The suspension of bone marrow cells was centrifuged at 250 g for 5 min. Red blood cells were lysed on ice by incubation with Tris 20 mM and NH_4Cl 150 mM during 5 minutes. Precursor BMMC were washed and cultured at a concentration of 0.5 to 1×10^6 cells/mL in RPMI-1640 supplemented with 10% fetal calf serum (Dominique Dutscher), 100 U/mL penicillin (Gibco®, Life Technologies™), 100 µg/mL streptomycin (Gibco®, Life Technologies™), 2 mM L-glutamine (Gibco®, Life Technologies™) and 1 mM sodium pyruvate (Gibco®, Life Technologies™), at 37 °C in a humidified atmosphere with 5% carbon dioxide. A combination of stem cell factor (SCF) (PeproTech®) 50 ng/mL and interleukin-3 (IL-3) (PeproTech®) 5 ng/ml was added weekly to the culture medium for two weeks and 5 ng/mL of IL-3 alone was supplemented weekly during the following weeks. Non-adherent cells were transferred to fresh medium at least once a week. Cells were used after 5–7 weeks of culture, when a mast cell purity of >95% was achieved, as assessed by toluidine blue staining and fluorescence-activated cell sorting (FACS) analysis of c-Kit expression. For BMM differentiation, cells were cultured at 2×10^6 cells/mL in Dulbecco's Modified Eagle Medium (DMEM) (Gibco®, Life Technologies™), supplemented with 10% fetal calf serum (Dominique Dutscher), 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco®, Life Technologies™), 100 U/mL penicillin (Gibco®, Life Technologies™) and 100 µg/mL streptomycin (Gibco®, Life Technologies™). Fresh medium was added at least once a week. A temperature of 37 °C and a humidified atmosphere with 5 % carbon dioxide were also used for incubation and cells were used at first passage.

1.2. Toluidine Blue Staining

In order to examine the heparin- and histamine-filled granules present in mastocytes, and thus evaluate the cells' quality, these were smeared into a microscope slide through cytocentrifugation at 300 g for 10 minutes and then immersed into a solution of toluidine blue in methanol for about 5 minutes. The sample was then dehydrated by passing it from ethanol 95º to absolute ethanol and, lastly, toluene, approximately 1 minute each.

1.3. Effector IL-6

Cells were seeded in a 96-well plate at 100 000 cells/well in 200 µL of complete culture medium. At t = –12h, cells were treated with 0,1% DMSO or Rosi 10 µmol/L. Then, at t = 0h, 0,1 µg/mL LPS or 10 ng/mL IL-33 were added for 24 hours. Cells were centrifuged at 250 g for 10 minutes and the supernatants collected. ELISA IL-6 (Quantikine®, R&D Systems®) was performed on the samples according to the manufacturer's instructions. The results were normalized to the amounts of either DNA (see *Hoechst Assay*) or protein (see *Protein Dosage*). For BMMC, six replicates per condition were made and the experience was performed twice, whereas for macrophages three replicates were used for all conditions and the experience was done once.

1.4. Hoechst Assay

The Hoechst assay was performed as previously described[197]. Cell pellets were diluted in a solution of Tris 10 mM, EDTA 1mM and NaCl 0,1M (Hoechst Buffer) and lysed by freeze/thaw cycles. 0,1 µg/mL of 2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole trihydrochloride trihydrate (Hoechst) (Thermo Scientific®), diluted in Hoechst Buffer, was added to the samples and standards and the fluorescence immediately read at 456 nm, with an excitation wavelength of 348 nm.

1.5. Protein Dosage

Protein was dosed by the Smith assay. Briefly, bicinchoninic acid and copper sulfate 50:1 were added to samples and standards. The reaction mixture was left incubating for approximately 45 minutes at 37 ºC, after which the absorbance was read at 562 nm.

2. ERAP1 studies

2.1. Mice colonies

All mice were treated according to the "Guide for the Care and Use of Laboratory Animals", published by the United States National Institutes of Health (NIH publication number 85-23, revised in 1996) and according to the French legislation (Décret n° 2013-118 du 1er février 2013 relatif à la protection des animaux utilisés à des fins scientifiques). Mice were bought from The Jackson Laboratory® and placed in reproductive conditions whenever necessary in order to maintain the colony. The animals were kept at temp of 20 ± 2 ºC, with a relative humidity of 50 ± 10 %, in a 12:12h light-dark cycle (light from 7 a.m. to 7 p.m.) and fed *ad libitum* with a standard SAFE diet (Scientific Animals Food & Engineering, France). The cages were changed once a week for hygiene purposes.

2.2. Genotyping

Mice were identified and 0,3 cm of each tail was collected and frozen in sterile RNase/DNase free eppendorfs at -80ºC until processing. DNA was extracted, through a hotshot method, as previously described[198]. Briefly, 75 µL of a solution containing 25 mM of sodium hydroxide (NaOH) and 0,2 mM of ethylenediaminetetraacetic acid (EDTA) was added to each tail and incubated at 98ºC for about 1,5 hours. The solution was then neutralized with 75 µL of 40 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCL), centrifuged for 1 minute at 10 000 g and diluted ten times, due to the presence of EDTA, before being separated in a 2 % agarose gel.

2.3. K/BxN arthritis induction

Eleven wild-type C57BL/6 (six females, five males) and ten (seven females, three males) ERAP1$^{-/-}$ mice, all with more than 40 weeks of age, were injected intraperitoneally with 200 µL of serum from K/BxN mice (kindly provided by Doctor Gaby Palma, Cem Gabay group, Medical University of Geneva), on days 0 and 2 of the experience. Throughout days 2 to 6 and 0 to 6, respectively, the clinical scoring of the mice and their paw's oedema were evaluated daily. The clinical scoring of each paw was conducted by Engineer Meriem Koufany, using a 0-3 grading scale based on swelling and the back limb's volume variations were assessed with a digital plethysmometer (LE7500, Bioseb). The mice's weight was also measured on days 0 and 6. On the last day of the experience

(day 6), mice were sacrificed by exsanguination, having previously been anesthetised with a ketamine (65 mg/kg), xylazine (13 mg/kg) and acepromazine (1,5 mg/kg) cocktail, also via intraperitoneal injection. The blood, four paws and skeletons were collected for further experiences.

2.4. Skeleton staining

After gathering the blood and paws, skeletons were cleaned, as much as possible, of skin, organs and muscle tissue, keeping the heart and/or kidneys to facilitate staining assessment. They were then kept in 95% ethanol until the beginning of the staining procedure. 0,03 % m/V of alcian blue, diluted in 76 % ethanol and 20 % acetic acid and 250 mm filtered, was added to each skeleton until cartilage colouring was evident (approximately 35 hours). Next, 95 % ethanol was added again for a minimum of 6 hours, followed by potassium hydroxide (KOH) 2 % until the remaining tissue was substantially but not completely digested (approximately 18 hours). Skeletons were then exposed to $3x10^{-4}$ % of red alizarin in 1 % KOH under mild shaking until bone staining was evident. Excess dye and tissue were removed with 2 % KOH while agitating. For skeleton preservation, these were passed through mixtures of ethanol/glycerol, starting with a glycerol concentration of 50 % until it progressively reached 100 %.

2.5. mRNA extraction

Back left paws were roughly cut with a scalpel and grinded in TRIzol® Reagent (QIAGEN®, Life Technologies™) with an ultra-turrax®. The mRNA extraction was performed according with the manufacturer's instructions. Summarily, clarification was achieved with supernatant collection after centrifugation at 5000 xg for 10 minutes at 4 ºC. Samples were incubated with chloroform and centrifuged 15 minutes at 12 000 xg and 4 ºC to allow for phase separation. RNA was precipitated by adding 100 % isopropanol to the aqueous phase during 10 minutes. After a centrifugation at 12 000 xg and 4 ºC during 10 minutes, the supernatant was discarded and the pellet washed with 75 % ethanol. RNA was finally diluted in RNase free water, quantified and reverse transcripted using M-MLV (Invitrogen™). The protocol was carried according to the manufacturer. RNA was ran through a 1 % agarose gel to verify its purity.

2.6. Real-Time PCR

cDNA was amplified and relatively quantified, using retinitis pigmentosa-29 (RP-29) signal as a reference. The primers follow. COX-2 forward (fwd): 5'-TAC-AAG-CAG-TGG-CAA-AGG-CC-3'; COX-2 reverse (rev):5'-CAG-TAT-TGA-GGA-GAA-CAG-ATG-GG-3'; Melting temperature (Tm) = 60 °C . IL-6 fwd: 5'-CCG-GAG-AGG-AGA-CTT-CAC-AG-3'; IL-6 rev: 5'-ACA-GTG-CAT-CAT-CGC-TGT-TC-3'; Tm =60°C. TNFα fwd: 5'-AGC-CCT-GGT-ATG-AGC-CCA-TTG-A-3'; TNFα rev: 5'-CCG-GAC-TCC-GTG-ATG-TCT-AAG-3'; Tm=59°C. RP-29 fwd:5'-GGA-GTC-ACC-CAC-GGA-AGT-T-3'; RP-29 rev: 5'-GCC-TAT-GTC-CTT-CGC-GTA-CT-3'; Tm=60°C.

2.7. Serum ELISA

Soluble TNFRI (Quantikine®, R&D Systems®) ELISA was performed according to the manufacturer's instructions.

2.8. Histology

Right back and front paws were kept in ethanol 98 % for histological analysis. The paws were then rinsed three times with distilled water for approximately ten minutes each time and decalcification was achieved overnight with Q Path™ Decalcifier DC3 (LABOnord). Following decalcification, samples were again washed with water and fixated with 10 % neutral buffered formalin until further used (minimum of 24 hours). These were then dehydrated in ascending grades of alcohol and impregnated in paraffin. 5 µm sagittal cuts were stained with safranin O and fast green FCF.

3. Graphics and statistical analysis

All graphics and statistical analysis (t-tests and ANOVA) were performed using GraphPad Prism® 5. Normality distribution was assessed and a confidence interval of 95 % was used every time.

III. Results and discussion

1. PPARγ

After an inflammatory stimulus, the expression and release of IL-6 from both bone marrow-derived mast cells (BMMC) and mastocytes (BMM) were measured. For both cell types, PPARγ was shown to have an effect on IL-6 production, although the results were opposite. BMMC that were knock-out for the receptor sported a significant decrease on IL-6 release, after stimulation with LPS or IL-33, when compared to wild-type (WT) cells (Figure 6 and Figure 7). The same tendency was observed with messenger IL-6 (Figure 5), although these results proved not to be statistically relevant, most likely due to a lack of replicates.

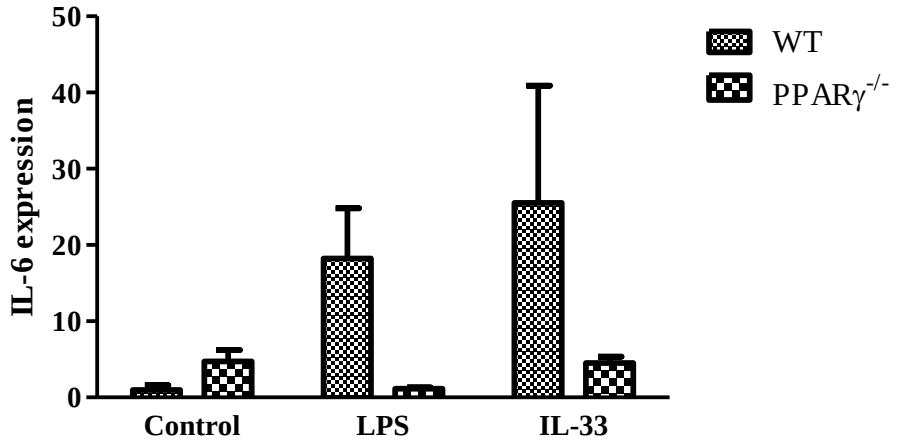


Figure 5: IL-6 mRNA in mast cells. Mice bone marrow-derived mast cells (BMMC) were treated with either LPS (0,1 µg/mL) or IL-33 (10 ng/mL) during 12 hours, the RNA extracted and analysed through real-time PCR (RT-PCR). No significant differences were detected ($p > 0,05$). $N = 3$.

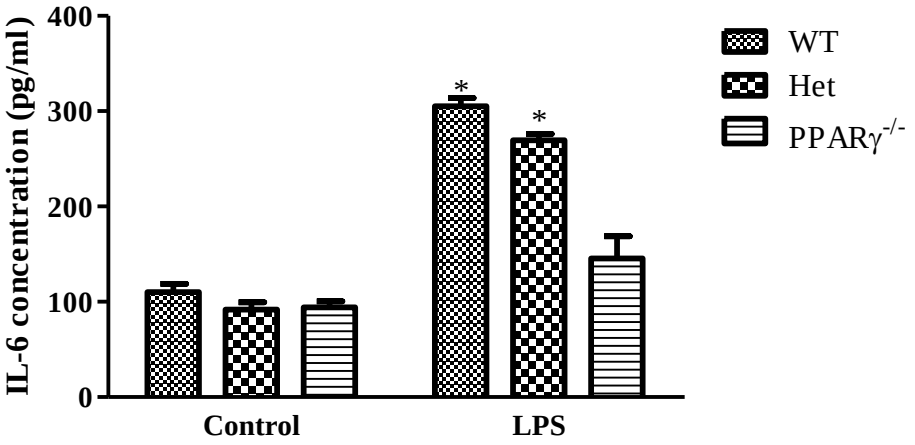


Figure 6: IL-6 in mast cells' supernatants treated with LPS. Mice bone marrow-derived mast cells (BMMC) were treated with LPS (0,1 µg/mL) during 24 hours and the supernatants analysed through ELISA. Non-treated samples (control) did not show any significant differences between groups ($p > 0,05$). LPS treatment, on the other hand, allowed for the significant ($p < 0,05$) separation of PPARγ$^{-/-}$ cells from both wild-type (WT) and heterozygote (Het) ones. WT and Het samples treated with LPS were also not statistically relevant ($p > 0,05$). $N = 6$ for control; $N = 3$ for LPS.

In order to further study the effect of PPARγ in BMMC, cells were treated with an agonist for the receptor prior to the inflammatory stimulus. Rosiglitazone (ROSI) treatment significantly reduced IL-6 release from LPS-stimulated PPARγ$^{-/-}$ cells but not WT ones (Figure 7). These results point towards a PPARγ-independent effect of the agonist, a phenomenon that has previously been reported by co-treating cells with a PPARγ antagonist[199,200]. Nonetheless, the use of antagonists presents the same limitations as working with agonists: secondary reactions are always present and the molecule's effect cannot be attributed to the intended target with certainty. By using KO cells, this work has fully targeted PPARγ and the obtained results are thus more reliable.

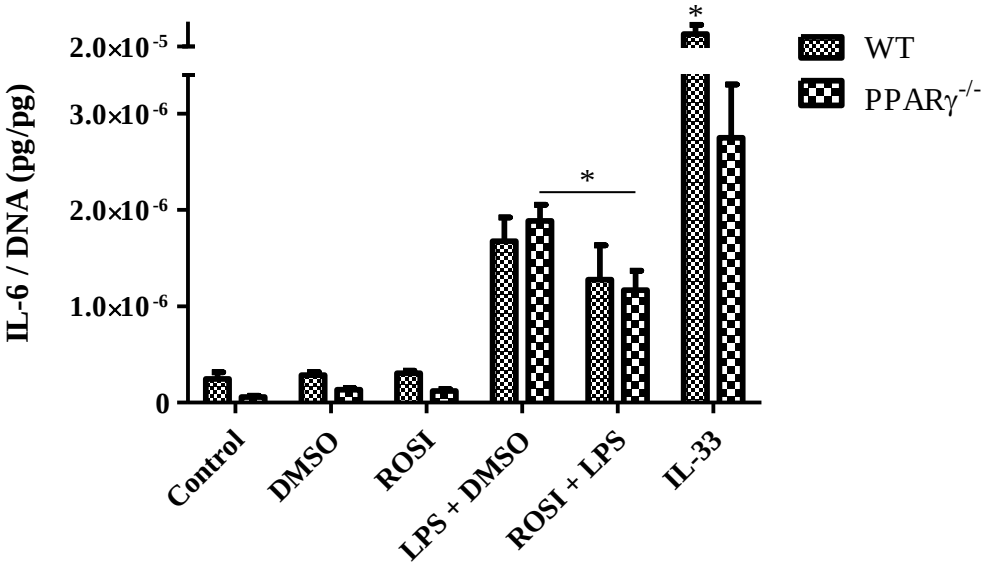


Figure 7: IL-6 in mast cells' supernatants treated with LPS, ROSI and IL-33. Mice bone marrow-derived mast cells (BMMC) were treated with DMSO (0,1 %) or rosiglitazone (ROSI) (10 µmol/L). After 12h, either LPS (0,1 µg/mL) or IL-33 (10 ng/mL) were added for stimulation during 24 hours. The supernatants and the cells were collected for ELISA and Hoechst analysis, respectively. IL-6 values were normalized to DNA concentration. The results from samples treated with LPS + DMSO and ROSI + LPS are statistically different from control, DMSO and ROSI groups ($p < 0,05$). Between these two groups (LPS + DMSO *vs* ROSI + LPS), only PPARγ$^{-/-}$ samples showed a significant difference. Treatment with IL-33 proved to be statistically relevant in comparison with all other treatments and WT cells reacted significantly more than PPARγ$^{-/-}$ ones. $N = 6$.

As for BMM, LPS stimulation originated a significantly higher release of IL-6 in PPARγ$^{-/-}$ cells compared to WT ones (Figure 8).

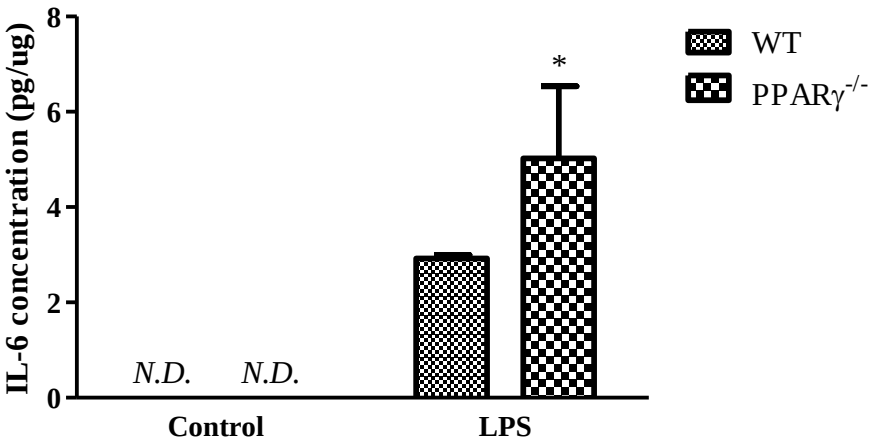


Figure 8: IL-6 in macrophages' supernatants. Mice bone marrow-derived macrophages (BMM) were treated with LPS (0,1 µg/mL) during 24h and the supernatants analysed through ELISA. All values were normalized to protein concentration. PPARγ$^{-/-}$ macrophages released significantly ($p < 0,05$) more IL-6 than wild-type (WT) ones. $N = 3$.

Despite the contradictory results, PPARγ effects on both BMMC[201] and BMM[139,202] are supported by previous literature. It could be that, in mast cells, PPARγ functions by binding to PPRE and activating genes' expression, whereas in macrophages it inhibits transcription through interference with inflammatory pathways (see *Introduction:* PPARγ). In fact, it has been shown that the anti-inflammatory action of PPARγ in macrophages is due to interaction with NF-κB[202]. The environmental conditions that prompt different actions in different cells types is yet unknown.

2. ERAP1

2.1. Genotyping

Due to the advanced age of ERAP1-/- mice, direct reproduction was not possible and so these were crossed with wild-type (WT) mice and re-crossed amongst each other to obtain homozygote animals KO for the protein. A genotyping result of WT, heterozygote (Het) and ERAP1$^{-/-}$ mice is shown as an example (Figure 9). Mutant mice have a 216-base pair (bp) gene, whereas WT animals bear the 361 bp allele and Het carry both.

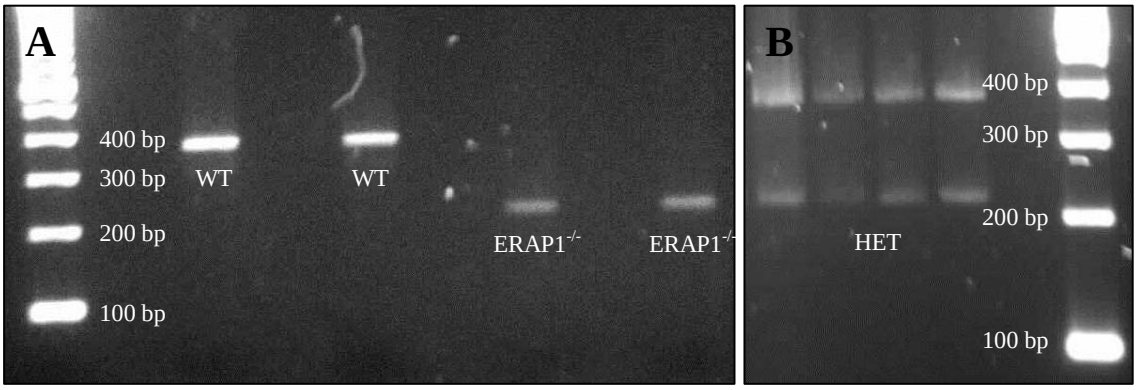


Figure 9: ERAP1 genotyping. Wild-type (WT) and ERAP1$^{-/-}$ mice have ERAP1 alleles at 361 bp and 216 bp, respectively **(A)**, whereas heterozygote (HET) animals carry both alleles **(B)**.

2.2. K/BxN arthritis

Differences between WT and ERAP1$^{-/-}$ mice, for both oedema (Graph 1) and clinical score (Graph 2), were not significant in the first three days (1-3) and became statistically relevant from day 4 until the end of the experiment (day 6). For WT mice, the time evolution of oedema is significant, whereas for ERAP1$^{-/-}$ it is not, suggesting that the latter did not develop arthritis during the six days of the experiment. This is further sustained by the significantly lower number of paws affected in ERAP1$^{-/-}$ mice, comparing with WT ones (Graph 3). The weight loss observations (Graph 4) accompany these results, being that WT mice lost significantly more weight than ERAP1$^{-/-}$ ones, consistent with the development of arthritis. The observed differences in arthritis development between females and males have proven not to be relevant. Likewise, arthritic development was random throughout the four paws.

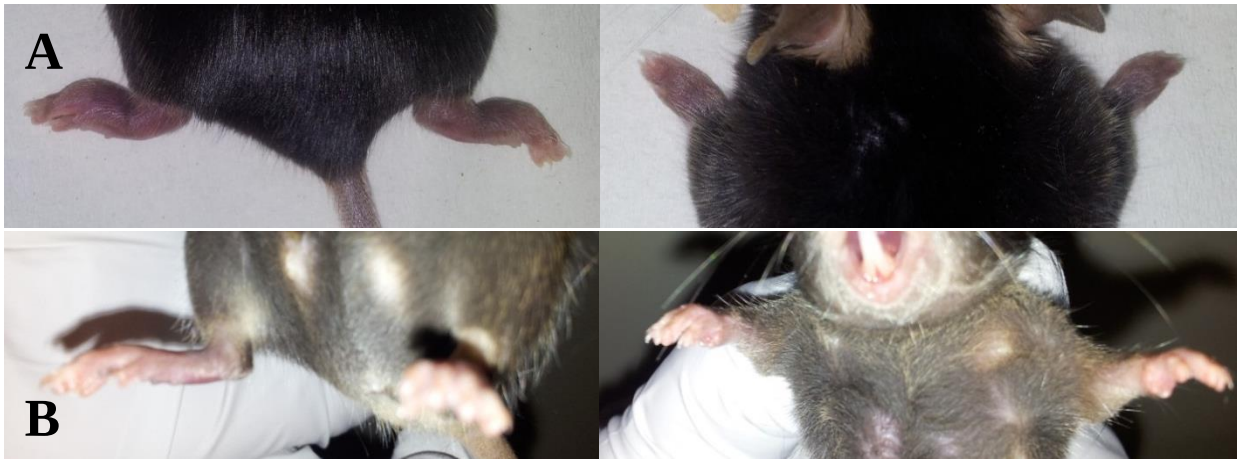
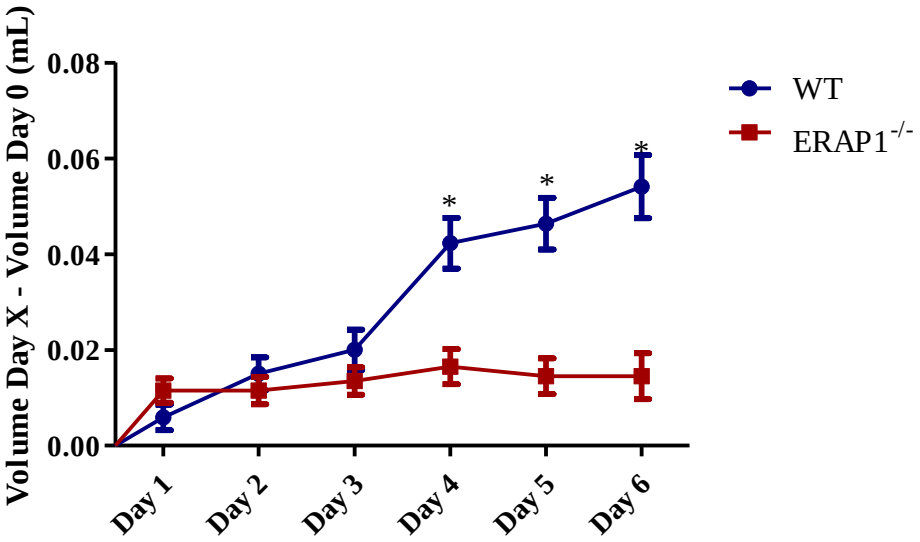
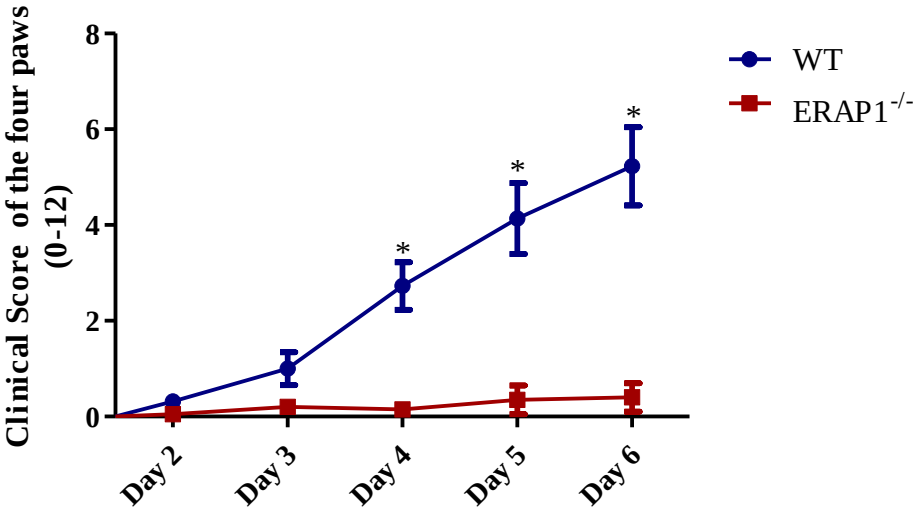


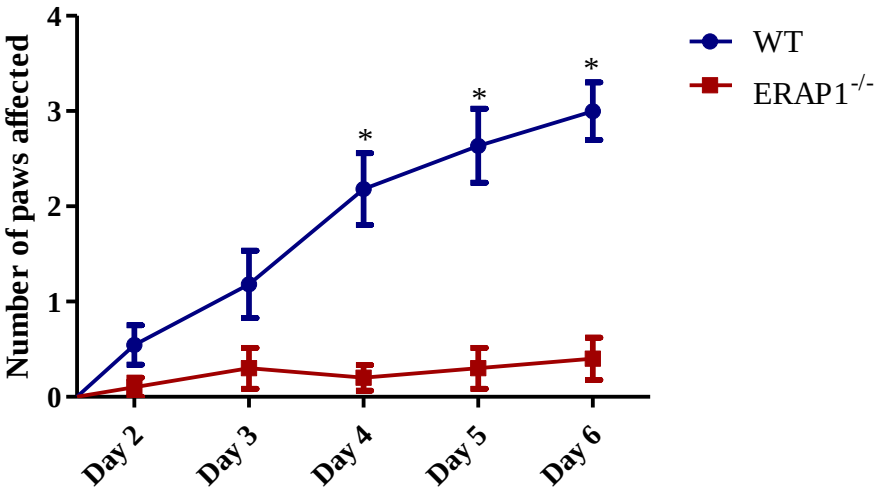
Figure 10: Paw appearance. Mice were injected intraperitoneally with 200 µL of K/BxN serum on days 0 and 2. (**A)** Wild-type and (**B)** ERAP1$^{-/-}$ mice at the day of the sacrifice (day 6).



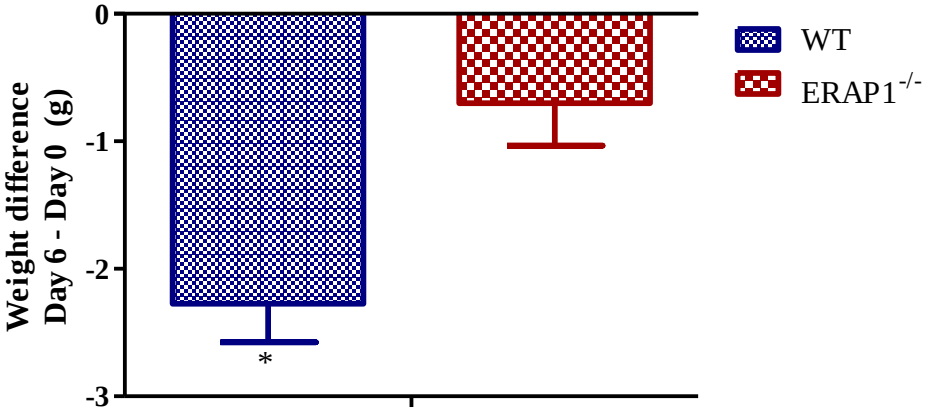
Graph 1: Paw oedema during the six days of the K/BxN serum transfer experience. Differences between wild-type (WT) and ERAP1$^{-/-}$ mice are not significant for days 1 to 3 and become so ($p < 0,05$) from day 4 to day 6. Time evolution is only significant for WT mice. No statistically relevant differences were noticed between male and female mice for both genetic backgrounds. *N* (WT) = 11; *N* (ERAP1$^{-/-}$) = 10.



Graph 2: Clinical score. Clinical scoring was made based on paw oedema, using a 0 to 3 scale. Differences between wild-type (WT) and ERAP1$^{-/-}$ mice are significant ($p < 0,05$) for days 4 to 6. Differences between paws are not significant. *N* (WT) = 11; *N* (ERAP1$^{-/-}$) = 10.



Graph 3: Number of paws affected per mouse. Based on the clinical scoring, wild-type (WT) mice had significantly ($p < 0,05$) more paws affected than ERAP1$^{-/-}$ during the six days of the experiment. *N* (WT) = 11; *N* (ERAP1$^{-/-}$) = 10.



Graph 4: Weight loss. Wild-type (WT) mice lost significantly ($p < 0,05$) more weight than ERAP1$^{-/-}$ during the six days of the experiment. *N* (WT) = 11; *N* (ERAP1$^{-/-}$) = 10.

Staining of the skeletons for cartilage and bone did not show any relevant differences between WT and ERAP1$^{-/-}$ animals (Figure 11), as expected since, in this model, arthritis is localised to the distal joints. However, inflammation was found in all examined mice from both groups, which is very likely due to their advanced age.

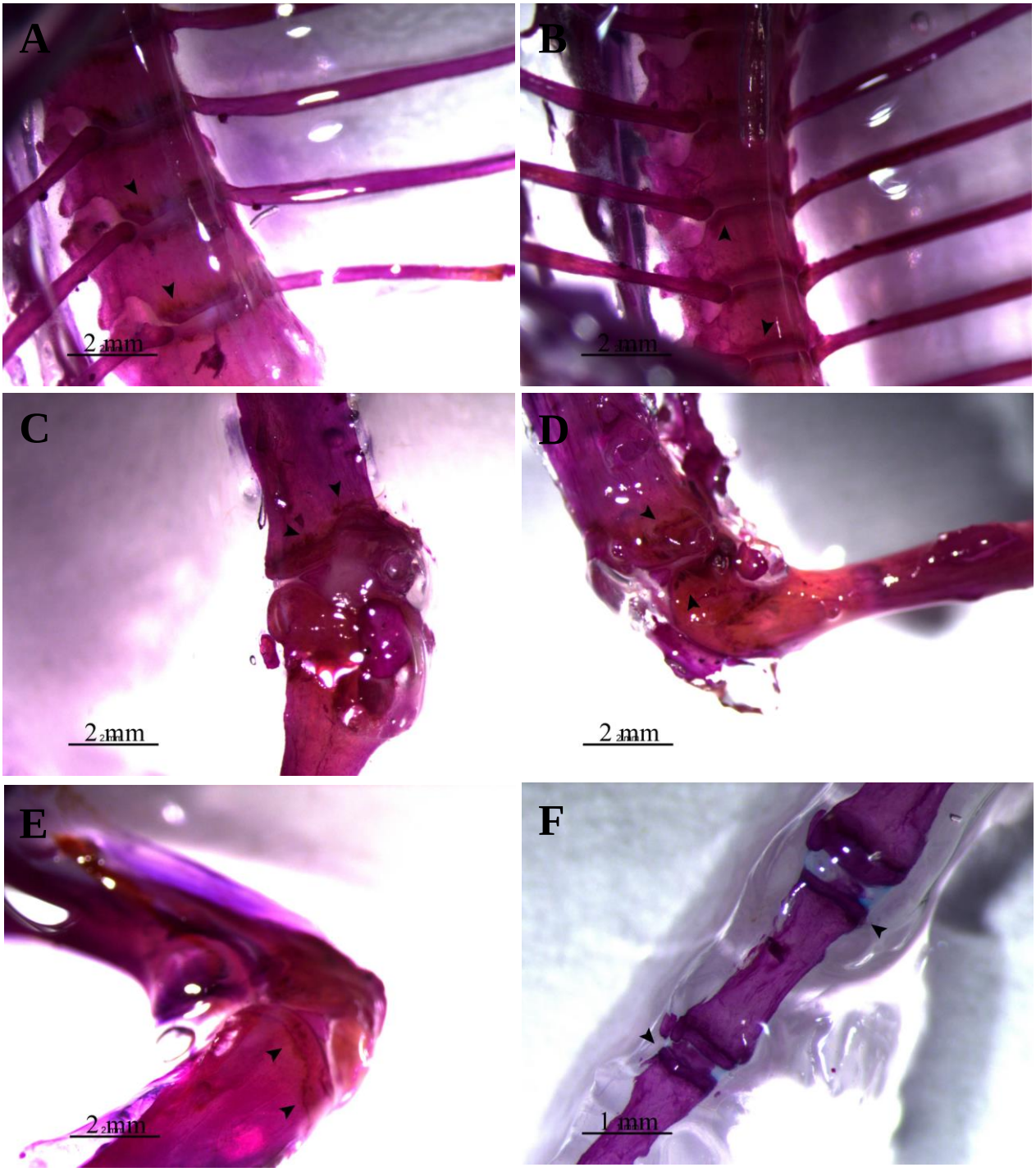


Figure 11: Skeleton inflammation. Both wild-type (WT) and ERAP1$^{-/-}$ mice presented with inflammation in the spine (A and B) and knees (C to E), without any significant difference between the two. A tail end is shown as a healthy joint (F). WT: A, C, E, F; ERAP1$^{-/-}$: B,D. *N* (WT) = 4; *N* (ERAP1$^{-/-}$) = 2.

In an ERAP1-deficient mouse, it was possible to observe the fusion of the vertebrae in the end of the spine (Figure 12), consistent with AS. A previous study aimed at examining joint fusion in untreated ERAP1$^{-/-}$ mice was not able to see this feature of the disease (Duval, F.; Moulin, D.; unpublished results), most likely because young mice were used instead of old ones. Despite the fact that, in this work, mice were treated in order to develop arthritis, it is unlikely that K/BxN treatment was responsible for spinal fusion, since this is a typical AS feature and the serum-transfer model originates symptoms that most resemble RA.

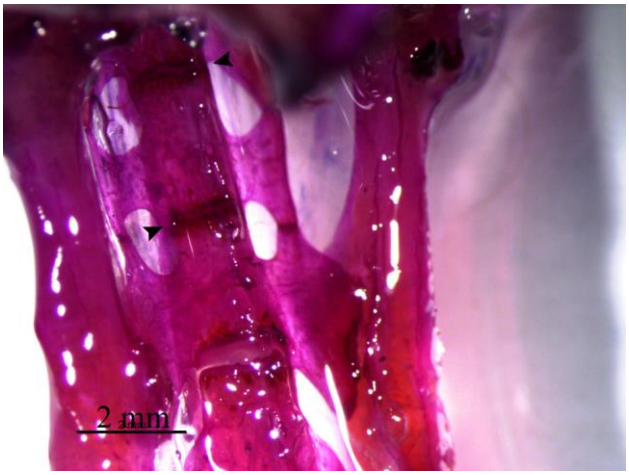


Figure 12: Vertebrae fusion observed in an ERAP1$^{-/-}$ mouse.

Despite the non-specificity of K/BxN serum arthritis induction in the remaining skeleton, histology of the back paws showed a more severe state of disease in WT animals, as demonstrated by the disorganization of the cartilage and loss of bone integrity (Figure 13).

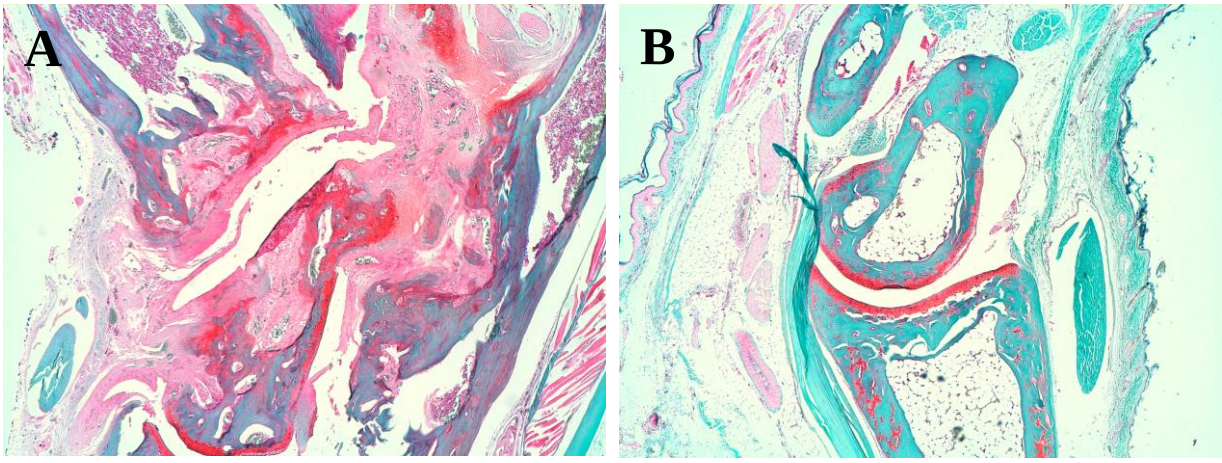
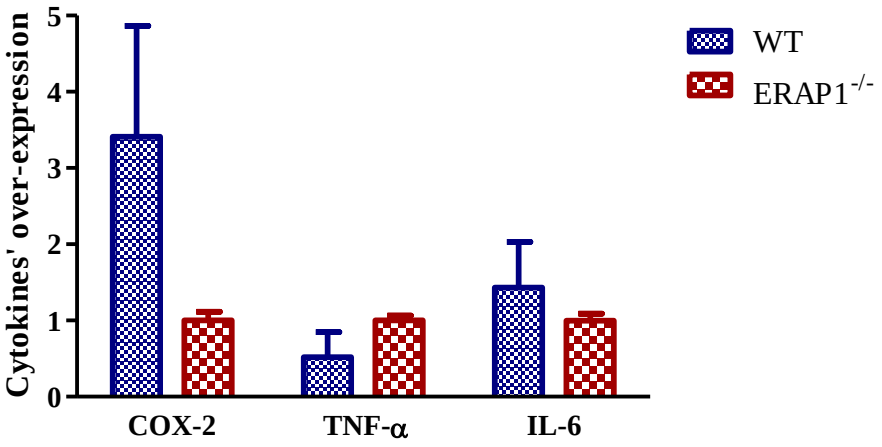


Figure 13: Paw histology. Back paws of WT (A) and ERAP1$^{-/-}$ (B) mice were cut sagittally and stained with safranin-O (red) and fast green FCF (blue), respectively showing cartilage and bone. *N* = 3.

Since COX-2, TNF-α and IL-6 are some of the cytokines associated with the K/BxN model, their presence in a messenger state was measured in the paws (Figure 5). There seems to be a tendency pointing to higher cytokines' expression in WT mice, which is consistent with the results shown this far, however the differences were not statistically significant for any of the cytokines, probably due to a small number of replicates. More samples would intuitively accentuate the differences between the two groups.



Graph 5: Cytokines' mRNA over-expression. Real Time-PCR of COX-2, TNF-α and IL-6 in paws from wild-type (WT) and ERAP1$^{-/-}$ mice injected with K/BxN serum. p > 0,05 for all cytokines. *N* = 3.

Therefore, mice KO for ERAP1 appeared resistant to the development of K/BxN-induced arthritis, during the six days of the experience. As a reminder, ERAP1 is responsible for the shedding of IL-6R, IL-1RII and TNFRI, respectively in a direct and indirect manner. It was previously mentioned that mice KO for IL-1RI do not develop any signs of arthritis, whereas TNF-α and IL-6 did not seem to have an important role in this model of arthritis (see *Introduction:* The K/BxN mouse model). As demonstrated by our experience, mIL-1RII might be crucial for IL-1 signalling – or lack thereof. By cleaving IL-1RII into its soluble form, IL-1β will preferentially bind to sIL-1RII or mIL-1RI, both with similar affinities towards the cytokine and both requiring the accessory protein for linkage stabilization. Regardless the fact that IL-1β affinity towards membrane type I receptor is close to that for sIL-1RII, the latter is still more likely to interact with the cytokine, if affinity is the only factor being considered. Hence, it is purposed that both the shedding of IL-1RII and the lack of sIL-1RAcP are responsible for the large difference in arthritic symptoms observed between WT and ERAP1$^{-/-}$ mice. If the presence of the accessory protein is mainly limited to the membrane-bound form, the interaction between IL-1β and mIL-1RI will be more favourable in detriment of that with sIL-1RI, therefore allowing IL-1 signalling. In fact, treatment with sIL-1RAcP improves the outcome of mice with collagen-induced arthritis (CIA)[203]. Correspondingly, IL-1Ra[204] and human anti-IL-1β[205-207] have also shown effectiveness in clinical trials of different forms of arthritis. So, for this particular model, the lack of ERAP1 is protective against the development of arthritis and drugs that target the enzyme's activity may qualify as a possible treatment. Campbell *et al.*[208] have also found a relationship between ERAP1 over-expression and ankylosing spondylitis, in a human study. Nonetheless, for years now that patients treated with TNFα blockers show improvements[209,210], which suggests that, in humans, arthritis is TNFα-dependent, raising more awareness to the fact that the murine K/BxN model does not fully overlap with human arthritis.

Another explanation for these results, complementary or not, is the increased macrophage activity induced by ERAP1. As mentioned, an inflammatory stimulus may cause the enzyme's over-expression and subsequent release into the extracellular medium where it activates macrophages. The elevated activity of these immune cells would thus be the factor responsible for an increased inflammation in ERAP1-containing mice.

3. PPARγ *vs.* ERAP1 *vs.* macrophages

The results presented in this work show that both PPARγ and ERAP1 might have an effect on macrophage function. These cells have, in turn, been associated with arthritis on their own, bring that macrophage depletion of arthritic mice reduces the severity of the disease[211]. So, it seems that macrophages might be a final player in arthritic development, with PPARγ and ERAP1 being capable of controlling their function. Ergo, a combination of molecules that target both of these proteins, with an activating effect on PPARγ and a silencing action on ERAP1, might be a promising arthritis treatment.

IV. Conclusion

PPARγ can have either a pro- or an anti-inflammatory effect, depending on the cell type, respectively BMMC or BMM. When exposed to LPS or IL-33, PPARγ$^{-/-}$ BMMC released significantly less effector IL-6 than cells from WT mice. On the contrary, LPS-stimulated macrophages need the receptor for inflammation control, possibly through interaction with NF-κB. The PPARγ agonist rosiglitazone seems to have an effect in IL-6 release from BMMC. However, it is independent from the receptor since KO cells also react to the agonist.

In the K/BxN arthritis transfer model, the presence of ERAP1 is crucial for the development of the disease. ERAP1$^{-/-}$ mice are resistant to arthritis development, as shown by the absence of paw swelling and weight loss, lower clinical scores and cytokines' expression and less severe histological features, comparing with WT mice. ERAP1's role in the disease is possibly due to IL-1RII shedding and/or macrophage activation. Dual treatment with PPARγ agonists and ERAP1 blockers could be advantageous for arthritis management.

Finally, ERAP1$^{-/-}$ mice might be a spontaneous model of AS if these are heavily aged, around 40-weeks-old.

V. Future work

Future work should continue to focus on the unveiling of mechanisms responsible for arthritis development, since research on this topic is not yet advanced enough to attempt new treatments. The K/BxN arthritis induction should be performed a second time, using younger mice and non-arthritic controls (*i.e.* non-injected WT and ERAP1$^{-/-}$ animals). For WT, ERAP1 could be dosed in an affected tissue to evaluate if it is over-expressed and if this could be a mechanism controlling macrophages' activation. To assess the macrophages' role in this model of arthritis, mice depleted of these cells can be exposed to the arthritogenic serum. If their part is confirmed, K/BxN-exposed mice can be treated with PPARγ agonists to verify if inflammation is indeed smaller. In a later stage, the therapeutic effects of combined PPARγ agonists and ERAP1 blockers can be appraised.

Finally, ERAP1$^{-/-}$ mice should be allowed to age naturally and their skeletons examined to determine whether this is a spontaneous model of AS. If so, various studies, namely disease progression with age, could be performed using this model.

VI. Bibliography

1 Australia, A. *What is arthritis*, <http://www.arthritisaustralia.com.au/index.php/arthritis-information.html>

2 Quintiles. *Arthritis by the numbers*, <http://www.clinicalresearch.com/conditions/arthritis/stats.aspx> (2011).

3 Center for Disease Control and Prevention (CDC). *Arthritis: FAQs - Cost Analysis*, <http://www.cdc.gov/arthritis/data_statistics/faqs/cost_analysis.htm#1> (2011).

4 Scott, D. L., Coulton, B. L., Symmons, D. P. M. & Popert, A. J. Long-term outcome of treating Rheumatoid Arthritis: Results after 20 years. *The Lancet* **329**, 1108-1111, doi:http://dx.doi.org/10.1016/S0140-6736(87)91672-2 (1987).

5 Pincus, T., Callahan, L. F., Sale, W. G., Brooks, A. L., Payne, L. E. & Vaughn, W. K. Severe functional declines, work disability, and increased mortality in seventy-five rheumatoid arthritis patients studied over nine years. *Arthritis Rheum* **27**, 864-872 (1984).

6 Symmons, D. P. M., Barret, E. M., Bankhead, C. R., Scott, D. G. L. & Silman, A. J. The Incidence of Rheumatoid Arthritis in the United Kingdom: Results from the Norfolk Arthritis Register. *Rheumatology* **33**, 735-739, doi:10.1093/rheumatology/33.8.735 (1994).

7 Gabriel, S. E., Crowson, C. S. & O'Fallon, M. The epidemiology of rheumatoid arthritis in Rochester, Minnesota, 1955–1985. *Arthritis & Rheumatism* **42**, 415-420, doi:10.1002/1529-0131(199904)42:3<415::aid-anr4>3.0.co;2-z (1999).

8 Aletaha, D., Neogi, T., Silman, A. J., Funovits, J., Felson, D. T., Bingham, C. O., Birnbaum, N. S., Burmester, G. R., Bykerk, V. P., Cohen, M. D., Combe, B., Costenbader, K. H., Dougados, M., Emery, P., Ferraccioli, G., Hazes, J. M., Hobbs, K., Huizinga, T. W., Kavanaugh, A., Kay, J., Kvien, T. K., Laing, T., Mease, P., Ménard, H. A., Moreland, L. W., Naden, R. L., Pincus, T., Smolen, J. S., Stanislawska-Biernat, E., Symmons, D., Tak, P. P., Upchurch, K. S., Vencovský, J., Wolfe, F. & Hawker, G. 2010 Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League Against Rheumatism collaborative initiative. *Annals of the Rheumatic Diseases* **69**, 1580-1588, doi:10.1136/ard.2010.138461 (2010).

9 Arnett, F. C., Edworthy, S. M., Bloch, D. A., McShane, D. J., Fries, J. F., Cooper, N. S., Healey, L. A., Kaplan, S. R., Liang, M. H., Luthra, H. S., Medsger, T. A., Mitchell, D. M., Neustadt, D. H., Pinals, R. S., Schaller, J. G., Sharp, J. T., Wilder, R. L. & Hunder, G. G. The american rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis & Rheumatism* **31**, 315-324, doi:10.1002/art.1780310302 (1988).

10 Gosset, P., Perez, T., Lassalle, P., Duquesnoy, B., Farre, J. M., Tonnel, A. B. & Capron, A. Increased TNF-alpha secretion by alveolar macrophages from patients with rheumatoid arthritis. *Am Rev Respir Dis* **143**, 593-597, doi:10.1164/ajrccm/143.3.593 (1991).

11 Buchan, G., Barrett, K., Turner, M., Chantry, D., Maini, R. N. & Feldmann, M. Interleukin-1 and tumour necrosis factor mRNA expression in rheumatoid arthritis: prolonged production of IL-1 alpha. *Clin Exp Immunol* **73**, 449-455 (1988).

12 Swaak, A. J., van Rooyen, A., Nieuwenhuis, E. & Aarden, L. A. Interleukin-6 (IL-6) in synovial fluid and serum of patients with rheumatic diseases. *Scand J Rheumatol* **17**, 469-474 (1988).

13 Nawata, Y., Eugui, E. M., Lee, S. W. & Allison, A. C. IL-6 Is the Principal Factor Produced by Synovia of Patients with Rheumatoid Arthritis That Induces B-Lymphocytes to Secrete Immunoglobulins. *Annals of the New York Academy of Sciences* **557**, 230-239, doi:10.1111/j.1749-6632.1989.tb24016.x (1989).

14 Iñiguez, M. A., Pablos, J. L., Carreira, P. E., Cabré, F. & Gomez-Reino, J. J. Detection of COX-1 and COX-2 isoforms in synovial fluid cells from inflammatory joint diseases. *Rheumatology* **37**, 773-778, doi:10.1093/rheumatology/37.7.773 (1998).

15 Lee, Y. H., Choi, S. J., Kim, A., Kim, C. H., Ji, J. D. & Song, G. G. Expression of cyclooxygenase-1 and -2 in rheumatoid arthritis synovium. *J Korean Med Sci* **15**, 88-92 (2000).

16 Aho, K., Heliovaara, M., Maatela, J., Tuomi, T. & Palosuo, T. Rheumatoid factors antedating clinical rheumatoid arthritis. *J Rheumatol* **18**, 1282-1284 (1991).

17 Nielen, M. M. J., van Schaardenburg, D., Reesink, H. W., van de Stadt, R. J., van der Horst-Bruinsma, I. E., de Koning, M. H. M. T., Habibuw, M. R., Vandenbroucke, J. P. & Dijkmans, B. A. C. Specific autoantibodies precede the symptoms of rheumatoid arthritis: A study of serial measurements in blood donors. *Arthritis & Rheumatism* **50**, 380-386, doi:10.1002/art.20018 (2004).

18 Rantapää-Dahlqvist, S., de Jong, B. A. W., Berglin, E., Hallmans, G., Wadell, G., Stenlund, H., Sundin, U. & van Venrooij, W. J. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis & Rheumatism* **48**, 2741-2749, doi:10.1002/art.11223 (2003).

19 Bartold, P. M., Marino, V., Cantley, M. & Haynes, D. R. Effect of Porphyromonas gingivalis-induced inflammation on the development of rheumatoid arthritis. *Journal of Clinical Periodontology* **37**, 405-411, doi:10.1111/j.1600-051X.2010.01552.x (2010).

20 Cantley, M. D., Haynes, D. R., Marino, V. & Bartold, P. M. Pre-existing periodontitis exacerbates experimental arthritis in a mouse model. *Journal of Clinical Periodontology* **38**, 532-541, doi:10.1111/j.1600-051X.2011.01714.x (2011).

21 Ogrendik, M., Kokino, S., Ozdemir, F., Bird, P. S. & Hamlet, S. Serum antibodies to oral anaerobic bacteria in patients with rheumatoid arthritis. *MedGenMed* **7**, 2 (2005).

22 Taylor, L. H., Twigg, S., Worthington, J., Emery, P., Morgan, A. W., Wilson, A. G. & Teare, M. D. Metaanalysis of the Association of Smoking and PTPN22 R620W Genotype on Autoantibody Status and Radiological Erosions in Rheumatoid Arthritis. *The Journal of Rheumatology*, doi:10.3899/jrheum.120784 (2013).

23 Di Giuseppe, D., Orsini, N., Alfredsson, L., Askling, J. & Wolk, A. Cigarette smoking and smoking cessation in relation to risk of rheumatoid arthritis in women. *Arthritis Research & Therapy* **15**, R56 (2013).

24 Eggert, F. M., McLeod, M. H. & Flowerdew, G. Effects of smoking and treatment status on periodontal bacteria: evidence that smoking influences control of periodontal bacteria at

the mucosal surface of the gingival crevice. *J Periodontol* **72**, 1210-1220, doi:10.1902/jop.2000.72.9.1210 (2001).

25 van Winkelhoff, A. J., Bosch-Tijhof, C. J., Winkel, E. G. & van der Reijden, W. A. Smoking affects the subgingival microflora in periodontitis. *J Periodontol* **72**, 666-671, doi:10.1902/jop.2001.72.5.666 (2001).

26 van der Heide, A., Jacobs, J. W., Bijlsma, J. W., Heurkens, A. H., van Booma-Frankfort, C., van der Veen, M. J., Haanen, H. C., Hofman, D. M., van Albada-Kuipers, G. A., ter Borg, E. J., Brus, H. L., Dinant, H. J., Kruize, A. A. & Schenk, Y. The effectiveness of early treatment with "second-line" antirheumatic drugs. A randomized, controlled trial. *Ann Intern Med* **124**, 699-707 (1996).

27 Sieper, J., Rudwaleit, M., Baraliakos, X., Brandt, J., Braun, J., Burgos-Vargas, R., Dougados, M., Hermann, K.-G., Landewé, R., Maksymowych, W. & van der Heijde, D. The Assessment of SpondyloArthritis international Society (ASAS) handbook: a guide to assess spondyloarthritis. *Annals of the Rheumatic Diseases* **68**, ii1-ii44, doi:10.1136/ard.2008.104018 (2009).

28 Helmick, C. G., Felson, D. T., Lawrence, R. C., Gabriel, S., Hirsch, R., Kwoh, C. K., Liang, M. H., Kremers, H. M., Mayes, M. D., Merkel, P. A., Pillemer, S. R., Reveille, J. D., Stone, J. H. & National Arthritis Data, W. Estimates of the prevalence of arthritis and other rheumatic conditions in the United States: Part I. *Arthritis & Rheumatism* **58**, 15-25, doi:10.1002/art.23177 (2008).

29 Reveille, J. D., Witter, J. P. & Weisman, M. H. Prevalence of axial spondylarthritis in the United States: Estimates from a cross-sectional survey. *Arthritis Care & Research* **64**, 905-910, doi:10.1002/acr.21621 (2012).

30 Braun, J., Bollow, M., Remlinger, G., Eggens, U., Rudwaleit, M., Distler, A. & Sieper, J. Prevalence of spondylarthropathies in HLA-B27 positive and negative blood donors. *Arthritis Rheum* **41**, 58-67, doi:10.1002/1529-0131(199801)41:1<58::aid-art8>3.0.co;2-g (1998).

31 Healey, E. L., Haywood, K. L., Jordan, K. P., Garratt, A. & Packham, J. C. Impact of ankylosing spondylitis on work in patients across the UK. *Scand J Rheumatol* **40**, 34-40, doi:10.3109/03009742.2010.487838 (2011).

32 Ramos-Remus, C., Hernandez-Rios, G., Duran-Barragan, S., Sanchez-Ortiz, A., Aceves-Avila, F. J., Castillo-Ortiz, J. D. & Gonzalez-Perez, O. Fifteen-year trends of long-term disability and sick leaves in ankylosing spondylitis. *Clin Rheumatol* **30**, 361-367, doi:10.1007/s10067-010-1669-y (2011).

33 Rohekar, S. & Pope, J. Assessment of work disability in seronegative spondyloarthritis. *Clin Exp Rheumatol* **28**, 35-40 (2010).

34 Boonen, A., Brinkhuizen, T., Landewé, R., van der Heijde, D. & Severens, J. L. Impact of ankylosing spondylitis on sick leave, presenteeism and unpaid productivity, and estimation of the societal cost. *Annals of the Rheumatic Diseases* **69**, 1123-1128, doi:10.1136/ard.2009.116764 (2010).

35 Fabreguet, I., Koumakis, E., Burki, V., Durnez, A., Elhai, M., Meyer, M., Paternotte, S., Payet, J., Roure, F., Fautrel, B., Dougados, M. & Gossec, L. Assessment of work

instability in spondyloarthritis: a cross-sectional study using the ankylosing spondylitis work instability scale. *Rheumatology* **51**, 333-337, doi:10.1093/rheumatology/ker319 (2012).

36 Brown, M. A., Laval, S. H., Brophy, S. & Calin, A. Recurrence risk modelling of the genetic susceptibility to ankylosing spondylitis. *Annals of the Rheumatic Diseases* **59**, 883-886, doi:10.1136/ard.59.11.883 (2000).

37 Brown, M. A., Kennedy, L. G., MacGregor, A. J., Darke, C., Duncan, E., Shatford, J. L., Taylor, A., Calin, A. & Wordsworth, P. Susceptibility to ankylosing spondylitis in twins: the role of genes, HLA, and the environment. *Arthritis Rheum* **40**, 1823-1828, doi:10.1002/1529-0131(199710)40:10<1823::AID-ART15>3.0.CO;2-1 (1997).

38 Geirsson, A. J., Eyjolfsdottir, H., Bjornsdottir, G., Kristjansson, K. & Gudbjornsson, B. Prevalence and clinical characteristics of ankylosing spondylitis in Iceland - a nationwide study. *Clin Exp Rheumatol* **28**, 333-340 (2010).

39 Evans, D. M., Spencer, C. C., Pointon, J. J., Su, Z., Harvey, D., Kochan, G., Oppermann, U., Dilthey, A., Pirinen, M., Stone, M. A., Appleton, L., Moutsianas, L., Leslie, S., Wordsworth, T., Kenna, T. J., Karaderi, T., Thomas, G. P., Ward, M. M., Weisman, M. H., Farrar, C., Bradbury, L. A., Danoy, P., Inman, R. D., Maksymowych, W., Gladman, D., Rahman, P., Morgan, A., Marzo-Ortega, H., Bowness, P., Gaffney, K., Gaston, J. S., Smith, M., Bruges-Armas, J., Couto, A. R., Sorrentino, R., Paladini, F., Ferreira, M. A., Xu, H., Liu, Y., Jiang, L., Lopez-Larrea, C., Diaz-Pena, R., Lopez-Vazquez, A., Zayats, T., Band, G., Bellenguez, C., Blackburn, H., Blackwell, J. M., Bramon, E., Bumpstead, S. J., Casas, J. P., Corvin, A., Craddock, N., Deloukas, P., Dronov, S., Duncanson, A., Edkins, S., Freeman, C., Gillman, M., Gray, E., Gwilliam, R., Hammond, N., Hunt, S. E., Jankowski, J., Jayakumar, A., Langford, C., Liddle, J., Markus, H. S., Mathew, C. G., McCann, O. T., McCarthy, M. I., Palmer, C. N., Peltonen, L., Plomin, R., Potter, S. C., Rautanen, A., Ravindrarajah, R., Ricketts, M., Samani, N., Sawcer, S. J., Strange, A., Trembath, R. C., Viswanathan, A. C., Waller, M., Weston, P., Whittaker, P., Widaa, S., Wood, N. W., McVean, G., Reveille, J. D., Wordsworth, B. P., Brown, M. A. & Donnelly, P. Interaction between ERAP1 and HLA-B27 in ankylosing spondylitis implicates peptide handling in the mechanism for HLA-B27 in disease susceptibility. *Nat Genet* **43**, 761-767, doi:10.1038/ng.873 (2011).

40 Brewerton, D. A., Hart, F. D., Nicholls, A., Caffrey, M., James, D. C. O. & Sturrock, R. D. Ankylosing Spondylitis and HL-A 27. *The Lancet* **301**, 904-907, doi:10.1016/s0140-6736(73)91360-3 (1973).

41 Schlosstein, L., Terasaki, P. I., Bluestone, R. & Pearson, C. M. High Association of an HL-A Antigen, W27, with Ankylosing Spondylitis. *New England Journal of Medicine* **288**, 704-706, doi:doi:10.1056/NEJM197304052881403 (1973).

42 Laval, S. H., Timms, A., Edwards, S., Bradbury, L., Brophy, S., Milicic, A., Rubin, L., Siminovitch, K. A., Weeks, D. E., Calin, A., Wordsworth, B. P. & Brown, M. A. Whole-Genome Screening in Ankylosing Spondylitis: Evidence of Non-MHC Genetic-Susceptibility Loci. *The American Journal of Human Genetics* **68**, 918-926, doi:10.1086/319509 (2001).

43 Burton, P. R., Clayton, D. G., Cardon, L. R., Craddock, N., Deloukas, P., Duncanson, A., Kwiatkowski, D. P., McCarthy, M. I., Ouwehand, W. H., Samani, N. J., Todd, J. A., Donnelly, P., Barrett, J. C., Davison, D., Easton, D., Evans, D. M., Leung, H. T., Marchini,

J. L., Morris, A. P., Spencer, C. C., Tobin, M. D., Attwood, A. P., Boorman, J. P., Cant, B., Everson, U., Hussey, J. M., Jolley, J. D., Knight, A. S., Koch, K., Meech, E., Nutland, S., Prowse, C. V., Stevens, H. E., Taylor, N. C., Walters, G. R., Walker, N. M., Watkins, N. A., Winzer, T., Jones, R. W., McArdle, W. L., Ring, S. M., Strachan, D. P., Pembrey, M., Breen, G., St Clair, D., Caesar, S., Gordon-Smith, K., Jones, L., Fraser, C., Green, E. K., Grozeva, D., Hamshere, M. L., Holmans, P. A., Jones, I. R., Kirov, G., Moskivina, V., Nikolov, I., O'Donovan, M. C., Owen, M. J., Collier, D. A., Elkin, A., Farmer, A., Williamson, R., McGuffin, P., Young, A. H., Ferrier, I. N., Ball, S. G., Balmforth, A. J., Barrett, J. H., Bishop, T. D., Iles, M. M., Maqbool, A., Yuldasheva, N., Hall, A. S., Braund, P. S., Dixon, R. J., Mangino, M., Stevens, S., Thompson, J. R., Bredin, F., Tremelling, M., Parkes, M., Drummond, H., Lees, C. W., Nimmo, E. R., Satsangi, J., Fisher, S. A., Forbes, A., Lewis, C. M., Onnie, C. M., Prescott, N. J., Sanderson, J., Matthew, C. G., Barbour, J., Mohiuddin, M. K., Todhunter, C. E., Mansfield, J. C., Ahmad, T., Cummings, F. R., Jewell, D. P., Webster, J., Brown, M. J., Lathrop, M. G., Connell, J., Dominiczak, A., Marcano, C. A., Burke, B., Dobson, R., Gungadoo, J., Lee, K. L., Munroe, P. B., Newhouse, S. J., Onipinla, A., Wallace, C., Xue, M., Caulfield, M., Farrall, M., Barton, A., Bruce, I. N., Donovan, H., Eyre, S., Gilbert, P. D., Hilder, S. L., Hinks, A. M., John, S. L., Potter, C., Silman, A. J., Symmons, D. P., Thomson, W., Worthington, J., Dunger, D. B., Widmer, B., Frayling, T. M., Freathy, R. M., Lango, H., Perry, J. R., Shields, B. M., Weedon, M. N., Hattersley, A. T., Hitman, G. A., Walker, M., Elliott, K. S., Groves, C. J., Lindgren, C. M., Rayner, N. W., Timpson, N. J., Zeggini, E., Newport, M., Sirugo, G., Lyons, E., Vannberg, F., Hill, A. V., Bradbury, L. A., Farrar, C., Pointon, J. J., Wordsworth, P., Brown, M. A., Franklyn, J. A., Heward, J. M., Simmonds, M. J., Gough, S. C., Seal, S., Stratton, M. R., Rahman, N., Ban, M., Goris, A., Sawcer, S. J., Compston, A., Conway, D., Jallow, M., Rockett, K. A., Bumpstead, S. J., Chaney, A., Downes, K., Ghori, M. J., Gwilliam, R., Hunt, S. E., Inouye, M., Keniry, A., King, E., McGinnis, R., Potter, S., Ravindrarajah, R., Whittaker, P., Widden, C., Withers, D., Cardin, N. J., Ferreira, T., Pereira-Gale, J., Hallgrimsdo'ttir, I. B., Howie, B. N., Su, Z., Teo, Y. Y., Vukcevic, D., Bentley, D., Mitchell, S. L., Newby, P. R., Brand, O. J., Carr-Smith, J., Pearce, S. H., Reveille, J. D., Zhou, X., Sims, A. M., Dowling, A., Taylor, J., Doan, T., Davis, J. C., Savage, L., Ward, M. M., Learch, T. L., Weisman, M. H. & Brown, M. Association scan of 14,500 nonsynonymous SNPs in four diseases identifies autoimmunity variants. *Nat Genet* **39**, 1329-1337, doi:10.1038/ng.2007.17 (2007).

44 Calin, A., Marder, A., Becks, E. & Burns, T. Genetic differences between B27 positive patients with ankylosing spondylitis and B27 positive healthy controls. *Arthritis Rheum* **26**, 1460-1464 (1983).

45 van der Linden, S., Valkenburg, H. & Cats, A. The risk of developing ankylosing spondylitis in HLA-B27 positive individuals: a family and population study. *Br J Rheumatol* **22**, 18-19 (1983).

46 Reveille, J. D., Sims, A. M., Danoy, P., Evans, D. M., Leo, P., Pointon, J. J., Jin, R., Zhou, X., Bradbury, L. A., Appleton, L. H., Davis, J. C., Diekman, L., Doan, T., Dowling, A., Duan, R., Duncan, E. L., Farrar, C., Hadler, J., Harvey, D., Karaderi, T., Mogg, R., Pomeroy, E., Pryce, K., Taylor, J., Savage, L., Deloukas, P., Kumanduri, V., Peltonen, L., Ring, S. M., Whittaker, P., Glazov, E., Thomas, G. P., Maksymowych, W. P., Inman, R. D., Ward, M. M., Stone, M. A., Weisman, M. H., Wordsworth, B. P. & Brown, M. A. Genome-wide association study of ankylosing spondylitis identifies non-MHC susceptibility loci. *Nat Genet* **42**, 123-127, doi:10.1038/ng.513 (2010).

47 Harvey, D., Pointon, J. J., Evans, D. M., Karaderi, T., Farrar, C., Appleton, L. H., Sturrock, R. D., Stone, M. A., Oppermann, U., Brown, M. A. & Wordsworth, B. P. Investigating the

genetic association between ERAP1 and ankylosing spondylitis. *Human Molecular Genetics* **18**, 4204-4212, doi:10.1093/hmg/ddp371 (2009).

48 Craiu, A., Gaczynska, M., Akopian, T., Gramm, C. F., Fenteany, G., Goldberg, A. L. & Rock, K. L. Lactacystin and clasto-Lactacystin β-Lactone Modify Multiple Proteasome β-Subunits and Inhibit Intracellular Protein Degradation and Major Histocompatibility Complex Class I Antigen Presentation. *Journal of Biological Chemistry* **272**, 13437-13445, doi:10.1074/jbc.272.20.13437 (1997).

49 Vinitsky, A., Cardozo, C., Sepp-Lorenzino, L., Michaud, C. & Orlowski, M. Inhibition of the proteolytic activity of the multicatalytic proteinase complex (proteasome) by substrate-related peptidyl aldehydes. *J Biol Chem* **269**, 29860-29866 (1994).

50 Rock, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D. & Goldberg, A. L. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. *Cell* **78**, 761-771, doi:10.1016/s0092-8674(94)90462-6 (1994).

51 Griffin, T. A., Nandi, D., Cruz, M., Fehling, H. J., Kaer, L. V., Monaco, J. J. & Colbert, R. A. Immunoproteasome Assembly: Cooperative Incorporation of Interferon γ (IFN-γ)–inducible Subunits. *The Journal of Experimental Medicine* **187**, 97-104, doi:10.1084/jem.187.1.97 (1998).

52 Murata, S., Sasaki, K., Kishimoto, T., Niwa, S., Hayashi, H., Takahama, Y. & Tanaka, K. Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science* **316**, 1349-1353, doi:10.1126/science.1141915 (2007).

53 Toes, R. E. M., Nussbaum, A. K., Degermann, S., Schirle, M., Emmerich, N. P. N., Kraft, M., Laplace, C., Zwinderman, A., Dick, T. P., Müller, J., Schönfisch, B., Schmid, C., Fehling, H.-J., Stevanovic, S., Rammensee, H. G. & Schild, H. Discrete Cleavage Motifs of Constitutive and Immunoproteasomes Revealed by Quantitative Analysis of Cleavage Products. *The Journal of Experimental Medicine* **194**, 1-12, doi:10.1084/jem.194.1.1 (2001).

54 Nitta, T., Murata, S., Sasaki, K., Fujii, H., Ripen, A. M., Ishimaru, N., Koyasu, S., Tanaka, K. & Takahama, Y. Thymoproteasome Shapes Immunocompetent Repertoire of CD8+ T Cells. *Immunity* **32**, 29-40, doi:10.1016/j.immuni.2009.10.009 (2010).

55 Mo, X. Y., Cascio, P., Lemerise, K., Goldberg, A. L. & Rock, K. Distinct Proteolytic Processes Generate the C and N Termini of MHC Class I-Binding Peptides. *The Journal of Immunology* **163**, 5851-5859 (1999).

56 Craiu, A., Akopian, T., Goldberg, A. & Rock, K. L. Two distinct proteolytic processes in the generation of a major histocompatibility complex class I-presented peptide. *Proceedings of the National Academy of Sciences* **94**, 10850-10855, doi:10.1073/pnas.94.20.10850 (1997).

57 Lucchiari-Hartz, M., van Endert, P. M., Lauvau, G., Maier, R., Meyerhans, A., Mann, D., Eichmann, K. & Niedermann, G. Cytotoxic T Lymphocyte Epitopes of HIV-1 Nef. *The Journal of Experimental Medicine* **191**, 239-252 (2000).

58 Cascio, P., Hilton, C., Kisselev, A. F., Rock, K. L. & Goldberg, A. L. 26S proteasomes and immunoproteasomes produce mainly N-extended versions of an antigenic peptide. *EMBO J* **20**, 2357-2366, doi:10.1093/emboj/20.10.2357 (2001).

59 Blanchard, N., Kanaseki, T., Escobar, H., Delebecque, F., Nagarajan, N. A., Reyes-Vargas, E., Crockett, D. K., Raulet, D. H., Delgado, J. C. & Shastri, N. Endoplasmic Reticulum Aminopeptidase Associated with Antigen Processing Defines the Composition and Structure of MHC Class I Peptide Repertoire in Normal and Virus-Infected Cells. *The Journal of Immunology* **184**, 3033-3042, doi:10.4049/jimmunol.0903712 (2010).

60 Paz, P., Brouwenstijn, N., Perry, R. & Shastri, N. Discrete Proteolytic Intermediates in the MHC Class I Antigen Processing Pathway and MHC I–Dependent Peptide Trimming in the ER. *Immunity* **11**, 241-251, doi:10.1016/s1074-7613(00)80099-0 (1999).

61 Knuehl, C., Spee, P., Ruppert, T., Kuckelkorn, U., Henklein, P., Neefjes, J. & Kloetzel, P.-M. The Murine Cytomegalovirus pp89 Immunodominant H-2Ld Epitope Is Generated and Translocated into the Endoplasmic Reticulum as an 11-Mer Precursor Peptide. *The Journal of Immunology* **167**, 1515-1521 (2001).

62 Goldberg, A. L., Cascio, P., Saric, T. & Rock, K. L. The importance of the proteasome and subsequent proteolytic steps in the generation of antigenic peptides. *Molecular Immunology* **39**, 147-164, doi:10.1016/s0161-5890(02)00098-6 (2002).

63 Kisselev, A. F., Akopian, T. N., Woo, K. M. & Goldberg, A. L. The Sizes of Peptides Generated from Protein by Mammalian 26 and 20 S Proteasomes. *Journal of Biological Chemistry* **274**, 3363-3371, doi:10.1074/jbc.274.6.3363 (1999).

64 Fruci, D., Niedermann, G., Butler, R. H. & van Endert, P. M. Efficient MHC class I-independent amino-terminal trimming of epitope precursor peptides in the endoplasmic reticulum. *Immunity* **15**, 467-476 (2001).

65 Momburg, F., Roelse, J., Hämmerling, G. J. & Neefjes, J. J. Peptide size selection by the major histocompatibility complex-encoded peptide transporter. *The Journal of Experimental Medicine* **179**, 1613-1623, doi:10.1084/jem.179.5.1613 (1994).

66 Uebel, S., Kraas, W., Kienle, S., Wiesmüller, K.-H., Jung, G. & Tampé, R. Recognition principle of the TAP transporter disclosed by combinatorial peptide libraries. *Proceedings of the National Academy of Sciences* **94**, 8976-8981 (1997).

67 Lauvau, G., Kakimi, K., Niedermann, G., Ostankovitch, M., Yotnda, P., Firat, H., Chisari, F. V. & van Endert, P. M. Human Transporters Associated with Antigen Processing (Taps) Select Epitope Precursor Peptides for Processing in the Endoplasmic Reticulum and Presentation to T Cells. *The Journal of Experimental Medicine* **190**, 1227-1240, doi:10.1084/jem.190.9.1227 (1999).

68 van Endert, P. M., Tampe, R., Meyer, T. H., Tisch, R., Bach, J. F. & McDevitt, H. O. A sequential model for peptide binding and transport by the transporters associated with antigen processing. *Immunity* **1**, 491-500 (1994).

69 Neisig, A., Roelse, J., Sijts, A. J., Ossendorp, F., Feltkamp, M. C., Kast, W. M., Melief, C. J. & Neefjes, J. J. Major differences in transporter associated with antigen presentation (TAP)-dependent translocation of MHC class I-presentable peptides and the effect of flanking sequences. *The Journal of Immunology* **154**, 1273-1279 (1995).

70 Guo, H. C., Jardetzky, T. S., Garrett, T. P., Lane, W. S., Strominger, J. L. & Wiley, D. C. Different length peptides bind to HLA-Aw68 similarly at their ends but bulge out in the middle. *Nature* **360**, 364-366, doi:10.1038/360364a0 (1992).

71 Fremont, D. H., Matsumura, M., Stura, E. A., Peterson, P. A. & Wilson, I. A. Crystal structures of two viral peptides in complex with murine MHC class I H-2Kb. *Science* **257**, 919-927 (1992).

72 Blanchard, N. & Shastri, N. Coping with loss of perfection in the MHC class I peptide repertoire. *Current Opinion in Immunology* **20**, 82-88, doi:10.1016/j.coi.2007.12.004 (2008).

73 Saric, T., Chang, S. C., Hattori, A., York, I. A., Markant, S., Rock, K. L., Tsujimoto, M. & Goldberg, A. L. An IFN-gamma-induced aminopeptidase in the ER, ERAP1, trims precursors to MHC class I-presented peptides. *Nat Immunol* **3**, 1169-1176, doi:10.1038/ni859 (2002).

74 Saveanu, L., Carroll, O., Lindo, V., Del Val, M., Lopez, D., Lepelletier, Y., Greer, F., Schomburg, L., Fruci, D., Niedermann, G. & van Endert, P. M. Concerted peptide trimming by human ERAP1 and ERAP2 aminopeptidase complexes in the endoplasmic reticulum. *Nat Immunol* **6**, 689-697, doi:10.1038/ni1208 (2005).

75 York, I. A., Brehm, M. A., Zendzian, S., Towne, C. F. & Rock, K. L. Endoplasmic reticulum aminopeptidase 1 (ERAP1) trims MHC class I-presented peptides in vivo and plays an important role in immunodominance. *Proceedings of the National Academy of Sciences* **103**, 9202-9207, doi:10.1073/pnas.0603095103 (2006).

76 Cagliani, R., Riva, S., Biasin, M., Fumagalli, M., Pozzoli, U., Lo Caputo, S., Mazzotta, F., Piacentini, L., Bresolin, N., Clerici, M. & Sironi, M. Genetic diversity at endoplasmic reticulum aminopeptidases is maintained by balancing selection and is associated with natural resistance to HIV-1 infection. *Human Molecular Genetics* **19**, 4705-4714, doi:10.1093/hmg/ddq401 (2010).

77 Rock, K. L., York, I. A. & Goldberg, A. L. Post-proteasomal antigen processing for major histocompatibility complex class I presentation. *Nat Immunol* **5**, 670-677, doi:10.1038/ni1089 (2004).

78 Tsui, F. W. L., Haroon, N., Reveille, J. D., Rahman, P., Chiu, B., Tsui, H. W. & Inman, R. D. Association of an ERAP1 ERAP2 haplotype with familial ankylosing spondylitis. *Annals of the Rheumatic Diseases* **69**, 733-736, doi:10.1136/ard.2008.103804 (2010).

79 Serwold, T., Gonzalez, F., Kim, J., Jacob, R. & Shastri, N. ERAAP customizes peptides for MHC class I molecules in the endoplasmic reticulum. *Nature* **419**, 480-483, doi:10.1038/nature01074 (2002).

80 Cui, X., Hawari, F., Alsaaty, S., Lawrence, M., Combs, C. A., Geng, W., Rouhani, F. N., Miskinis, D. & Levine, S. J. Identification of ARTS-1 as a novel TNFR1-binding protein that promotes TNFR1 ectodomain shedding. *J Clin Invest* **110**, 515-526, doi:10.1172/jci13847 (2002).

81 Schomburg, L., Kollmus, H., Friedrichsen, S. & Bauer, K. Molecular characterization of a puromycin-insensitive leucyl-specific aminopeptidase, PILS-AP. *Eur J Biochem* **267**, 3198-3207 (2000).

82 Hattori, A., Matsumoto, H., Mizutani, S. & Tsujimoto, M. Molecular cloning of adipocyte-derived leucine aminopeptidase highly related to placental leucine aminopeptidase/oxytocinase. *J Biochem* **125**, 931-938 (1999).

83 Rawlings, N. D., Barrett, A. J. & Bateman, A. MEROPS: the peptidase database. *Nucleic Acids Research* **38**, D227-D233, doi:10.1093/nar/gkp971 (2010).

84 Nguyen, T. T., Chang, S. C., Evnouchidou, I., York, I. A., Zikos, C., Rock, K. L., Goldberg, A. L., Stratikos, E. & Stern, L. J. Structural basis for antigenic peptide precursor processing by the endoplasmic reticulum aminopeptidase ERAP1. *Nat Struct Mol Biol* **18**, 604-613, doi:10.1038/nsmb.2021 (2011).

85 Goto, Y., Tanji, H., Hattori, A. & Tsujimoto, M. Glutamine-181 is crucial in the enzymatic activity and substrate specificity of human endoplasmic-reticulum aminopeptidase-1. *Biochem J* **416**, 109-116, doi:10.1042/bj20080965 (2008).

86 Gandhi, A., Lakshminarasimhan, D., Sun, Y. & Guo, H. C. Structural insights into the molecular ruler mechanism of the endoplasmic reticulum aminopeptidase ERAP1. *Sci Rep* **1**, 186, doi:10.1038/srep00186 (2011).

87 Kochan, G., Krojer, T., Harvey, D., Fischer, R., Chen, L., Vollmar, M., von Delft, F., Kavanagh, K. L., Brown, M. A., Bowness, P., Wordsworth, P., Kessler, B. M. & Oppermann, U. Crystal structures of the endoplasmic reticulum aminopeptidase-1 (ERAP1) reveal the molecular basis for N-terminal peptide trimming. *Proceedings of the National Academy of Sciences* **108**, 7745-7750, doi:10.1073/pnas.1101262108 (2011).

88 Thompson, M. W., Archer, E. D., Romer, C. E. & Seipelt, R. L. A conserved tyrosine residue of Saccharomyces cerevisiae leukotriene A4 hydrolase stabilizes the transition state of the peptidase activity. *Peptides* **27**, 1701-1709, doi:10.1016/j.peptides.2006.02.006 (2006).

89 Schomburg, L., Kollmus, H., Friedrichsen, S. & Bauer, K. Molecular characterization of a puromycin-insensitive leucyl-specific aminopeptidase, PILS-AP. *European Journal of Biochemistry* **267**, 3198-3207, doi:10.1046/j.1432-1327.2000.01348.x (2000).

90 Chang, S.-C., Momburg, F., Bhutani, N. & Goldberg, A. L. The ER aminopeptidase, ERAP1, trims precursors to lengths of MHC class I peptides by a “molecular ruler” mechanism. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 17107-17112, doi:10.1073/pnas.0500721102 (2005).

91 York, I. A., Chang, S. C., Saric, T., Keys, J. A., Favreau, J. M., Goldberg, A. L. & Rock, K. L. The ER aminopeptidase ERAP1 enhances or limits antigen presentation by trimming epitopes to 8-9 residues. *Nat Immunol* **3**, 1177-1184, doi:10.1038/ni860 (2002).

92 Evnouchidou, I., Kamal, R. P., Seregin, S. S., Goto, Y., Tsujimoto, M., Hattori, A., Voulgari, P. V., Drosos, A. A., Amalfitano, A., York, I. A. & Stratikos, E. Cutting Edge: Coding Single Nucleotide Polymorphisms of Endoplasmic Reticulum Aminopeptidase 1 Can Affect Antigenic Peptide Generation In Vitro by Influencing Basic Enzymatic Properties of the Enzyme. *The Journal of Immunology* **186**, 1909-1913, doi:10.4049/jimmunol.1003337 (2011).

93 Hearn, A., York, I. A. & Rock, K. L. The Specificity of Trimming of MHC Class I-Presented Peptides in the Endoplasmic Reticulum. *The Journal of Immunology* **183**, 5526-5536, doi:10.4049/jimmunol.0803663 (2009).

94 Evnouchidou, I., Momburg, F., Papakyriakou, A., Chroni, A., Leondiadis, L., Chang, S. C., Goldberg, A. L. & Stratikos, E. The internal sequence of the peptide-substrate determines its N-terminus trimming by ERAP1. *PLoS One* **3**, e3658, doi:10.1371/journal.pone.0003658 (2008).

95 Brouwenstijn, N., Serwold, T. & Shastri, N. MHC Class I Molecules Can Direct Proteolytic Cleavage of Antigenic Precursors in the Endoplasmic Reticulum. *Immunity* **15**, 95-104, doi:10.1016/s1074-7613(01)00174-1 (2001).

96 Serwold, T., Gaw, S. & Shastri, N. ER aminopeptidases generate a unique pool of peptides for MHC class I molecules. *Nat Immunol* **2**, 644-651, doi:10.1038/89800 (2001).

97 Lysell, J., Padyukov, L., Kockum, I., Nikamo, P. & Stahle, M. Genetic Association with ERAP1 in Psoriasis Is Confined to Disease Onset after Puberty and Not Dependent on HLA-C*06. *J Invest Dermatol*, doi:10.1038/jid.2012.280 (2012).

98 Gaczynska, M., Rock, K. L. & Goldberg, A. L. Gamma-interferon and expression of MHC genes regulate peptide hydrolysis by proteasomes. *Nature* **365**, 264-267, doi:10.1038/365264a0 (1993).

99 Cariaso, M. & Lennon, G. SNPedia: a wiki supporting personal genome annotation, interpretation and analysis. *Nucleic Acids Research* **40**, D1308-D1312, doi:10.1093/nar/gkr798 (2012).

100 Sherry, S. T., Ward, M.-H., Kholodov, M., Baker, J., Phan, L., Smigielski, E. M. & Sirotkin, K. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Research* **29**, 308-311, doi:10.1093/nar/29.1.308 (2001).

101 Chen, R., Yao, L., Meng, T. & Xu, W. The association between seven ERAP1 polymorphisms and ankylosing spondylitis susceptibility: a meta-analysis involving 8,530 cases and 12,449 controls. *Rheumatol Int* **32**, 909-914, doi:10.1007/s00296-010-1712-y (2012).

102 Goto, Y., Hattori, A., Ishii, Y. & Tsujimoto, M. Reduced activity of the hypertension-associated Lys528Arg mutant of human adipocyte-derived leucine aminopeptidase (A-LAP)/ER-aminopeptidase-1. *FEBS Letters* **580**, 1833-1838, doi:10.1016/j.febslet.2006.02.041 (2006).

103 Guerini, F. R., Cagliani, R., Forni, D., Agliardi, C., Caputo, D., Cassinotti, A., Galimberti, D., Fenoglio, C., Biasin, M., Asselta, R., Scarpini, E., Comi, G. P., Bresolin, N., Clerici, M. & Sironi, M. A Functional Variant in ERAP1 Predisposes to Multiple Sclerosis. *PLoS One* **7**, e29931, doi:10.1371/journal.pone.0029931 (2012).

104 Lee, Y. H., Choi, S. J., Ji, J. D. & Song, G. G. Associations between ERAP1 polymorphisms and ankylosing spondylitis susceptibility: a meta-analysis. *Inflamm Res* **60**, 999-1003, doi:10.1007/s00011-011-0374-x (2011).

105 Hammer, G. E., Gonzalez, F., James, E., Nolla, H. & Shastri, N. In the absence of aminopeptidase ERAAP, MHC class I molecules present many unstable and highly immunogenic peptides. *Nat Immunol* **8**, 101-108, doi:10.1038/ni1409 (2007).

106 Yan, J., Parekh, V. V., Mendez-Fernandez, Y., Olivares-Villagómez, D., Dragovic, S., Hill, T., Roopenian, D. C., Joyce, S. & Van Kaer, L. In vivo role of ER-associated peptidase activity in tailoring peptides for presentation by MHC class Ia and class Ib molecules. *The Journal of Experimental Medicine* **203**, 647-659, doi:10.1084/jem.20052271 (2006).

107 Köhler, A., Cascio, P., Leggett, D. S., Woo, K. M., Goldberg, A. L. & Finley, D. The Axial Channel of the Proteasome Core Particle Is Gated by the Rpt2 ATPase and Controls Both Substrate Entry and Product Release. *Molecular Cell* **7**, 1143-1152, doi:10.1016/s1097-2765(01)00274-x (2001).

108 Goto, Y., Ogawa, K., Hattori, A. & Tsujimoto, M. Secretion of Endoplasmic Reticulum Aminopeptidase 1 Is Involved in the Activation of Macrophages Induced by Lipopolysaccharide and Interferon-γ. *Journal of Biological Chemistry* **286**, 21906-21914, doi:10.1074/jbc.M111.239111 (2011).

109 Tanioka, T., Hattori, A., Masuda, S., Nomura, Y., Nakayama, H., Mizutani, S. & Tsujimoto, M. Human Leukocyte-derived Arginine Aminopeptidase: the third member of the oxytocinase subfamily of aminopeptidases. *Journal of Biological Chemistry* **278**, 32275-32283, doi:10.1074/jbc.M305076200 (2003).

110 Haroon, N. & Inman, R. D. Endoplasmic reticulum aminopeptidases: Biology and pathogenic potential. *Nat Rev Rheumatol* **6**, 461-467, doi:10.1038/nrrheum.2010.85 (2010).

111 Hattori, A., Goto, Y. & Tsujimoto, M. Exon 10 Coding Sequence Is Important for Endoplasmic Reticulum Retention of Endoplasmic Reticulum Aminopeptidase 1. *Biological and Pharmaceutical Bulletin* **35**, 601-605 (2012).

112 Hattori, A., Kitatani, K., Matsumoto, H., Miyazawa, S., Rogi, T., Tsuruoka, N., Mizutani, S., Natori, Y. & Tsujimoto, M. Characterization of Recombinant Human Adipocyte-Derived Leucine Aminopeptidase Expressed in Chinese Hamster Ovary Cells. *The Journal of Biochemistry* **128**, 755-762 (2000).

113 Islam, A., Adamik, B., Hawari, F. I., Ma, G., Rouhani, F. N., Zhang, J. & Levine, S. J. Extracellular TNFR1 Release Requires the Calcium-dependent Formation of a Nucleobindin 2-ARTS-1 Complex. *Journal of Biological Chemistry* **281**, 6860-6873, doi:10.1074/jbc.M509397200 (2006).

114 Adamik, B., Islam, A., Rouhani, F. N., Hawari, F. I., Zhang, J. & Levine, S. J. An association between RBMX, a heterogeneous nuclear ribonucleoprotein, and ARTS-1 regulates extracellular TNFR1 release. *Biochemical and Biophysical Research Communications* **371**, 505-509, doi:10.1016/j.bbrc.2008.04.103 (2008).

115 Haroon, N., Tsui, F. W. L., Chiu, B., Tsiu, H. W. & Inmam, R. D. Serum Cytokine Receptors in Ankylosing Spondylitis: Relationship to Inflammatory Markers and Endoplasmic Reticulum Aminopeptidase Polymorphisms. *The Journal of Rheumatology* **37**, 1907-1910, doi:10.3899/jrheum.100019 (2010).

116 Cui, X., Rouhani, F. N., Hawari, F. & Levine, S. J. An Aminopeptidase, ARTS-1, Is Required for Interleukin-6 Receptor Shedding. *Journal of Biological Chemistry* **278**, 28677-28685, doi:10.1074/jbc.M300456200 (2003).

117 Cui, X., Rouhani, F. N., Hawari, F. & Levine, S. J. Shedding of the Type II IL-1 Decoy Receptor Requires a Multifunctional Aminopeptidase, Aminopeptidase Regulator of TNF Receptor Type 1 Shedding. *The Journal of Immunology* **171**, 6814-6819 (2003).

118 Raposo, G., van Santen, H. M., Leijendekker, R., Geuze, H. J. & Ploegh, H. L. Misfolded major histocompatibility complex class I molecules accumulate in an expanded ER-Golgi intermediate compartment. *J Cell Biol* **131**, 1403-1419 (1995).

119 Turner, M. J., Sowders, D. P., DeLay, M. L., Mohapatra, R., Bai, S., Smith, J. A., Brandewie, J. R., Taurog, J. D. & Colbert, R. A. HLA-B27 Misfolding in Transgenic Rats Is Associated with Activation of the Unfolded Protein Response. *The Journal of Immunology* **175**, 2438-2448 (2005).

120 Turner, M. J., DeLay, M. L., Bai, S., Klenk, E. & Colbert, R. A. HLA–B27 up-regulation causes accumulation of misfolded heavy chains and correlates with the magnitude of the unfolded protein response in transgenic rats: Implications for the pathogenesis of spondylarthritis-like disease. *Arthritis & Rheumatism* **56**, 215-223, doi:10.1002/art.22295 (2007).

121 Zhang, K., Shen, X., Wu, J., Sakaki, K., Saunders, T., Rutkowski, D. T., Back, S. H. & Kaufman, R. J. Endoplasmic Reticulum Stress Activates Cleavage of CREBH to Induce a Systemic Inflammatory Response. *Cell* **124**, 587-599, doi:http://dx.doi.org/10.1016/j.cell.2005.11.040 (2006).

122 Yamamoto, N., Nakayama, J., Yamakawa-Kobayashi, K., Hamaguchi, H., Miyazaki, R. & Arinami, T. Identification of 33 polymorphisms in the adipocyte-derived leucine aminopeptidase (ALAP) gene and possible association with hypertension. *Human Mutation* **19**, 251-257, doi:10.1002/humu.10047 (2002).

123 Fung, E. Y., Smyth, D. J., Howson, J. M., Cooper, J. D., Walker, N. M., Stevens, H., Wicker, L. S. & Todd, J. A. Analysis of 17 autoimmune disease-associated variants in type 1 diabetes identifies 6q23/TNFAIP3 as a susceptibility locus. *Genes Immun* **10**, 188-191, doi:10.1038/gene.2008.99 (2009).

124 Taranta, A., Gianviti, A., Palma, A., De Luca, V., Mannucci, L., Procaccino, M. A., Ghiggeri, G. M., Caridi, G., Fruci, D., Ferracuti, S., Ferretti, A., Pecoraro, C., Gaido, M., Penza, R., Edefonti, A., Murer, L., Tozzi, A. E. & Emma, F. Genetic risk factors in typical haemolytic uraemic syndrome. *Nephrology Dialysis Transplantation* **24**, 1851-1857, doi:10.1093/ndt/gfn720 (2009).

125 Williams, P. J. & Broughton Pipkin, F. The genetics of pre-eclampsia and other hypertensive disorders of pregnancy. *Best Pract Res Clin Obstet Gynaecol* **25**, 405-417, doi:10.1016/j.bpobgyn.2011.02.007 (2011).

126 Mehta, A. M., Jordanova, E. S., van Wezel, T., Uh, H.-W., Corver, W. E., Kwappenberg, K. M. C., Verduijn, W., Kenter, G. G., van der Burg, S. H. & Fleuren, G. J. Genetic variation of antigen processing machinery components and association with cervical carcinoma. *Genes, Chromosomes and Cancer* **46**, 577-586, doi:10.1002/gcc.20441 (2007).

127 Mehta, A. M., Jordanova, E. S., Kenter, G. G., Ferrone, S. & Fleuren, G. J. Association of antigen processing machinery and HLA class I defects with clinicopathological outcome in cervical carcinoma. *Cancer Immunol Immunother* **57**, 197-206, doi:10.1007/s00262-007-0362-8 (2008).

128 Mehta, A. M., Jordanova, E. S., Corver, W. E., van Wezel, T., Uh, H.-W., Kenter, G. G. & Jan Fleuren, G. Single nucleotide polymorphisms in antigen processing machinery component ERAP1 significantly associate with clinical outcome in cervical carcinoma. *Genes, Chromosomes and Cancer* **48**, 410-418, doi:10.1002/gcc.20648 (2009).

129 Strange, A., Capon, F., Spencer, C. C., Knight, J., Weale, M. E., Allen, M. H., Barton, A., Band, G., Bellenguez, C., Bergboer, J. G., Blackwell, J. M., Bramon, E., Bumpstead, S. J., Casas, J. P., Cork, M. J., Corvin, A., Deloukas, P., Dilthey, A., Duncanson, A., Edkins, S., Estivill, X., Fitzgerald, O., Freeman, C., Giardina, E., Gray, E., Hofer, A., Huffmeier, U., Hunt, S. E., Irvine, A. D., Jankowski, J., Kirby, B., Langford, C., Lascorz, J., Leman, J., Leslie, S., Mallbris, L., Markus, H. S., Mathew, C. G., McLean, W. H., McManus, R., Mossner, R., Moutsianas, L., Naluai, A. T., Nestle, F. O., Novelli, G., Onoufriadis, A., Palmer, C. N., Perricone, C., Pirinen, M., Plomin, R., Potter, S. C., Pujol, R. M., Rautanen, A., Riveira-Munoz, E., Ryan, A. W., Salmhofer, W., Samuelsson, L., Sawcer, S. J., Schalkwijk, J., Smith, C. H., Stahle, M., Su, Z., Tazi-Ahnini, R., Traupe, H., Viswanathan, A. C., Warren, R. B., Weger, W., Wolk, K., Wood, N., Worthington, J., Young, H. S., Zeeuwen, P. L., Hayday, A., Burden, A. D., Griffiths, C. E., Kere, J., Reis, A., McVean, G., Evans, D. M., Brown, M. A., Barker, J. N., Peltonen, L., Donnelly, P. & Trembath, R. C. A genome-wide association study identifies new psoriasis susceptibility loci and an interaction between HLA-C and ERAP1. *Nat Genet* **42**, 985-990, doi:10.1038/ng.694 (2010).

130 Yamada, Y., Ando, F. & Shimokata, H. Association of candidate gene polymorphisms with bone mineral density in community-dwelling Japanese women and men. *Int J Mol Med* **19**, 791-801 (2007).

131 Blanchard, N., Gonzalez, F., Schaeffer, M., Joncker, N. T., Cheng, T., Shastri, A. J., Robey, E. A. & Shastri, N. Immunodominant, protective response to the parasite Toxoplasma gondii requires antigen processing in the endoplasmic reticulum. *Nat Immunol* **9**, 937-944, doi:10.1038/ni.1629 (2008).

132 Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. & Wahli, W. Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. *Cell* **68**, 879-887, doi:http://dx.doi.org/10.1016/0092-8674(92)90031-7 (1992).

133 Jones, J. R., Barrick, C., Kim, K.-A., Lindner, J., Blondeau, B., Fujimoto, Y., Shiota, M., Kesterson, R. A., Kahn, B. B. & Magnuson, M. A. Deletion of PPARγ in adipose tissues of mice protects against high fat diet-induced obesity and insulin resistance. *Proceedings of the National Academy of Sciences* **102**, 6207-6212, doi:10.1073/pnas.0306743102 (2005).

134 Mukherjee, R., Jow, L., Croston, G. E. & Paterniti, J. R. Identification, Characterization, and Tissue Distribution of Human Peroxisome Proliferator-activated Receptor (PPAR) Isoforms PPARγ2 versus PPARγ1 and Activation with Retinoid X Receptor Agonists and Antagonists. *Journal of Biological Chemistry* **272**, 8071-8076, doi:10.1074/jbc.272.12.8071 (1997).

135 Fajas, L., Fruchart, J.-C. & Auwerx, J. PPARγ3 mRNA: a distinct PPARγ mRNA subtype transcribed from an independent promoter. *FEBS Letters* **438**, 55-60, doi:http://dx.doi.org/10.1016/S0014-5793(98)01273-3 (1998).

136 Ricote, M., Huang, J., Fajas, L., Li, A., Welch, J., Najib, J., Witztum, J. L., Auwerx, J., Palinski, W. & Glass, C. K. Expression of the peroxisome proliferator-activated receptor γ (PPARγ) in human atherosclerosis and regulation in macrophages by colony stimulating factors and oxidized low density lipoprotein. *Proceedings of the National Academy of Sciences* **95**, 7614-7619 (1998).

137 Gearing, K. L., Göttlicher, M., Teboul, M., Widmark, E. & Gustafsson, J. A. Interaction of the peroxisome-proliferator-activated receptor and retinoid X receptor. *Proceedings of the National Academy of Sciences* **90**, 1440-1444, doi:10.1073/pnas.90.4.1440 (1993).

138 Lemay, D. G. & Hwang, D. H. Genome-wide identification of peroxisome proliferator response elements using integrated computational genomics. *Journal of Lipid Research* **47**, 1583-1587, doi:10.1194/jlr.M500504-JLR200 (2006).

139 Ricote, M., Li, A. C., Willson, T. M., Kelly, C. J. & Glass, C. K. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* **391**, 79-82, doi:10.1038/34178 (1998).

140 Pascual, G., Fong, A. L., Ogawa, S., Gamliel, A., Li, A. C., Perissi, V., Rose, D. W., Willson, T. M., Rosenfeld, M. G. & Glass, C. K. A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma. *Nature* **437**, 759-763, doi:10.1038/nature03988 (2005).

141 Boyault, S., Simonin, M.-A., Bianchi, A., Compe, E., Liagre, B., Mainard, D., Bécuwe, P., Dauça, M., Netter, P., Terlain, B. & Bordji, K. 15-Deoxy-Δ12,14-PGJ2, but not troglitazone, modulates IL-1β effects in human chondrocytes by inhibiting NF-κB and AP-1 activation pathways. *FEBS Letters* **501**, 24-30, doi:http://dx.doi.org/10.1016/S0014-5793(01)02614-X (2001).

142 Ji, J. D., Cheon, H., Jun, J. B., Choi, S. J., Kim, Y. R., Lee, Y. H., Kim, T. H., Chae, I. J., Song, G. G., Yoo, D. H., Kim, S. Y. & Sohn, J. Effects of Peroxisome Proliferator-activated Receptor-γ (PPAR-γ) on the Expression of Inflammatory Cytokines and Apoptosis Induction in Rheumatoid Synovial Fibroblasts and Monocytes. *Journal of Autoimmunity* **17**, 215-221, doi:http://dx.doi.org/10.1006/jaut.2001.0542 (2001).

143 Meier, C. A., Chicheportiche, R., Juge-Aubry, C. E., Dreyer, M. G. & Dayer, J. M. Regulation of the interleukin-1 receptor antagonist in THP-1 cells by ligands of the peroxisome proliferator-activated receptor gamma. *Cytokine* **18**, 320-328 (2002).

144 Jiang, C., Ting, A. T. & Seed, B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* **391**, 82-86, doi:10.1038/34184 (1998).

145 Bordji, K., Grillasca, J.-P., Gouze, J.-N., Magdalou, J., Schohn, H., Keller, J.-M., Bianchi, A., Dauça, M., Netter, P. & Terlain, B. Evidence for the Presence of Peroxisome Proliferator-activated Receptor (PPAR) α and γ and Retinoid Z Receptor in Cartilage: PPARγ activation modulates the effects of interleukin-1β on rat chondrocytes. *Journal of Biological Chemistry* **275**, 12243-12250, doi:10.1074/jbc.275.16.12243 (2000).

146 Afif, H., Benderdour, M., Mfuna-Endam, L., Martel-Pelletier, J., Pelletier, J.-P., Duval, N. & Fahmi, H. Peroxisome proliferator-activated receptor gamma1 expression is diminished in human osteoarthritic cartilage and is downregulated by interleukin-1beta in articular chondrocytes. *Arthritis Research & Therapy* **9**, R31 (2007).

147 Kawahito, Y., Kondo, M., Tsubouchi, Y., Hashiramoto, A., Bishop-Bailey, D., Inoue, K.-i., Kohno, M., Yamada, R., Hla, T. & Sano, H. 15-deoxy-Δ12,14-PGJ2 induces synoviocyte apoptosis and suppresses adjuvant-induced arthritis in rats. *The Journal of Clinical Investigation* **106**, 189-197, doi:10.1172/jci9652 (2000).

148 Butt, C., Gladman, D. & Rahman, P. PPAR-gamma gene polymorphisms and psoriatic arthritis. *The Journal of Rheumatology* **33**, 1631-1633 (2006).

149 Bongartz, T., Coras, B., Vogt, T., Schölmerich, J. & Müller-Ladner, U. Treatment of active psoriatic arthritis with the PPARγ ligand pioglitazone: an open-label pilot study. *Rheumatology* **44**, 126-129, doi:10.1093/rheumatology/keh423 (2005).

150 Pishvaian, M. J., Marshall, J. L., Wagner, A. J., Hwang, J. J., Malik, S., Cotarla, I., Deeken, J. F., He, A. R., Daniel, H., Halim, A.-B., Zahir, H., Copigneaux, C., Liu, K., Beckman, R. A. & Demetri, G. D. A phase 1 study of efatutazone, an oral peroxisome proliferator-activated receptor gamma agonist, administered to patients with advanced malignancies. *Cancer* **118**, 5403-5413, doi:10.1002/cncr.27526 (2012).

151 Murakami, K., Wada, J., Ogawa, D., Horiguchi, C. S., Miyoshi, T., Sasaki, M., Uchida, H. A., Nakamura, Y. & Makino, H. The effects of telmisartan treatment on the abdominal fat depot in patients with metabolic syndrome and essential hypertension: Abdominal fat Depot Intervention Program of Okayama (ADIPO). *Diabetes and Vascular Disease Research* **10**, 93-96, doi:10.1177/1479164112444640 (2013).

152 Neuschwander-Tetri, B. A., Brunt, E. M., Wehmeier, K. R., Sponseller, C. A., Hampton, K. & Bacon, B. R. Interim results of a pilot study demonstrating the early effects of the PPAR-γ ligand rosiglitazone on insulin sensitivity, aminotransferases, hepatic steatosis and body weight in patients with non-alcoholic steatohepatitis. *Journal of Hepatology* **38**, 434-440, doi:http://dx.doi.org/10.1016/S0168-8278(03)00027-8 (2003).

153 Tsubouchi, Y., Kawahito, Y., Kohno, M., Inoue, K.-i., Hla, T. & Sano, H. Feedback Control of the Arachidonate Cascade in Rheumatoid Synoviocytes by 15-deoxy-Δ12,14-Prostaglandin J2. *Biochemical and Biophysical Research Communications* **283**, 750-755, doi:http://dx.doi.org/10.1006/bbrc.2001.4847 (2001).

154 Bianchi, A., Moulin, D., Sebillaud, S., Koufany, M., Galteau, M.-M., Netter, P., Terlain, B. & Jouzeau, J.-Y. Contrasting effects of peroxisome-proliferator-activated receptor (PPAR)gamma agonists on membrane-associated prostaglandin E2 synthase-1 in IL-1beta-stimulated rat chondrocytes: evidence for PPARgamma-independent inhibition by 15-deoxy-Delta12,14prostaglandin J2. *Arthritis Research & Therapy* **7**, R1325 - R1337 (2005).

155 Hawari, F. I., Shykoff, B. E. & Izzo, J. L. Heparin attenuates norepinephrine-induced venoconstriction. *Vascular Medicine* **3**, 95-100, doi:10.1177/1358836x9800300202 (1998).

156 St. John, A. L. & Abraham, S. N. Innate Immunity and Its Regulation by Mast Cells. *The Journal of Immunology* **190**, 4458-4463, doi:10.4049/jimmunol.1203420 (2013).

157 Aoki, R., Kawamura, T., Goshima, F., Ogawa, Y., Nakae, S., Nakao, A., Moriishi, K., Nishiyama, Y. & Shimada, S. Mast Cells Play a Key Role in Host Defense against Herpes Simplex Virus Infection through TNF-alpha and IL-6 Production. *J Invest Dermatol*, doi:10.1038/jid.2013.150 (2013).

158 Galli, S. J. & Tsai, M. Mast cells in allergy and infection: Versatile effector and regulatory cells in innate and adaptive immunity. *European Journal of Immunology* **40**, 1843-1851, doi:10.1002/eji.201040559 (2010).

159 Goodman, M. L., Rivers, R. E. & Irwin, J. W. Mast cells in allergy. *Bibl Anat* **13**, 188-189 (1975).

160 Nigrovic, P. & Lee, D. Mast cells in inflammatory arthritis. *Arthritis Res Ther* **7**, 1 - 11 (2005).

161 Kitamura, Y., Go, S. & Hatanaka, K. Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. *Blood* **52**, 447-452 (1978).

162 Kitamura, Y., Yokoyama, M., Matsuda, H., Ohno, T. & Mori, K. J. Spleen colony-forming cell as common precursor for tissue mast cells and granulocytes. *Nature* **291**, 159-160 (1981).

163 Huang, E., Nocka, K., Beier, D. R., Chu, T.-Y., Buck, J., Lahm, H.-W., Wellner, D., Leder, P. & Besmer, P. The hematopoietic growth factor KL is encoded by the SI locus and is the ligand of the c-kit receptor, the gene product of the W locus. *Cell* **63**, 225-233, doi:http://dx.doi.org/10.1016/0092-8674(90)90303-V (1990).

164 Zsebo, K. M., Wypych, J., McNiece, I. K., Lu, H. S., Smith, K. A., Karkare, S. B., Sachdev, R. K., Yuschenkoff, V. N., Birkett, N. C., Williams, L. R., Satyagal, V. N., Tung, W., Bosselman, R. A., Mendiaz, E. A. & Langley, K. E. Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* **63**, 195-201, doi:http://dx.doi.org/10.1016/0092-8674(90)90300-4 (1990).

165 Metcalfe, D. D., Baram, D. & Mekori, Y. A. Mast cells. *Physiological Reviews* **77**, 1033-1079 (1997).

166 Nocka, K., Buck, J., Levi, E. & Besmer, P. Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. *EMBO J* **9**, 3287-3294 (1990).

167 Tsai, M., Takeishi, T., Thompson, H., Langley, K. E., Zsebo, K. M., Metcalfe, D. D., Geissler, E. N. & Galli, S. J. Induction of mast cell proliferation, maturation, and heparin synthesis by the rat c-kit ligand, stem cell factor. *Proceedings of the National Academy of Sciences* **88**, 6382-6386 (1991).

168 Bressler, R. B., Thompson, H. L., Keffer, J. M. & Metcalfe, D. D. Inhibition of the growth of IL-3-dependent mast cells from murine bone marrow by recombinant granulocyte macrophage-colony-stimulating factor. *The Journal of Immunology* **143**, 135-139 (1989).

169 Rottem, M., Hull, G. & Metcalfe, D. D. Demonstration of differential effects of cytokines on mast cells derived from murine bone marrow and peripheral blood mononuclear cells. *Exp Hematol* **22**, 1147-1155 (1994).

170 Broide, D. H., Wasserman, S. I., Alvaro-Gracia, J., Zvaifler, N. J. & Firestein, G. S. Transforming growth factor-beta 1 selectively inhibits IL-3-dependent mast cell proliferation without affecting mast cell function or differentiation. *The Journal of Immunology* **143**, 1591-1597 (1989).

171 Ihle, J. N., Keller, J., Oroszlan, S., Henderson, L. E., Copeland, T. D., Fitch, F., Prystowsky, M. B., Goldwasser, E., Schrader, J. W., Palaszynski, E., Dy, M. & Lebel, B. Biologic properties of homogeneous interleukin 3. I. Demonstration of WEHI-3 growth factor activity, mast cell growth factor activity, p cell-stimulating factor activity, colony-stimulating factor activity, and histamine-producing cell-stimulating factor activity. *The Journal of Immunology* **131**, 282-287 (1983).

172 Hültner, L., Druez, C., Moeller, J., Uyttenhove, C., Schmitt, E., Rüde, E., Dörmer, P. & van Snick, J. Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). *European Journal of Immunology* **20**, 1413-1416, doi:10.1002/eji.1830200632 (1990).

173 Thompson-Snipes, L., Dhar, V., Bond, M. W., Mosmann, T. R., Moore, K. W. & Rennick, D. M. Interleukin 10: a novel stimulatory factor for mast cells and their progenitors. *The Journal of Experimental Medicine* **173**, 507-510, doi:10.1084/jem.173.2.507 (1991).

174 Matsuda, H., Kannan, Y., Ushio, H., Kiso, Y., Kanemoto, T., Suzuki, H. & Kitamura, Y. Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. *The Journal of Experimental Medicine* **174**, 7-14, doi:10.1084/jem.174.1.7 (1991).

175 Zhang, X., Goncalves, R. & Mosser, D. M. in *Current Protocols in Immunology* (John Wiley & Sons, Inc., 2001).

176 Weischenfeldt, J. & Porse, B. Bone Marrow-Derived Macrophages (BMM): Isolation and Applications. *Cold Spring Harbor Protocols* **2008**, pdb.prot5080, doi:10.1101/pdb.prot5080 (2008).

177 Hume Macrophage Lab, R. I. *Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood and subsequent culture of macrophage cells.*, <Isolation of peripheral blood mononuclear cells (PBMCs) from whole blood and subsequent culture of macrophage cells.> (

178 Warren, M. K. & Vogel, S. N. Bone marrow-derived macrophages: development and regulation of differentiation markers by colony-stimulating factor and interferons. *The Journal of Immunology* **134**, 982-989 (1985).

179 Miller, A. Role of IL-33 in inflammation and disease. *Journal of Inflammation* **8**, 22 (2011).

180 Besnard, A.-G., Togbe, D., Guillou, N., Erard, F., Quesniaux, V. & Ryffel, B. IL-33-activated dendritic cells are critical for allergic airway inflammation. *European Journal of Immunology* **41**, 1675-1686, doi:10.1002/eji.201041033 (2011).

181 Kouskoff, V., Korganow, A.-S., Duchatelle, V., Degott, C., Benoist, C. & Mathis, D. Organ-Specific Disease Provoked by Systemic Autoimmunity. *Cell* **87**, 811-822, doi:http://dx.doi.org/10.1016/S0092-8674(00)81989-3 (1996).

182 Schaller, M., Burton, D. R. & Ditzel, H. J. Autoantibodies to GPI in rheumatoid arthritis: linkage between an animal model and human disease. *Nat Immunol* **2**, 746-753 (2001).

183 Lindqvist, A. K., Nakken, B., Sundler, M., Kjellen, P., Jonsson, R., Holmdahl, R. & Skarstein, K. Influence on spontaneous tissue inflammation by the major histocompatibility complex region in the nonobese diabetic mouse. *Scand J Immunol* **61**, 119-127, doi:10.1111/j.0300-9475.2005.01550.x (2005).

184 Lane, L., Argoud-Puy, G., Britan, A., Cusin, I., Duek, P. D., Evalet, O., Gateau, A., Gaudet, P., Gleizes, A., Masselot, A., Zwahlen, C. & Bairoch, A. neXtProt: a knowledge platform for human proteins. *Nucleic Acids Research* **40**, D76-D83, doi:10.1093/nar/gkr1179 (2012).

185 Matsumoto, I., Maccioni, M., Lee, D. M., Maurice, M., Simmons, B., Brenner, M., Mathis, D. & Benoist, C. How antibodies to a ubiquitous cytoplasmic enzyme may provoke joint-specific autoimmune disease. *Nat Immunol* **3**, 360-365, doi:10.1038/ni772 (2002).

186 Korganow, A.-S., Ji, H., Mangialaio, S., Duchatelle, V., Pelanda, R., Martin, T., Degott, C., Kikutani, H., Rajewsky, K., Pasquali, J.-L., Benoist, C. & Mathis, D. From Systemic T Cell Self-Reactivity to Organ-Specific Autoimmune Disease via Immunoglobulins. *Immunity* **10**, 451-461, doi:http://dx.doi.org/10.1016/S1074-7613(00)80045-X (1999).

187 Ji, H., Pettit, A., Ohmura, K., Ortiz-Lopez, A., Duchatelle, V., Degott, C., Gravallese, E., Mathis, D. & Benoist, C. Critical Roles for Interleukin 1 and Tumor Necrosis Factor α in Antibody-induced Arthritis. *The Journal of Experimental Medicine* **196**, 77-85, doi:10.1084/jem.20020439 (2002).

188 Kyburz, D., Carson, D. A. & Corr, M. The role of CD40 ligand and tumor necrosis factor α signaling in the transgenic K/BxN mouse model of rheumatoid arthritis. *Arthritis & Rheumatism* **43**, 2571-2577, doi:10.1002/1529-0131(200011)43:11<2571::aid-anr26>3.0.co;2-4 (2000).

189 Corr, M. & Crain, B. The Role of FcγR Signaling in the K/B × N Serum Transfer Model of Arthritis. *The Journal of Immunology* **169**, 6604-6609 (2002).

190 Nigrovic, P. A., Binstadt, B. A., Monach, P. A., Johnsen, A., Gurish, M., Iwakura, Y., Benoist, C., Mathis, D. & Lee, D. M. Mast cells contribute to initiation of autoantibody-mediated arthritis via IL-1. *Proceedings of the National Academy of Sciences* **104**, 2325-2330, doi:10.1073/pnas.0610852103 (2007).

191 Wipke, B. T. & Allen, P. M. Essential Role of Neutrophils in the Initiation and Progression of a Murine Model of Rheumatoid Arthritis. *The Journal of Immunology* **167**, 1601-1608 (2001).

192 Dinarello, C. A. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood* **117**, 3720-3732, doi:10.1182/blood-2010-07-273417 (2011).

193 Halappanavar, S., Nikota, J., Wu, D., Williams, A., Yauk, C. L. & Stampfli, M. IL-1 receptor regulates microRNA-135b expression in a negative feedback mechanism during cigarette smoke-induced inflammation. *J Immunol* **190**, 3679-3686, doi:10.4049/jimmunol.1202456 (2013).

194 Karkar, A. M., Tam, F. W., Steinkasserer, A., Kurrle, R., Langner, K., Scallon, B. J., Meager, A. & Rees, A. J. Modulation of antibody-mediated glomerular injury in vivo by IL-1ra, soluble IL-1 receptor, and soluble TNF receptor. *Kidney Int* **48**, 1738-1746 (1995).

195 Dinarello, C. A. The IL-1 family and inflammatory diseases. *Clin Exp Rheumatol* **20**, S1-13 (2002).

196 Shelburne, C. P., McCoy, M. E., Piekorz, R., Sexl, V., Roh, K.-H., Jacobs-Helber, S. M., Gillespie, S. R., Bailey, D. P., Mirmonsef, P., Mann, M. N., Kashyap, M., Wright, H. V., Chong, H. J., Bouton, L. A., Barnstein, B., Ramirez, C. D., Bunting, K. D., Sawyer, S., Lantz, C. S. & Ryan, J. J. Stat5 expression is critical for mast cell development and survival. *Blood* **102**, 1290-1297, doi:10.1182/blood-2002-11-3490 (2003).

197 Weisblum, B. & Haenssler, E. Fluorometric properties of the bibenzimidazole derivative Hoechst 33258, a fluorescent probe specific for AT concentration in chromosomal DNA. *Chromosoma* **46**, 255-260 (1974).

198 Truett, G. E., Heeger, P., Mynatt, R. L., Truett, A. A., Walker, J. A. & Warman, M. L. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). *Biotechniques* **29**, 52, 54 (2000).

199 Han, S. & Roman, J. Rosiglitazone suppresses human lung carcinoma cell growth through PPARγ-dependent and PPARγ-independent signal pathways. *Molecular Cancer Therapeutics* **5**, 430-437, doi:10.1158/1535-7163.mct-05-0347 (2006).

200 Chana, R. S. & Brunskill, N. J. Thiazolidinediones inhibit albumin uptake by proximal tubular cells through a mechanism independent of peroxisome proliferator activated receptor gamma. *Am J Nephrol* **26**, 67-74, doi:10.1159/000091807 (2006).

201 Diaz, B. L., Fujishima, H., Kanaoka, Y., Urade, Y. & Arm, J. P. Regulation of Prostaglandin Endoperoxide Synthase-2 and IL-6 Expression in Mouse Bone Marrow-Derived Mast Cells by Exogenous But Not Endogenous Prostanoids. *The Journal of Immunology* **168**, 1397-1404 (2002).

202 Necela, B. M., Su, W. & Thompson, E. A. Toll-like receptor 4 mediates cross-talk between peroxisome proliferator-activated receptor γ and nuclear factor-κB in macrophages. *Immunology* **125**, 344-358, doi:10.1111/j.1365-2567.2008.02849.x (2008).

203 Smeets, R. L., Joosten, L. A. B., Arntz, O. J., Bennink, M. B., Takahashi, N., Carlsen, H., Martin, M. U., van den Berg, W. B. & van de Loo, F. A. J. Soluble interleukin-1 receptor accessory protein ameliorates collagen-induced arthritis by a different mode of action from that of interleukin-1 receptor antagonist. *Arthritis & Rheumatism* **52**, 2202-2211, doi:10.1002/art.21108 (2005).

204 Bresnihan, B., Alvaro-Gracia, J. M., Cobby, M., Doherty, M., Domljan, Z., Emery, P., Nuki, G., Pavelka, K., Rau, R., Rozman, B., Watt, I., Williams, B., Aitchison, R., McCabe, D. & Musikic, P. Treatment of rheumatoid arthritis with recombinant human interleukin-1 receptor antagonist. *Arthritis Rheum* **41**, 2196-2204, doi:10.1002/1529-0131(199812)41:12<2196::aid-art15>3.0.co;2-2 (1998).

205 Ruperto, N., Quartier, P., Wulffraat, N., Woo, P., Ravelli, A., Mouy, R., Bader-Meunier, B., Vastert, S. J., Noseda, E., D'Ambrosio, D., Lecot, J., Chakraborty, A., Martini, A., Chioato, A. & Paediatric Rheumatology International Clinical Trials, O. A phase II,

multicenter, open-label study evaluating dosing and preliminary safety and efficacy of canakinumab in systemic juvenile idiopathic arthritis with active systemic features. *Arthritis & Rheumatism* **64**, 557-567, doi:10.1002/art.33342 (2012).

206 Ruperto, N., Brunner, H. I., Quartier, P., Constantin, T., Wulffraat, N., Horneff, G., Brik, R., McCann, L., Kasapcopur, O., Rutkowska-Sak, L., Schneider, R., Berkun, Y., Calvo, I., Erguven, M., Goffin, L., Hofer, M., Kallinich, T., Oliveira, S. K., Uziel, Y., Viola, S., Nistala, K., Wouters, C., Cimaz, R., Ferrandiz, M. A., Flato, B., Gamir, M. L., Kone-Paut, I., Grom, A., Magnusson, B., Ozen, S., Sztajnbok, F., Lheritier, K., Abrams, K., Kim, D., Martini, A. & Lovell, D. J. Two Randomized Trials of Canakinumab in Systemic Juvenile Idiopathic Arthritis. *New England Journal of Medicine* **367**, 2396-2406, doi:doi:10.1056/NEJMoa1205099 (2012).

207 Alten, R., Gomez-Reino, J., Durez, P., Beaulieu, A., Sebba, A., Krammer, G., Preiss, R., Arulmani, U., Widmer, A., Gitton, X. & Kellner, H. Efficacy and safety of the human anti-IL-1beta monoclonal antibody canakinumab in rheumatoid arthritis: results of a 12-week, Phase II, dose-finding study. *BMC Musculoskelet Disord* **12**, 153, doi:10.1186/1471-2474-12-153 (2011).

208 Campbell, E. C., Fettke, F., Bhat, S., Morley, K. D. & Powis, S. J. Expression of MHC class I dimers and ERAP1 in an ankylosing spondylitis patient cohort. *Immunology* **133**, 379-385, doi:10.1111/j.1365-2567.2011.03453.x (2011).

209 Kavanaugh, A., St Clair, E. W., McCune, W. J., Braakman, T. & Lipsky, P. Chimeric anti-tumor necrosis factor-alpha monoclonal antibody treatment of patients with rheumatoid arthritis receiving methotrexate therapy. *J Rheumatol* **27**, 841-850 (2000).

210 Huang, Z., Yang, B., Shi, Y., Cai, B., Li, Y., Feng, W., Fu, Y., Luo, L. & Wang, L. Anti-TNF-α therapy improves Treg and suppresses Teff in patients with rheumatoid arthritis. *Cellular Immunology* **279**, 25-29, doi:http://dx.doi.org/10.1016/j.cellimm.2012.09.001 (2012).

211 Li, J., Hsu, H.-C., Yang, P., Wu, Q., Li, H., Edgington, L. E., Bogyo, M., Kimberly, R. P. & Mountz, J. D. Treatment of arthritis by macrophage depletion and immunomodulation: Testing an apoptosis-mediated therapy in a humanized death receptor mouse model. *Arthritis & Rheumatism* **64**, 1098-1109, doi:10.1002/art.33423 (2012).