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The genetics, structure and function of the M1 aminopeptidase oxytocinase subfamily and their therapeutic potential in immune-mediated disease

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ABSTRACT

The oxytocinase subfamily of M1 aminopeptidases plays an important role in processing and trimming of peptides for presentation on major histocompatibility (MHC) Class I molecules. Several large-scale genomic studies have identified association of members of this family of enzymes, most notably ERAP1 and ERAP2, with immune-mediated diseases including ankylosing spondylitis, psoriasis and birdshot chorioretinopathy. Much is now known about the genetics of these enzymes and how genetic variants alter their function, but how these variants contribute to disease remains largely unresolved. Here we discuss what is known about their structure and function and highlight some of the knowledge gaps that affect development of drugs targeting these enzymes.

1. M1 aminopeptidase oxytocinase subfamily – protein structure and function

Adaptive cellular immune responses require the cell-surface presentation of processed self-proteins and foreign-derived antigens by the Major Histocompatibility Complex (MHC) molecules. Dedicated antigen-presenting cells (APC) of the immune system express MHC class II ligands (MHC-II), which are used to activate CD4+ T-cells in response to internalised and presented exogenous proteins sourced from extracellular pathogens. In contrast, almost all cells carry MHC class I (MHC-I), which typically displays endogenous peptides derived from normal cellular proteins. Screening against MHC-I and II complexes displaying self-peptides facilitates the selection of self-tolerant T-cells during maturation in the thymus, with the deletion of autoreactive cells with a low threshold of activation against self. In contrast self-MHC-I recognition by natural killer (NK) cells suppresses cytotoxic NK function via signalling through inhibitory receptors for MHC-I ligands on the lymphocyte. Infected or transformed cells produce altered arrays of cellular proteins and hence altered MHC-I complexes, activating CD8+ cytotoxic T lymphocytes (CTLs) or NK cells to eliminate the aberrant cells. The generation of antigenic peptides, however, can also be disturbed in ways that lead to immune system evasion or to autoimmune reactions.

While some antigenic peptides can be generated by other cytosolic proteases, most are derived from endogenous proteins through proteolytic processing by the proteasome [1] which typically produces only a limited percentage of peptide fragments of the appropriate length for MHC presentation (8–10 residues) [2]. While the proteasome-generated C-termini of the peptides are always maintained for MHC binding, some sequences require further N-terminal proteolysis to trim the peptides to the correct length. Two aminopeptidases in the endoplasmic reticulum (ER), endoplasmic reticulum aminopeptidases 1 and 2 (ERAP1 and ERAP2) are responsible for this N-terminal antigen trimming [3], which determines the peptide repertoire dispayed by MHC -I molecules [4]. The repertoire of cleaved, MHC-I-bound endogenous peptides presented to the immune system not only provides a means for lymphocytes to effectively monitor for infection, but is imperative in shaping tolerogenic T-cell populations in the negative selection of autoreactive cells. This mechanism of self-recognition through MHC-I presentation of endogenous peptides allows infected and transformed cells to be identified through the altered array of MHC-I – peptide (pMHC-I) complexes on the cell surface, leading to

Abbreviations: ERAP, endoplasmic reticulum aminopeptidase; IRAP, insulin-regulated aminopeptidase; HLA, human leukocyte antigen; AS, ankylosing spondylitis; CD, cluster of differentiation; ER, Endoplasmic reticulum

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elimination of the aberrant cells.

While recognition of aberrant cells through pMHC-I can occur for all cell types, the actual activation of effector CTLs is dependent on professional APCs. Subsequently, most cancers and infections that have specific cell tropism not involving APCs require another process to activate the immune response. This process, known as "cross-presentation", allows exogenous antigens to be assembled in MHC-I complexes by dendritic cells (DC) [5]. Following phagocytosis of exogenous antigens by DCs the antigens will either remain within the phagosome and be proteolysed by lysosomal proteases, particularly cathepsin S, or be exported to the cytosol where they are processed by the proteasome. Processed fragments may be transported to the ER for further processing by ERAP1 and ERAP2 [6] or are returned to the phagosome where they are N-terminally trimmed by the insulin-regulated aminopeptidase (IRAP) [7,8], a closely related enzyme to ERAP1 and ERAP2. Given that cytosolic and ER aminopeptidases directly sculpt the peptidome that educates and instructs immune responses, it is easy to conceptualise that genetic polymorphisms that impact on their expression or function may predispose to immune-mediated pathologies. In recent years, deconvolution of the genetic contributors to complex immune-mediated diseases has revealed that, in many distinct conditions, these enzymes are implicated in the altered biological processes that likely culminate in autoimmunity [9–14].

ERAP1, ERAP2 and IRAP are members of the oxytocinase subfamily of M1 aminopeptidases and are characterised by the presence of two key sequence motifs; the HEXXH zinc-binding and GXMEN substrate recognition sequences [15–17]. The *ERAP1, ERAP2* and *IRAP* genes are, in humans, located on chromosome 5q15, suggesting recent gene duplication events and subsequent divergence; interestingly mice only have genes for *ERAP1* (chromosome 13, known as *ERAAP* in rodents) and *IRAP* (chromosome 17). At the amino acid sequence level the proteins are closely related, with IRAP showing 43 and 49% identity to ERAP1 and ERAP2 respectively, while the two ERAP enzymes are 49% identical.

The crystal structures of ERAP1 [18,19], the first of the family to be determined, revealed a four domain structure enclosing a large internal cavity containing the catalytic zinc ion (Fig. 1). The catalytic zinc is bound to Domain II, which has a thermolysin-like fold and contains both the characteristic HEXXH and GXMEN motifs. Domain I and Domain IV enclose the active site cavity, Domain I has a cap on the amino terminal end of the site and Domain IV a bowl that forms most of the actual cavity. Domain III is a β-sandwich structure that links Domains II and IV. These first structures highlighted the importance of conformational flexibility in the function of these proteins, with different 'open' and 'closed' forms identified (Fig. 1). This result was emphasised with the structure of IRAP being found in an intermediate 'semi-closed' conformation [20] (Fig. 1). Along with structures of ERAP2 [21,22], these data indicated that the 'closed' form of the enzyme is the active conformation, with the 'open' form representing an inactive state [22] potentially involved in substrate exchange.

Further structures of ERAP1, ERAP2 and IRAP, some complexed with inhibitors or other ligands, have subsequently been determined (summarised in Table 1). As with the initial structures, the key insight from these data is the role of flexibility in the activity of the proteins. For example, significant rearrangements of the GAMEN motif in IRAP were observed upon ligand binding, which coupled to the transition between the original partially open and a new, fully closed conformation of the protein [23]. Similarly, co-crystal structures of ERAP2 with various inhibitors showed a mixture of single conformation complexes and those with two or more alternate binding configurations [24] due to rearrangement of the active site residues. The structures also shed light on the differential substrate specificity of the enzymes, in particular the 'molecular ruler' aspect peculiar to ERAP1 activity where the enzyme is highly active on model substrates of more than 9 residues but effectively inactive on shorter peptides. A hydrophobic pocket distinct from the active site region was postulated to anchor C-terminal hydrophobic residues on a peptide, positioning the substrate correctly in the active site with peptides less than 9 residues in length unable to span the critical distance between these sites [18,19].

The role of conformational flexibility in the function of the M1 aminopeptidases has also been explored through molecular dynamics simulations of both IRAP [23] and ERAP1 [25]. While the IRAP simulations focused on local adaptation to the binding of a pseudopeptidic ligand, the ERAP1 simulations were intended to explore large scale motions of the protein. This analysis showed that the three structural states seen in crystal structures (open, closed and intermediate 'semi-closed') are accessible, in the absence of ligand, with very low energy barriers for transition between the conformations. The simulations also provided a working model for the interaction of ERAP1 with an MHC-I antigen complex, an interaction that is hypothesised to occur during antigen trimming [26], but that has been difficult to model on the basis of experimental structures. A 'wide-open' conformation of ERAP1 was shown to be potentially accessible, in which the angle between Domain IV and the rest of the protein increases to a degree that provides room for the insertion of a peptide-MHC-I complex. The complex can pack close enough to the active site to bring the N-terminal end of the peptide into the active site pocket [25]. Whether trimming of MHC-I bound peptides by ERAP1 or 2 occurs *in vivo* remains controversial, particularly in light of the structure of the MHC-I peptide-loading complex (PLC) where the MHC-I-peptide is significantly enclosed by the components of the PLC [27].

2. Role of aminopeptidases in the pathogenesis of immune mediated inflammatory diseases

While there are abundant data to associate the genetics of

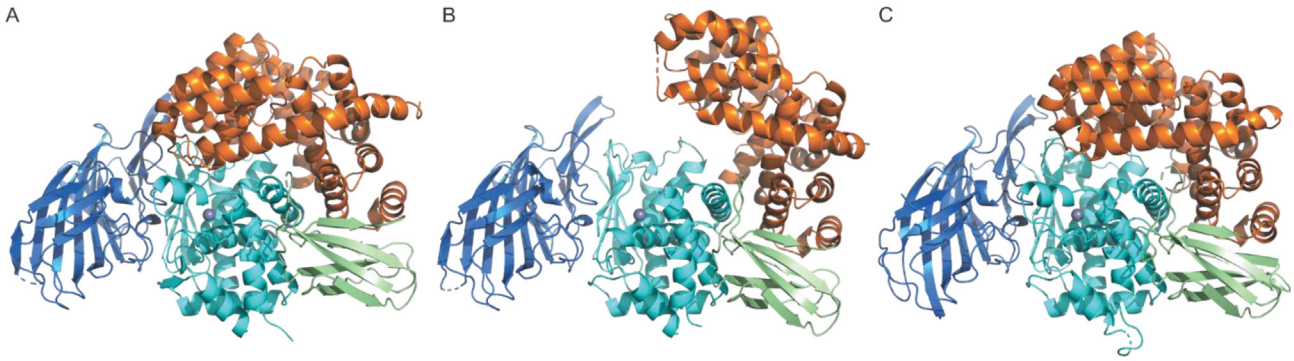

Fig. 1. Diagrammatic representation of the (A) closed and (B) open forms of ERAP1 and (C) the semi-closed conformations of IRAP seen in the crystal structures of the proteins. The structures are shown as cartoons coloured by domain with DI blue, DII cyan, DIII light green and DIV orange. The active site zinc is shown in each structure as a grey sphere. Structures shown are (A) 2YD0, (B) 3MDJ and (C) 2P8Q.

Table 1
Summary of the crystal structures for ERAP1, ERAP2 and IRAP available in the PDB.

Protein	PDB ID	Resolution (Å)	Active Site Ligand	State[a]	Notes	References
ERAP1	3MDJ	2.95	Bestatin	Open		[19]
ERAP1	3QNF	3	–	Open		[18]
ERAP1	2YD0	2.7	Bestatin	Closed		[18]
ERAP1	3RJO	2.3	–	Open	Truncated to Domains III and IV	[108]
ERAP1	5J5E	2.8	–	Open	Truncated to Domains III and IV	[108]
ERAP2	3SE6	3.08	Lysine	Closed		[22]
ERAP2	4E36	3.22	Lysine	Closed		[55]
ERAP2	4JBS	2.79	P52	Closed		[73]
ERAP2	5CU5	3.02	–	Closed	Zinc-free structure	[109]
ERAP2	5AB0	2.5	DG025	Closed		[109]
ERAP2	5AB2	2.73	GPI	Closed	Antigenic epitope sequence GPGRAFVTI	[109]
ERAP2	5J6S	2.8	6GA	Closed		[24]
ERAP2	5K1V	2.9	6PX	Closed		[24]
IRAP	4P8Q	3.02	UNK	Semi	Modelled as Alanine	[20]
IRAP	4PJ6	2.96	Lysine	Semi		[20]
IRAP	4Z7I	3.31	DG025	Semi		[23]
IRAP	5C97	3.37	–	Semi		[23]
IRAP	5MJ6	2.53	7O2	Closed		[23]

[a] State describes which of the three conformational states the structure represents, the 'open', 'closed' or 'semi-closed' conformations.

aminopeptidases with increased susceptibility to certain immune-mediated diseases (summarised in Table 2), paradoxically the mechanisms through which aminopeptidases affect disease pathogenesis remain poorly understood. Over the past decade, genome-wide association studies (GWAS) of immune-mediated disease have emphasised the contribution of tens to hundreds of independent loci to the genetic risk of these multifactorial conditions. The strongest genetic associations seen are those with specific class 1 and class 2 human leukocyte antigen (HLA) alleles [28], often with concurrent disease risk polymorphisms in the endoplasmic reticulum aminopeptidase genes *ERAP1* and *ERAP2*. Disease-associated SNPs in *ERAP1* and/or *ERAP2* have been identified in ankylosing spondylitis (AS) [9], Behcet's disease [11], Crohn's disease [13], multiple sclerosis [14], birdshot chorioretinopathy [29], Type I diabetes [30], Kawasaki disease [31], and psoriasis [10], although elucidating precisely how these genetic changes contribute to a phenotype of immune dysregulation has proven difficult. As reviewed below, there is now substantial evidence that ERAP polymorphisms alter the HLA-presented peptidome by changing the activity and specificity of the enzyme, adding weight to the observation that their genetic effects are often restricted to a specific HLA background. When first identified, the restriction of *ERAP1* associations to HLA-B*27 positive individuals with AS was the most robust evidence of genetic epistasis observed in any complex disease [32]; a phenomenon later also detected in those carrying the AS risk allele HLA-B*40 [33]. Epistatic interactions have also been identified between ERAP1 and HLA-Cw*06 in psoriasis [10], HLA-B*51 in Behcet's disease [11] and recently between ERAP2 and HLA-A*29:02 in birdshot chorioretinopathy [12]. Given the distinct and differing peptide specificities of HLA alleles, it is fathomable that changes in the peptide pool are exacerbated when those changes can be translated to the immune system through the appropriately inherited HLA, thus the co-dependence of these two loci in conferring risk.

2.1. Ankylosing spondylitis

The motivation to understand the role of ERAP enzymes in instructing the immune system has firmly oriented the field of AS research in particular. The finding that the *HLA-B*27* allele is almost essential for AS development still lacks an immunological explanation, despite being made nearly 50 years ago [34,35]. A landmark study published in 2007 first identified two nonsynonymous SNPs in *ERAP1* (then termed *ARTS1,* situated on chromosome 5q15*)* with strong AS associations in a European cohort, rs30187 and rs27044 [9]. The rs30187 association, and that with three further nonsynonymous *ERAP1* polymorphisms (rs17482078, rs10050860, rs2287987), was validated in a North American Caucasian replication study, tagging the gene as an excellent functional candidate for driving disease, possibly by means of altering the HLA-B*27 restricted peptidome [9]. Further fine mapping of *ERAP1* revealed it as a highly polymorphic gene; 11 non-synonymous coding variants (3 novel in codons for highly conserved amino acids), and a number of non-coding SNPs near intron-exon splice sites and within the 5′UTR identified in just 48 individuals with AS [36]. *ERAP1* SNP associations have been repeatedly replicated in population studies of varying sizes across varying ethnicities [37–44], and the first to identify a strong disease associated haplotype (rs27044[C])/rs10050860[C]/rs30187[T]) in three independent cohorts suggested that haplotypic combinations of susceptibility alleles contribute substantially to disease risk [38]. Ten ERAP1 haplotypes, encoding functionally distinct allotypes, have since been identified at > 1% frequency across the human population [45]. The AS risk association remains largely attributed to common haplotypes containing the risk alleles rs30187[T] and rs10050860[C] [46].

It is now understood that *ERAP1* associations can be partitioned into a primary SNP effect at rs30187, and a secondary independent association with rs10050860 that retains significance upon conditioning on the primary signal. HLA-B*27 positive individuals homozygous for protective variants at both SNPs are afforded a 3–4 times lower disease risk than HLA-B*27 carriers co-inheriting the risk alleles, yet, remarkably, these associations do not prevail in HLA-B*27/HLA-B*40 negative disease [32]. Three more aminopeptidase associations have been revealed in recent years, driven by SNPs in the 5q15 locus aminopeptidases *ERAP2* and *LNPEP* (encoding IRAP), and the cytosolic aminopeptidase *NPEPPS* on chromosome 17q21 [47]. *ERAP2* and *LNPEP* associations are only seen upon correction for the nearby *ERAP1* association. The association of *ERAP2* with AS was initially reported in HLA-B*27 negative individuals [47] and a loss of function variant in *ERAP2* is protective in both HLA-B*27 positive and negative AS cases [48]. It should be noted that, although the identified epistasis between *HLA-B*27* and *ERAP1* has propelled research into the molecular mechanisms underlying disease aetiology, much is still to be understood about the genetic contributors to AS and how they interact in a biological system. AS has been estimated to have a heritability of > 90% and only 20.1% of this attributed to the carriage of HLA-B*27, with a small additional fraction (4.3%) to polymorphisms at other loci including the aminopeptidase associations. That altered peptide processing may initiate damaging immunological processes in this disease is the crux of many hypotheses addressing the root cause of pathology, but these need to be addressed in the context of the immunological background upon

Table 2
Key 5q15 locus SNP associations with immune-mediated diseases.

Gene	SNP ID	Hg19 Position	Amino Acid Position*	Risk Allele (RA)**	RA Frequency (ExAC)	Functional Effect of Risk Allele	Associated Conditions
ERAP1	rs30187	96,124,330	Arg528**Lys**	T	0.380	Increased ERAP1 expression, increased rate of substrate trimming, increased destruction of peptides with ERAP1-sensitive P1 residues, increased production peptides with ERAP1-resistant P1 residues [32,57,70]	AS [9,47], MS [14], psoriasis [110,111], PsA [112]
ERAP1	rs27044	96,118,852	Glu730**Gln**	G	0.306	Unknown	AS [9], psoriasis [113], PsA [112]
ERAP1	rs17482078	96,118,866	Gln725**Arg**	C	0.850	Increased rate of substrate trimming [32]	AS [9]
ERAP1	rs10050860	96,122,210	Asn575**Asp**	C	0.845	In complete linkage disequilibrium with rs17482078 [32]	AS [9]
ERAP1	rs2287987	96,129,535	Val349**Met**	T	0.846	Unknown	AS [9]
ERAP2	rs2549782	96,231,000	Lys392**Asn**	T	0.547	Increased rate of substrate trimming [55]	AS [47]
ERAP2	rs2549794	96,244,549	Intronic	C	0.340 [+]	Unknown	Crohn's disease [13]
ERAP2	rs2248374	96,235,896	Intronic	A	0.452	ERAP2 expression relative to lack of expression [68], lower amount of peptide with N-terminal basic residues and decreased HLA-B*27 peptidome affinity [56]	AS [47], PsA [112], birdshot chorioretinopathy [29][#]

*Disease associated amino acids are in boldface.
**Alleles are quotes as on the forward strand.
[+] 1000 Genomes minor allele frequency.
[#] The ERAP2 SNP association reported with birdshot chorioretinopathy is with rs7705093, tagging rs2248374.

which they act, the genetic moderators of which have been only partially devised.

2.2. Psoriasis

There are strong associations between psoriasis and polymorphisms in *ERAP1* [10] and *ERAP2* [49]. Analogous to the *HLA-B*27/ERAP1* interactions in AS, *HLA-Cw*06/ERAP1* epistasis was identified in psoriasis [10]. Moreover, the *ERAP1* associations with AS and psoriasis are concordant (same haplotype with the same direction of association). It is highly likely, therefore, that ERAP1 and probably ERAP2 play similar roles in pathogenesis of AS and psoriasis. However, few studies have examined the HLA-Cw*06 peptidome in detail and only a small number of Cw6 ligands are known. In the absence of detail, we can only speculate as to the functions of ERAP1/2 in psoriasis but the highly analogous nature of the HLA-aminopeptidase genetics and gene-gene interactions in AS and psoriasis suggests that common modes of action operate in both diseases.

2.3. Birdshot chorioretinopathy (BSCR)

This rare form of autoimmune posterior uveitis is strongly associated with *HLA-A*29* [50] but large-scale genetic studies in BSCR are difficult due to the low prevalence of disease. Nonetheless BSCR has recently been associated with *ERAP2* polymorphisms [29]. BSCR has not yet been genetically linked with *ERAP1* variants but *in vitro* biochemical analysis of the HLA-A*29 peptidome in cells expressing different functional variants of ERAP1 demonstrated that peptide length, sequence and HLA-binding affinity were affected [51] in a manner similar to that observed elsewhere for HLA-B*27. The influence of ERAP2 on the HLA-A*29 peptidome is yet unknown.

2.4. Behcet's disease

Behcet's disease, a form of vasculitis common in Turkey, East Asia and the middle East, is strongly associated with *HLA-B*51* [52]. GWAS revealed epistasis between *ERAP1* and *HLA-B*51* in Behcet's patients [11] supporting a role for ERAP1 in sculpting the HLA-B*51 peptidome. Interestingly though, one of the strongest associated SNPs in *ERAP1*, rs17482078 showed opposite directions of association in Behcet's to that in AS. Q725 increases the risk for Behçet's disease but is protective for AS. The HLA-B*51 peptidome has been described [53] but the influence of ERAP1 variants on this has yet to be defined.

3. Functional consequences of polymorphisms in aminopeptidases

With thorough genomic dissection of the 5q15 locus confirming its relevance to several immune mediated conditions, functional studies isolating causal mutations and their direction of effect are pertinent. The rs30187 C allele, a Lys528Arg non-synonymous amino acid change protective for both AS and psoriasis [10,47], shows a significantly reduced rate of substrate trimming both in recombinant protein and in vitro assays [18,32,54]. Similarly, the *ERAP2* protective allele rs2549782 (G, Asn392Lys) excises N-terminal residues from peptide epitopes, particularly those with a positively charged or hydrophobic N-terminal, up to 165-fold slower than the risk associated variant [55]. This may be of little practical relevance however, since the SNP encoding this substitution is tightly associated with rs2248374 that abolishes ERAP 2 expression. While they share homology there is little redundancy in the cooperative function of ERAP1 and 2, each enzyme showing a unique substrate specificity and catalytic potential.

Highly active variants of ERAP1 carrying a combination of disease-risk polymorphisms have been shown to over-trim peptide substrates [56,57]. In vitro these alleles generate a HLA-B*27 peptidome skewed towards nonamers over longer peptides and with a reduction in

Table 3
Summary of screen results for ERAP1, ERAP2 and/or IRAP inhibitors.

Class of inhibitor chemical structure	IC_{50} range (μM)									Reference
	ERAP1			ERAP2			IRAP			
	< 1	1–100	> 100	< 1	1–100	> 100	< 1	1–100	> 100	
Benzopyran	–	–	3	–	–	3	3	2	–	[77]
Disulfide cyclized tripeptide Angiotensin (Ang) IV analogue	Nd[1]			nd			11	2	–	[79]
Macrocyclic analogues of AngIV	nd			nd			11	4	–	[78]
Benzopyran	nd			nd			4	19	23	[83]
Arylsulfonamides	nd			nd			–	14	5	[80]
Small-molecule library[2]	nd			nd			1	18	–	[82]
Arylsulfonamides	nd			nd			3	–	–	[81]
Aminobenzamide	–	4	7[a]	–	4	3 + 4[a]	–	9	2	[84]
Phosphinic pseudopeptide transition state analogues[3]	3	1	–	3	1	–	3	1	–	[73]
Diaminobenzoic acid derivatives	2	24	16 + 35[a]	5	43	14 + 15 [a]	6	54	10 + 7 [a]	[85]
Phosphonic acids	2	15	2	5	7	7	nd			[87]
Phosphinic acids	–	–	29	2	18	9	nd			
Phosphinic psuedotripeptides	16	13	2	26	5	–	28	3	–	[74]
Virtual screening then testing	1	3	49[b]	–	1	1	–	1	1	[86]

Notes:
1. Not determined.
2. 10,500 compound primary screen.
3. Original DG013A article.
a. No inhibition detected to 50 μM.
b. Compounds with IC_{50} values > 25 μM.

peptides with an alanine at position 1 (P1), a residue highly susceptible to ERAP1 cleavage. Several recent studies have examined the effects of ERAP1 haplotypes on the HLA-B*27 peptidome [57–59]. Combined, these studies highlight some key features of ERAP1 biology that influence HLA-B*27 antigen presentation: ERAP1 polymorphisms influence trimming and presentation of many peptides; most, but not all, ERAP1 variants affect peptides at the P1 residue; and, the influence of ERAP1 on the HLA-B*27 peptidome is very diverse due to the multiplicity of ERAP1 variants and the complexity of their patterns of inheritance in various haplotypic combinations. Conversely, ERAP2 preferentially destroys peptides with N-terminal basic residues that are generated by highly active ERAP1 variants, lowering the affinity of the HLA-B*27 peptidome in which basic P1 residues are favoured [56]. It has further been proposed that ERAP2 may influence peptide length by allosteric activation of ERAP1 in ERAP1-ERAP2 heterodimers [26,60].

In AS, ERAP proteins are proposed to influence immune function in three possible ways: (i) by altering the HLA-B*27 peptidome in such a manner as to generate an arthritogenic peptide; (ii) by disrupting folding of peptide:MHC complexes resulting in an unfolded protein response (UPR) and induction of ER stress; (iii) by contributing to the formation of cell surface HLA-B*27 homodimers as a result of abnormal trimming of peptide and subsequent formation of unstable peptide:HLA-B*27 complexes. HLA-B*27 homodimers are subsequently recognised by specific CD4 T cells [61]. Evidence supporting roles for ERAP proteins in all three models exists (reviewed elsewhere [62,63]). However, evidence demonstrating the UPR model has been difficult to prove in humans [64,65] despite strong supportive evidence in HLA-B*27-transgenic rats [66,67]. In other ERAP associated conditions it is plausible that similar disruption to the nature of peptide-HLA constructs underpin disease.

Complementary to the pathogenic nature of over-active ERAP variants, a substantial overlap exists between disease risk SNPs and expression quantitative trait loci (eQTLs) associated with increased *ERAP1* and *ERAP2* transcript and protein expression [36]. Most staggering is the strong association with the null variant rs2248374(G) in *ERAP2*, protective for AS, that completely abolishes protein expression [47,68]. Cell lines carrying the rs30187(T) risk polymorphism as well as the rs30187/rs17482078/rs10050860(T/C/C) risk haplotype have similarly been shown to exhibit higher ERAP1 protein and transcript expression relative to those expressing the protective alleles [57,69]. Recently a thorough eQTL analysis of 1221 genotyped and imputed variants spanning the 5q15 locus confirmed that risk variants in linkage with rs30187 substantially increase *ERAP1* transcript expression (lead eQTL rs39840 conferring a 34.3% increase in transcript levels) and *ERAP2* transcript expression in the ~75% of individuals that do express *ERAP2* (lead eQTL rs2927608 conferring a 148% increase in transcript levels) [70]. Further, SNPs situated on the rs10050860 disease associated haplotype were shown to be strongly associated with the alternate expression of two ERAP1 isoforms differing in the inclusion of exon 20, evidently due to their effect on isoform splicing. The most significant splice-altering disease-risk variant (rs7063, AS association P-value 1.3×10^{-41}), situated between exon 19 and 20, drives strong preferential expression of the 19-exon transcript and significantly higher ERAP1 protein expression overall given the predominance of this protein isoform over all others [70]. Increased ERAP1 and ERAP2 expression likely exacerbates the effect of the overactive variants in these genes that tie them to a range of pathologies, varying in the site of immune driven damage but nevertheless underpinned by a similar immunogenetic architecture [71].

In vivo, loss of ERAP1 expression also affects peptide handling and the HLA-B*27 peptidome. Homozygous deletion of *Erap1* in HLA-B*27 transgenic rats affected approximately one-third of the B*27 peptidome but left most unchanged, suggesting that some of the HLA-B*27 immunopeptidome is not dependent on Erap1 processing. In this model, loss of Erap1 increased mean peptide length and increased the frequency of C-terminal hydrophobic residues and of N-terminal Ala, Ser, or Lys. The presence of ERAP1 on the other hand increased the frequency of C-terminal Lys and Arg, of Glu and Asp at intermediate residues, and of N-terminal Gly [72]. Changes in the function and expression of these aminopeptidases likely culminate in a peptide repertoire that is, by some means, immunogenic, or promote immune system activation by poorly tolerising cytotoxic lymphocytes with an immense capacity to elicit cell and tissue damage.

4. Translating aminopeptidase biology to new drugs

Whatever their mechanism(s) of action, the best characterised disease-associated variants of ERAP1 implicated in AS result in a gain of

Table 4
IC_{50} values for key inhibitors identified in various screens.

Name	IC_{50} (μM)			Assays tested	References
	ERAP1	ERAP2	IRAP		
Bestatin	> 5			L-AMC	[114]
Tosedostat	> 5			L-AMC	[114]
Purpurin	~10			L-AMC, *in vivo* angiogenesis in zebrafish	[115]
DG002A	0.52	0.547	0.218	L-AMC or R-AMC, HLA-B*27	[73]
DG002B	0.513	0.571	0.344	surface expression, GSW11	[73]
DG013A	0.033	0.011	0.03	epitope surface presentation	[73]
DG013B	3.6	1.7	2.2		[73]
DG023	0.043	0.037	0.002	L-AMC or R-AMC	[74]
DG026	3.694	0.74	0.032	L-AMC or R-AMC, DC^3-cell induced IL-2 production from T cells	[74]
Thimerosal	0.24	> 50	> 50	L-AMC, DC-cell induced IL-2 production from T cells	[86]

L-AMC: L-leucine-7-amido-4-methyl coumarin; R-AMC: L-arginyl-7-amido-4-methyl coumarin.

function [39] implying that inhibiting ERAP1, and likely also ERAP2, function is an important avenue of drug development for treatment of this disease. While less is known about aminopeptidase biology in psoriasis and birdshot chorioretinopathy, ERAP drug targeting strategies in these diseases are also likely to be of value. The availability of ERAP1 crystal structures has enabled recent advances in development, and particularly rational design, of ERAP1/2 inhibitors (Tables 3 and 4) [73,74]. Bestatin and tosedostat, two compounds that inhibit a broad spectrum of aminopeptidases including aminopeptidase N, have shown efficacy in Phase II clinical trials in the treatment of lung cancer and acute myeloid leukaemia, respectively [75,76], demonstrating that the aminopeptidase family is targetable for clinical effect. A number of screens have been performed to discover high potency inhibitors of IRAP [77–83], or ERAP1, ERAP2 and IRAP [73,74,84–87].

From the screens reported by the Stratikos group [73,74] a compound designated DG013A has been identified with high potency against ERAP1 (IC_{50}: 33 nM), ERAP2 (IC_{50}: 11 nM) and IRAP (IC_{50}: 30 nM) but low selectivity. DG013A has also been shown to affect a number of cellular processes. Incubation of cells expressing HLA-B*27 with increasing amounts of DG013A results in greater levels of peptide-bound cell surface HLA-B*27 expression [73]. Similarly, cell surface presentation of the GSW11 epitope by CT26 cells increased with a dose-dependent inhibition by DG013A [73]. The presence of DG013A reduced CD107α expression from human CD56+ CD3- NK cells incubated with an R528/Q730 mutant of ERAP1 [88]. DG013A also reduced RAW264.7 cell phagocytic activity in a dose dependent fashion and decreased IL-1β, IL-6 and TNFα expression from K528/E730 ERAP1 treated human peripheral blood mononuclear cells [88]. Also, DG013A was shown to reduce the ligation of KIR3DL2 to HLA-B*27 free heavy chains through the decrease in IL-2 production, and to decrease Th17 expansion and IL17A production from CD4+ T cells incubated with HLA-B*27 expressing antigen presenting cells [89]. These results suggest that inhibition of the M1 aminopeptidases may prove beneficial to treatment of diseases in which they are implicated. It is also important to note that ERAAP silencing in mouse lymphoma cells promoted NK cell-mediated anti0tumour effects [90] implying that ERAP silencing may have applications beyond inflammatory diseases.

5. Targeting aminopeptidases in inflammatory diseases

A challenge for the use of aminopeptidase inhibitors in treatment of chronic immune-mediated diseases will be ensuring specificity of those inhibitors such that all M1 aminopeptidases are not targeted. Non-specific targeting is likely to result in side-effects that may be manageable for short-term treatment but which may pose greater risks in long-term treatment of chronic conditions. Encouragingly though humans carrying the loss of function variant of ERAP2 appear healthy and there are no reports of increased disease susceptibilities in this genetic cohort. ERAAP-/- mice appear to only display increased susceptibility to *T. gondii* [91] and some lymphocytic choriomeningitis virus (LCMV) [92] infections. One caveat of those animals being housed in controlled specific pathogen free environments must be considered. Importantly, a key difference between knockout transgenic experiments and in vivo pharmacological inhibition is the timing of the inhibition with respect to generation of immunological tolerance. Pioneering work by the Shastri group elegantly demonstrated that, in ERAAP—mice, MHC Class-I presents many unstable and immunogenic peptides, reflecting failures of tolerance mechanisms in these animals [93]. Drugs targeting ERAP proteins will not impact tolerance mechanisms in a fully developed immune system and are likely, therefore, to be less harmful to an individual than the effects seen in ERAAP−/− mice.

ERAP1 and ERAP2 are expressed in many tumour types [94–97] but we do not yet understand what role(s) aminopeptidases might play in tumour growth or immune responses to cancer. In cervical cancer, for example, ERAP1 expression correlates with clinical outcome [97] but *ERAP1* polymorphisms do not contribute to genetic susceptibility to cervical cancer [98] so is altered ERAP1 biology permissive of cervical cancer development or a potential biomarker of a failed immune response? *ERAP1* variants are associated with elevated blood pressure [99] and the AS, psoriasis and birdshot chorioretinopathy risk variant of *ERAP1* at rs31087 confers protection against hypertension [100] raising the likelihood that aminopeptidase inhibition will affect cardiovascular function. As long as doubts remain about the functions of aminopeptidases in health and disease careful screening should accompany any aminopeptidase-targeted clinical development programs.

Strategies to target one or more aminopeptidases require careful validation *in vitro* and *in vivo* and a greater understanding of the precise physiological role(s) played by aminopeptidases in each disease. Humans carry nine M1 aminopeptidases and the exact physiological role of those is not yet clearly defined. For example, cell surface expression of MHC Class I free heavy chains has been reported to be both increased [101] and decreased [102] in monocytes in the presence of the AS protective variant 730Glu of ERAP1. Pre-clinical assessments of the effect of aminopeptidase inhibition on disease phenotypes also poses significant challenges. Few animal models exist for the chronic diseases in which aminopeptidases likely play important roles. Those that do exist have not been shown to be ERAP1 dependent to date. The HLA-B*27 rat, a widely-accepted model of some features of spondyloarthopathy, showed no clinical benefits of reduced ERAP1 expression [72]. Other commonly used animal models, including SKG mice [103], mice overexpressing TNF [104,105] or IL-23 [106] are driven by pathogenic cytokines and are likely independent of peptide handling processes, although this has not been formally tested in such models. Similarly, models of psoriasis are unlikely to involve aminopeptidase-dependent mechanisms since most are chemically induced and phenocopy disease symptoms rather than immune processes. Perhaps the strongest candidate for a valuable pre-clinical screening model of aminopeptidase inhibitors is the *HLA-A*29* transgenic mouse which spontaneously develops symptoms of birdshot chorioretinopathy [107]. Aminopeptidase dependency has not yet been demonstrated in this model but warrants further investigation. At a minimum the field would benefit from generation of animals expressing relevant human HLA transgenes, for example HLA-B*27, along with human ERAP1 and/or ERAP2. Such a tool would permit investigation of the effects of aminopeptidase inhibitors on peptide handling in the context of human HLA Class I biology and while also enabling screening of effects of aminopeptidase inhibition on tumour development, cardiovascular disease and response to infections.

6. Conclusions

There is convincing genetic evidence linking M1 aminopeptidases, particularly ERAP1 and ERAP2, with several chronic inflammatory diseases. Biochemical and immunological interrogation of aminopeptidase biology has described some ways in which genetics influences aminopeptidase activity, but much is still to be learnt about the genetic complexities of ERAP1 and ERAP2 and the shared and unique features of these enzymes. There is a strong need to develop animal models to allow better evaluation of how aminopeptidases function in vivo and to determine the whole animal consequences of inhibiting aminopeptidase functions.

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