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Intracellular antigen processing by ERAP2: Molecular mechanism and roles in health and disease

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A B S T R A C T

Endoplasmic Reticulum Aminopeptidase 2 (ERAP2) is an intracellular enzyme localized in the ER that has been shown to play roles in the generation of peptides that serve as ligands for MHC class I (MHC-1) molecules. Although ERAP2 has been primarily described as an accessory and complementary enzyme to the homologous ERAP1, several lines of evidence during the last few years suggest that it can play distinct and important roles in processing antigenic peptides and influencing cellular cytotoxic immune responses. Such emerging evidence has been shaping ERAP2 as a potentially tractable target for regulating select autoimmune and anti-cancer responses for therapeutic purposes. Here, we review the state-of-the-art knowledge on the role of ERAP2 in antigen processing, its structure and molecular mechanism, influence on shaping MHC-I-bound immunopeptidomes and its involvement in disease pathogenesis.

1. Discovery of ERAP2 and role in antigen processing

ERAP2 was first described as leukocyte-derived arginine aminopeptidase by the group of Dr. Tsujimoto as a novel member of the M1 family of aminopeptidases that was highly homologous to placental leucine aminopeptidase (now most often referred to as insulin-regulated aminopeptidase, IRAP) and adipocyte-derived leucine aminopeptidase (now most often referred to as ER aminopeptidase 1, ERAP1) [1]. The same group later assigned ERAP2 to be part of the oxytocinase subfamily of M1 aminopeptidases based on homology and function [2]. ERAP2 is upregulated by interferon gamma, is localized in the ER and has unique substrate specificity for positively charged amino acids [1,3]. Although it can process hormonal substrates such as angiotensin III and kallidin *in vitro* [1], most literature to date has focused on its ability to process N-terminally extended precursors of antigenic peptides and therefore on its role in cellular immunity [4].

The role of ERAP2 in antigen processing hinges on the generation of peptide ligands for Major Histocompatibility Class I (MHC-I) molecules [5]. In all somatic cells, endogenous proteins can be digested in the cytosol, often by the action of the proteasome, generating peptides that can be transported into the ER. There, further processing by aminopeptidases can customize the length and sequence of these peptides, before they are loaded onto nascent MHC-I through the action of a multi component protein complex termed the peptide loading complex [6]. The peptide-MHC-I complexes are then transported on the cell surface where they can be recognized by specialized receptors on T-lymphocytes and NK cells, which trigger cytotoxic responses against antigenically altered targets, such as infected and cancer cells. ERAP1 has been shown to be important for this process, both by helping generate optimized MHC-I peptide cargo and by over-trimming some peptides, making them unable to be bound by MHC-I [7]. The role of ERAP2 in this process was first directly demonstrated in 2005 by Saveanu et al. [4] in a seminal study in which the authors demonstrated that in certain cases ERAP1 was not sufficient to generate the mature epitopes for MHCI and that ERAP2 was necessary to supplement activity for correct epitope generation. Interestingly, that study demonstrated that ERAP2 functioned as a part of a bimolecular complex with ERAP1. The function and roles of such a complex is discussed in more detail in another review in this issue by Peter van Endert et al. Regardless, the literature since then has been more focused on the function of ERAP1, whereas ERAP2 has been studied less intensely. This may have been due to either ERAP2 being viewed as an accessory – and perhaps secondary – enzyme next to ERAP1's dominant function or to the absence of the ERAP2 gene in rodents – limiting the options for detailed immunological experiments. Several findings during the recent years however have highlighted an emerging distinct role of ERAP2 in peptide processing and immunity that warrants further study and suggests novel emerging opportunities for pharmacological interventions aiming at regulating immune responses.

2. Structure, enzymatic activity and mechanism of function

Crystallographic structures of ERAP2 have provided key insights on its function. The first crystal structure was solved in 2012 [8] and

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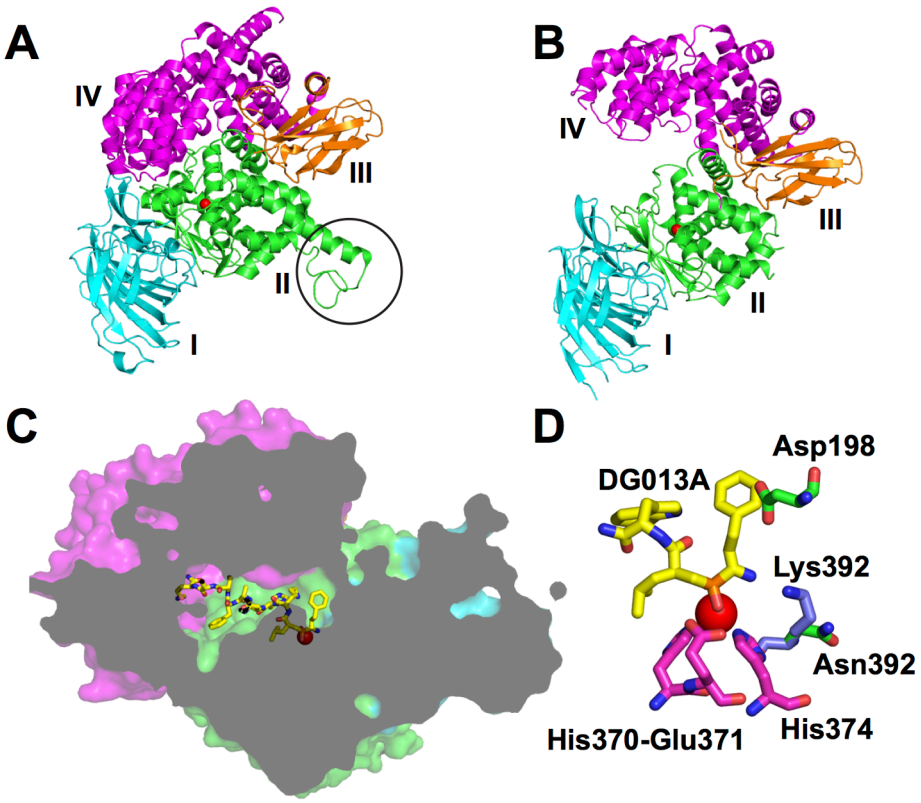

Fig. 1. (A) Schematic representation of the structure of ERAP2 (PDB code 3SE6) with the four protein domains indicated. The exon10 loop is indicated by a circle. (B) Schematic representation of the open conformation of ERAP1 (PDB code 3MDJ) in a similar orientation. (C) Cutaway view of the crystal structure of ERAP2 (PDB code 4Z7I) showing a peptide analogue trapped inside the central cavity of the enzyme. (D) Detail from the active site of ERAP2 (PDB code 4JBS) showing an active-site inhibitor (yellow sticks), the amino acids that stabilize the Zn (II) atom (magenta sticks), the key residue that determines ERAP2 selectivity (Asp198, in green sticks) and the polymorphic residue 392 (SNP rs2549782) in blue and green sticks. Catalytic Zn(II) atoms are shown as red spheres. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

revealed a four-domain organization forming a large internal cavity that contained the catalytic center (Fig. 1A). The crystal structure of a polymorphic variant of ERAP2 was solved later the same year and showed a similar fold with only localized differences around the polymorphic amino acid that however accounted for some drastic changes in enzymatic activity and selectivity [9]. The structure of ERAP2 has also been solved in complex with three active-site inhibitors and two peptidic ligands [10–12]. All currently known ERAP2 crystal structures have a similar domain organization to the closed conformation of ERAP1 [13]. This conformation features a very large internal cavity that contains the catalytic center but has no direct access to the external solvent, therefore precluding subsequent catalytic cycles and substrate-product exchange. As a result, it has been hypothesized that ERAP2 also exists in alternate conformations resembling the open conformation of ERAP1, in which domain IV has moved away from domain II (which contains the catalytic center) allowing peptidic substrates to enter and access the catalytic site (Fig. 1B). Although this conformation is conceptually mandatory for substrate access and has been computationally predicted, it has not yet been experimentally observed.

The active site of ERAP2 contains a Zinc(II) atom coordinated by the conserved HELAH motif, a hallmark of M1 aminopeptidases, next to the GAMEN motif that helps form the catalytic site and guide selectivity [8,14]. The unique selectivity of ERAP2 for positively charged amino acids was first proposed to be due to Glu181, a residue that lines the side of the S1 specificity pocket of the enzyme [15]. This was further supported by mutagenesis and structural studies [8,16]. Two crystal structures of ERAP2 with non-cleavable peptidic substrates later provided insight on how the enzyme recognizes peptides [11]. Both peptides were found to have their N-terminus stabilized in the active site and their C-terminal moieties extend away through the internal cavity and towards the domain II/IV junction (Fig. 1C). The crystal structure of the larger of the two, a 10mer phosphinic transition-state analogue, was of sufficient quality to map several interactions between side-chains of the peptide and residues of the interior of the cavity. In several cases shallow or moderately deep pockets in the cavity accommodated peptide side-chains and contributed to peptide recognition. Overall, the interactions of the peptide with the substrate cavity of ERAP2, apart from the S1 pocket, appear shallow and opportunistic and although contribute to substrate recognition, are permissive enough to allow recognition of a very large variety of peptide sequences, something thought to be consistent ~~to~~ with the ability of ERAP2 to process a variety of peptide substrates [11]. The same crystal structure revealed a previously unidentified feature of the oxytocinase subfamily of M1 aminopeptidases: a loop, encoded by exon 10 of the gene, consisting of 23 amino acids that has been “unseen” in crystallographic structures of ERAP1 and considered to be unstructured [17]. This loop has been proposed to be important for ER retention and a similar loop in ERAP1 has been shown to control ERAP1 secretion through formation of mixed disulfides with ERp44 [18,19]. In the case of ERAP2, the loop adopted a particular secondary/tertiary structure including an internal disulfide bond and a helical component (Fig. 1A). Interactions with an adjacent ERAP2 molecule in the crystal were also evident, suggesting a structural template for protein-protein interactions [11].

The lack of access of the substrate to the internal cavity of ERAP2 as well as the inability of the trapped substrate to access the external solvent has led to a hypothesis of a multi-step mechanism of peptide trimming, similar to the one proposed for the homologous ERAP1 [11,17,20]. According to this mechanism, the enzyme cycles between a closed and open conformation, the latter of which can capture peptidic substrates, which in term promote enzyme closing. Opportunistic interactions between peptide side-chains and residues that line the internal cavity promote substrate capture, which induces a conformational change of the enzyme and influence substrate specificity. Catalysis of N-terminal peptide bond cleavage proceeds by the well characterized Zn(II)-catalyzed thermolysin-like mechanism shared by all M1 aminopeptidases [20]. The products of this catalysis are i) a free amino acid and ii) a new shorter peptide. In order for more cleavages to take place, the free amino acid has to be released from the catalytic center, something that almost certainly necessitates an “opening” conformational change that will provide access to the external solvent.

After the amino acid diffuses away, the remaining peptide can either "slide" towards the catalytic center for further trimming or be released altogether to allow binding and processing of a new peptide. Interestingly, although both ERAP1 and ERAP2 appear to share many similarities in mechanism, it seems that subtle and not well-characterized details in their structure drive distinct substrate preferences. Most strikingly, ERAP1 appears to prefer long (more than 10 amino acid) substrates and this selectivity has been proposed to arise by a regulatory site that activates the enzyme when long substrates are present [17]. ERAP2 does not share this property and this is consistent with a preference for more moderate-length substrates (7-8mers) something that has led to the hypothesis that ERAP2 may be better suited to destroy antigenic peptides that generally need to have a length of at least 8–9 residues in order to bind onto MHC-I [11].

3. ERAP2 variation in sequence, expression and function

Unlike ERAP1, which displays a significant diversity at the protein level [21], the polymorphism of ERAP2 affecting its amino acid sequence seems to be very limited. In particular, the K392N dimorphism, determined by the rs2549782 SNP, is distributed at the genotypic level with an almost equal frequency in most populations and drastically affects the enzymatic activity and specificity of the enzyme [9]. The N392 variant was similar to the K392 counterpart in the trimming of peptide substrates with N-terminal basic residues, but was much more efficient in trimming hydrophobic ones, although not as much as ERAP1. The basis for these specificity changes were in the alterations induced by the K392N change in the catalytic site, affecting the transition-state stabilization, as well as in the binding site of the N-terminal side-chain. The drastic functional effects of this polymorphism can potentially diversify the antigen processing capacity of individuals in the populations where both enzyme variants are expressed [22]. Yet rs2549782 is usually in strong linkage disequilibrium (LD) with another SNP, rs2248374, which determines ERAP2 protein expression. This polymorphism (A/G) is a splice-site variant, whose G allele is considered to induce differential splicing of an extended exon 10 leading to generation of premature stop codons. The resulting transcript is degraded by nonsense mediated decay and usually results in undetectable protein expression [23]. Yet a truncated ERAP2 protein can sometimes be observed in cell lines [24]. SNP rs2248374 tags two haplotypes that include numerous polymorphisms, among them other splice-altering variants, in very tight LD [25]. Both haplotypes have been maintained at nearly equal frequency by balancing selection. In a recent study, dendritic cells from individuals carrying the haplotype tagged by the rs2248374 G allele expressed RNA corresponding to novel forms of truncated ERAP2 proteins, lacking the catalytic aminopeptidase domain, upon influenza infection [26]. These forms presumably arise from linked splice-site SNPs. Although unable to perform direct antigen processing, the up-regulation of these proteins upon viral infection might raise the question of a hypothetical, as yet unknown function, of these seemingly aberrant ERAP2 forms. Thus, balancing selection of the rs2248374-tagged haplotypes might result from a selective advantage of variability in ERAP2 expression and antigen processing at the population level, or from an advantage in the expression of alternative ERAP2 forms with unforeseen function in virally infected individuals.

In most populations, the tight LD between the G allele of rs2248374 and the rs2549782 allele coding for the N392 variant implies that only the K392 allotype is generally expressed. In addition, since the frequencies of the non-expressing and expressing ERAP2 haplotypes are very similar due to balancing selection, about 25% of individuals, those homozygous for the non-expressing haplotype, fail to express ERAP2 protein. In contrast, individuals homozygous for the rs2248374 A allele express about 50% more ERAP2 protein compared to heterozygous individuals for this SNP.

Among the polymorphisms with the largest effect on ERAP2 expression is rs10044354 (C/T), where homozygous individuals for the C allele show very low levels of ERAP2 protein [24,27]. Despite its rather strong LD with rs2248374, this variant has an independent influence on ERAP2 protein expression [24], which was observed, for instance, in some cell lines [28]. Since this polymorphism is located in an intronic region of the adjacent LNPEP gene, the effects of rs10044354 are most likely due to other polymorphisms in tight LD [25].

Recently, the polymorphism rs7586269 (A/G) in the promoter region of the ERAP2 gene was associated with changes in ERAP2 expression in the Sardinian population [29]. In this study, the G allele of rs7586269 resulted in lower ERAP2 expression apparently due to a double effect: lower transcription and higher mRNA degradation. Interestingly, these effects correlated with higher ERAP1 expression in a number of cell lines, raising the possibility of a concerted regulation of the expression of both genes, with opposite effects, by this polymorphism. However, unlike rs2549782 and rs10044354, which show a similar distribution of both alleles in multiple populations, the minor allele frequency of rs7586269 is no more than 14%, and often much lower in the major populations of the 1000 Genomes (www.internationalgenome.org).

Deeper understanding of the relationship and relative importance of these and other expression quantitative trait loci (eQTLs), let alone the mechanism by which they modulate ERAP2 expression, is hampered by the tight LD of many polymorphisms across the ERAP2 locus and neighbor region [25]. It is conceivable that multiple polymorphisms, besides those discussed above, regulate ERAP2 expression in multiple ways. The article by T. Kenna and M. Brown in this issue addresses these questions in depth.

4. ERAP2 and disease

ERAP2 is a risk factor for at least three MHC-I-associated diseases: ankylosing spondylitis (AS), birdshot chorioretinopathy (BSCR) and psoriasis [27,30,31]. In contrast to ERAP1, which is in epistasis with the MHC-I susceptibility alleles in these diseases [32–35], the association of ERAP2 is not epistatic. It is also associated with Crohn's disease [35], a complex inflammatory disorder of the gut, primarily considered to be an MHC-II-associated disease, but with likely additional involvement of MHC-I genes [36]. In addition, ERAP2 is associated with risk of hypertension [37] and preeclampsia [38–41], a complex pathological condition of pregnancy, of which hypertension is a hallmark.

4A. Ankylosing spondylitis

The association of ERAP2 with AS was initially reported in HLA-B*27-negative individuals using rs2910686, located in the ERAP2-LNPEP region as the leading SNP [41] and subsequently confirmed in HLA-B*27-positive patients [30], using rs2248374. Protection from AS, was conferred by the G allele of the latter SNP, leading to loss of ERAP2 protein expression. This situation seems analogous to the protective effect of low activity ERAP1 variants, such as the Hap10 allotype [21,32], and strongly suggests that impaired ERAP1/2-mediated antigen processing is protective for this disease. Consistent with this conclusion, an analysis of eQTLs controlling the expression level of ERAP2 revealed that the AS risk genotype resulted in as much as 148% increased transcript expression, independently of the contribution of the splice-site variant rs2248374 [25]. Therefore the ERAP2-mediated risk of AS depends not only on the presence of the enzyme but also on its expression level.

4B. Birdshot chorioretinopathy

This eye-specific inflammatory disorder is unique among MHC-I-associated diseases in its organ specificity and in that its association with A*29:02 is virtually 100% [42]. The association of ERAP2 with BSCR was initially reported in one study [27], where the expression of this protein, as tagged by rs10044354 (T allele), was associated with

disease risk, similarly as in AS. This association was confirmed in a more recent study [24] that also determined that the contribution of this SNP to BSCR was independent of rs2248374. A very interesting outcome of this study is that risk of BSCR was not only favored by the presence and increased expression of ERAP2, but also by the low activity Hap10 haplotype of ERAP1. This is in strong contrast with AS, where Hap10 is protective. In fact, the combined association of rs10044354, determining increased ERAP2 expression, and Hap10 of ERAP1, with low expression and activity, was associated with BSCR more strongly than either gene alone. Therefore, although ERAP2 expression is associated with risk of both diseases the ERAP1/ERAP2 haplotype determining disease risk is different in both cases.

4C. Psoriasis

The involvement of ERAP2 in psoriasis was initially reported in a large meta-analysis [31], after controlling for the contribution of ERAP1. This study, which used as the leading SNP the eQTL rs2910686, determined that the risk allele for this disease (C) was the one resulting in higher ERAP2 expression. Yet ERAP1 and ERAP2 alleles with opposite effects on disease co-existed in the same haplotype, which poses considerable difficulty to assessing the actual contribution of ERAP2 to this disease, both at the genetic and functional levels. The results of this study suggest that the association of ERAP2 with psoriasis shows a similar pattern as in AS, as does the association of ERAP1, where the high activity of this enzyme is also a risk factor [21,33].

Psoriasis is a heterogeneous disease, with subsets differing in the age at onset and association with C*06:02 [43,44]. It is possible that the relevance of ERAP1 and ERAP2 may be different among disease subsets [45,46], but the few studies addressing this issue usually included relatively small cohorts. Thus, their conclusions might benefit from larger scale analyses allowing for robust statistical assessments of the association of these enzymes with disease subsets.

4D. Crohn's disease

This is, together with ulcerative colitis, one of the major forms of inflammatory bowel disease. It is a genetically complex disorder in which a very large number of susceptibility genes have been identified [47], including many related with innate immunity. ERAP2 was identified early as one of such genes [35], using rs2549794, an intronic eQTL in the ERAP2 gene. As in other diseases, it is possible that regulatory effects on the expression of the enzyme, promoted either by this SNP or other(s) in tight LD with it, might influence this disease. It is also possible that, at least part of the effect might be due to concurrent mutations in exon sequences of some patients potentially affecting the enzyme function [48]. Although Crohn's disease is considered to be mainly an MHC-II-associated disorder, several MHC-I alleles are also susceptibility factors [36]. The relevance of the latter may perhaps be highlighted by the involvement of ERAP2 as a risk factor in this disease.

4E. Hypertension and preeclampsia

A possible role of ERAP2 in regulating blood pressure, by mechanisms possibly independent of antigen processing, was suggested by early *in vitro* digestion studies demonstrating its capacity to catalyze the conversion of kallidin and angiotensin III to bradykinin and angiotensin IV, respectively [1]. A recent study reported an association of several eQTL tagging ERAP2 with baseline diastolic blood pressure (rs3733904, rs4869315 and rs2549782, all three associated with reduced ERAP2 mRNA) and incident hypertension (rs2927615) in a very large cohort of women [37] supporting a role of this enzyme, along with ERAP1 [37,49] in the blood pressure homeostasis.

The mechanism by which ERAP2 may modulate hypertension is unknown and poses the difficulty that, as far as it is known, this enzyme is only found in the ER. However, it has been shown that the redox state of ERp44, a resident protein of the ER involved in thiol-mediated protein retention [50], modulates hypertension, possibly by controlling the release of proteins, including ERAP1 [19], from the ER. This mechanism might hypothetically apply as well to ERAP2, although, to our knowledge, an interaction of this enzyme with ERp44 has not yet been demonstrated.

A regulatory role of ERAP2 on blood pressure might underlie, at least to some extent, the association of this gene with preeclampsia, a pregnancy disorder characterized by high blood pressure, with complex immune, inflammatory and other components [51]. The initial studies relating ERAP2 with preeclampsia were carried out in Caucasian Women from USA [38] and in Australian and Norwegian populations [39]. Two different missense SNPs, rs2549782 (coding for K392N) and rs17408150 (coding for L646Q), showing moderate or no LD, were associated with preeclampsia in the Australian and Norwegian cohorts, respectively. In another study [40], rs2549782 was also associated with preeclampsia in African Americans. Since rs2549782 is in tight LD with rs2248374, which determines ERAP2 protein expression in most populations [23], the risk allele of rs2549782 (G) for preeclampsia, coding for K362, is the one leading to the expression of ERAP2.

Neither rs2549782 nor rs17408150 were associated with this disease in the Chilean population [40]. Yet, lack of LD between rs2549782 (G/T, coding for the K362N variants) and rs2248374 (A/G, determining protein expression or lack of it respectively) among Chileans, allowed an independent assessment of both SNPs with preeclampsia in this population [22]. Although none of the two SNPs showed association with this disease, the double homozygous genotype rs2549782 (TT), rs2248374 (AA), leading to the exclusive and high expression of the N392 variant, was not found in Chileans fetuses or mothers, raising the very interesting question of the deleterious effects of this particularly active variant. For instance, it was hypothesized that its high enzymatic activity might alter the presentation of paternal antigens in the trophoblast, compromising the immune tolerance towards paternal-derived antigens in the fetal tissue by the maternal immune system. Of note, in that study, the TT, AG genotype, expressing only the N392 variant of ERAP2 but at lower levels, was tolerated, suggesting that the deleterious effect of this variant was critically dependent on its expression level.

Consistent with this hypothesis, a significantly higher level of immune-mediated apoptosis was observed in an HLA-C-positive, ERAP2-negative choriocarcinoma cell line transfected with the N362 variant of ERAP2, relative to either the mock- or K392-transfected cell line, suggesting a specific role of the N362 variant in activating NK and T-cell responses against choriocarcinoma cells [52]. Indeed, the observation that many tumors, including a high percentage of choriocarcinomas, fail to express ERAP2 [52,53] suggests a role of this enzyme in anti-tumor activity, including, or perhaps consisting of, promoting immune-mediated tumor clearance [54]. The role of ERAP1 and ERAP2 in cancer is reviewed by Doriana Fruci and colleagues in this issue.

5. ERAP2 and the cellular immunopeptidome

Although the enzymatic specificity o ERAP2 has been analyzed *in vitro* in multiple studies using both non-peptidic and peptidic substrates [1,4,9,11,16,55], the actual role of this enzyme in shaping MHC-I-bound peptidomes in live cells is less known. Yet, the implication of ERAP2 as risk factor for multiple inflammatory diseases underlines the importance of assessing its role in antigen processing *in vivo*. Because of its complementary N-terminal cleavage specificity and substrate length preferences compared to ERAP1, and because it improves ERAP1-mediated peptide processing through concerted trimming *in vitro* [4,56,57] it is generally assumed that ERAP2 plays a subsidiary role to that of ERAP1 in antigen processing. This idea is also supported by lack of an orthologous enzyme to ERAP2 in mice and by absence of ERAP2, but not ERAP1, in a significant percentage (≈25%) of human individuals.

The analysis of the effects of ERAP2 on MHC-I peptidomes provides an opportunity to determine the actual role of this enzyme and its relationship to ERAP1-mediated processing in live cells and is probably essential to understand its relevance in MHC-I-associated diseases. For this reason the relationship of ERAP2 with the MHC-I peptidome was initially studied in HLA-B*27 and HLA-A*29:02, two molecules with highly disparate peptidomes, and showing extremely high association with distinct inflammatory diseases, AS and BSCR, respectively, in which ERAP2 is also involved. In both cases, the focus was on the quantitative changes induced in the peptidome by ERAP2, rather than on the qualitative generation or destruction of specific epitopes, and the approach adopted was the comparative analysis of MHC-I-bound peptidomes in ERAP1-concordant lymphoid cell lines differing in their expression or not of ERAP2.

*5A. ERAP2 and the HLA-B*27 peptidome*

The HLA-B*27:05 peptidome is characterized by a very stringent restriction at peptide position (P) 2, with Arg in > 95% of the peptides, a preference for aliphatic/aromatic residues at P3, and aliphatic, aromatic and basic C-terminal residues. Still, a small part of the HLA-B*27:05 peptidome also includes peptides with Gln and Lys at P2 [58–61]. In addition, basic (Arg, Lys) and small residues (Gly, Ala, Ser) are frequent at P1. As it is usually the case with human MHC-I molecules, the HLA-B*27 peptidome consists mainly of 9-mers and a lower percentage of 10-mers, with shorter or longer peptides being much less frequent [61–64].

Two studies examined the effects of ERAP2 expression on the HLA-B*27 peptidome. In the first one [64], the peptidomes of two ERAP2-negative cell lines expressing high activity ERAP1 variants (Hap1 and Hap2, respectively) were compared with the peptidome of an ERAP2-positive cell line expressing the Hap2 variant of ERAP1. A statistically significant decrease (about 3–4%) in the number of peptides with basic P1 residues was observed in the ERAP2-positive cell line compared to any of the ERAP2-negative ones, suggesting some destruction of these B*27:05 ligands. Quantitative differences in peptide amounts were observed at two levels. First, nonamers were increased, at the expense of longer peptides, in the presence of ERAP2, suggesting a more efficient trimming and an improved generation of ligands with optimal length, with this enzyme. The effect was statistically significant, but not dramatic, and affected only a fraction of the peptidome. The most important differences were found in the distribution of P1 residue frequencies when the peptides predominant in the absence of ERAP2 were compared with those predominant in the presence of this enzyme. Whereas ERAP2-negative cells showed no differences in P1 residue frequencies of their B*27:05 ligands, a selective diminishment in the amounts of peptides with basic P1 residues occurred in the presence of ERAP2. This reflected a direct action of the enzyme, essentially independent of ERAP1, in destroying HLA-B*27 ligands with ERAP2-susceptible P1 residues and demonstrated a significant effect of this enzyme on the HLA-B*27 peptidome, even in a high activity ERAP1 context.

It must be noted that the HLA-B*27 peptidome is relatively rich in peptides with N-terminal basic residues and therefore it would be expected to be, at least in principle, particularly sensitive to this enzyme.

The second study [65] addressed the effects of ERAP2 on the HLA-B*27 peptidome as a function of the ERAP1 context in which the former enzyme is expressed. Initially the peptidomes from two ERAP2-positive cells expressing high (Hap2) or low activity (Hap10) ERAP1 variants were compared. In the more active ERAP1 context increased amounts of 9-mers, at the expense of longer peptides, were found, but P1 residue frequencies were very similar, except for a selective decrease of peptides with Ala1, an ERAP1-susceptible residue, in the more active ERAP1 context. When the peptidomes from an ERAP2-negative cell line expressing the Hap2 variant of ERAP1 and an ERAP2-positive cell line expressing Hap10 were compared the same effects in peptide length and Ala1 frequency found across the same ERAP1 mismatch and presence of ERAP2 were observed, but, in addition, a significant decrease in the amount of peptides with basic P1 residues was evident in the Hap10/ERAP2-positive cell line, relative to Hap2/ERAP2-negative cells. Therefore, the effects of ERAP2 could be distinguished from those of ERAP1 and the same diminishment in the amounts of peptides with basic P1 residues initially observed in a highly active ERAP1 context was reproduced in a low activity ERAP1 background.

Taken together both studies demonstrate a significant role of ERAP2 in shaping the HLA-B*27 peptidome, mainly by increased destruction of HLA-B*27 ligands with basic P1 residues, along with a more moderate effect on optimizing peptide length. These effects are distinguishable from those of ERAP1, indicating that ERAP2 acts, to a large extent, as a separate enzyme and not just through influencing ERAP1 processing. Yet, in these studies the effect of ERAP2 in distinct ERAP1 backgrounds was always compared with the peptidomes of cells expressing highly active ERAP1 variants. A direct comparison of the effects of ERAP2 in the low activity Hap10 context has not been carried out. It is therefore possible that the significance of ERAP2 expression in the processing of the HLA-B*27 peptidome may be different depending on the ERAP1 context, as suggested by *in vitro* digestion experiments [65].

*5B. ERAP2 and the HLA-A*29:02 peptidome*

The A*29:02 peptidome has a single major motif consisting of aromatic residues, mainly Tyr, at the C-terminal position. Much looser preferences for aliphatic/aromatic residues at the secondary anchor positions P3 and P7 are also observed. Basic P1 residues are much less frequent than in HLA-B*27. Instead, Tyr and Phe are relatively frequent at P1 [28,66]. Overall, A*29:02 ligands are significantly more hydrophobic than those of HLA-B*27.

The effects of ERAP2 on the A*29:02 peptidome were examined in a recent study [67] through the comparative analyses of the peptidomes expressed in two pairs of ERAP1 concordant cell lines, expressing highly active ERAP1 variants, but differing, within each cell line pair, in the expression or not of ERAP2. In both cases, effects on peptide length and P1 residue frequencies were observed, but they were different from those in the HLA-B*27 peptidome.

In the presence of ERAP2, 9-mers were decreased, and longer peptides increased. This apparently paradoxical variation was moderate but statistically significant, and suggested a protective effect, mediated by ERAP2, from ERAP1 over-trimming of A*29:02 ligands, a phenomenon that was not observed in HLA-B*27. The basis for this protective effect is unclear, but may be due to unproductive binding to ERAP2 of peptides not susceptible to trimming by this enzyme.

The effects of ERAP2 on P1 residue frequencies were less dramatic than in HLA-B*27, presumably because of the lower frequency of basic P1 residues among A*29:02 ligands. Yet, a lower frequency of ERAP2-susceptible residues and higher global hydrophobicity at this position was observed in the presence of this enzyme, reflecting a direct effect of ERAP2 in destroying A*29:02 ligands.

6. The nature of ERAP1/ERAP2 interaction in the processing of MHC-I peptidomes

In vitro studies have shown that the concerted peptide trimming mediated by ERAP1/ERAP2 heterodimers is much more efficient compared to the trimming by the uncoupled enzymes, ERAP1 was found to be more active in the heterodimeric complexes than in the free form [4,56,57]. These interesting findings are reviewed elsewhere in this issue by Peter van Endert and colleagues and will not be further discussed. Instead, we will briefly address here to what extent the immunopeptidomics studies, at their current state, are compatible either with a prominent role of ERAP1/ERAP2 heterodimers, or with alternative modes of cooperation between both enzymes.

A general improvement of peptide processing by ERAP2 is

suggested, especially in HLA-B*27, by the peptide length optimization induced by this enzyme. In addition, the increased hydrophobicity at P1 observed among HLA-A*29:02 ligands in the presence of ERAP2 is reminiscent of analogous observations with highly active ERAP1 variants [28]. However, none of these effects necessarily imply an activation of ERAP1 by interaction with ERAP2 or, for that matter, the involvement of physical heterodimers. Indeed, the strongest evidence against a mechanism implying a prominent role of ERAP2-mediated activation of ERAP1 in live cells is that the alterations in P1 residue frequencies observed both in the HLA-B*27 and A*29:02 peptidomes are unlike those found across ERAP1 activity differences, which involve changes in P1 residue usage related to their susceptibility to ERAP1. Instead, in the presence of ERAP2, changes in P1 residue frequencies relate mainly to their susceptibility to ERAP2 and not, or very little, to ERAP1 susceptibility.

Indeed, ERAP1/ERAP2 heterodimers account for a limited percentage of the pool of these enzymes in the ER [4] and, although weaker interactions not easily detectable by standard biochemical methods cannot be ruled out, monomeric forms of ERAP1 and ERAP2 are predominant in live cells.

The complementary residue specificity and length preferences of both enzymes allow for an alternative mode of interaction in peptide processing. For instance, octamers, which are produced by over-trimming of 9-mers, can inhibit ERAP1 processing of longer peptides [68] through unproductive binding to this enzyme. Further digestion of octamers by ERAP2 would counteract this inhibition and promote ERAP1 activity. In addition, as suggested by the length effects observed in the A*29:02 peptidome, long peptides with ERAP1-susceptible and ERAP2-resistant P1 residues may bind ERAP2 in a non-productive way, slowing down their processing by ERAP1 and also inhibiting ERAP2 trimming of short substrates. Again, ERAP1 processing of these longer peptides would counteract ERAP2 inhibition. Thus, a possible mode of reciprocal modulation of the activity of both enzymes may be through competitive binding and reciprocal digestion of substrates that are appropriate by one of the enzymes but inhibitory by the other. There is still limited evidence for this mechanism, which remains largely hypothetical, but it is amenable to experimental testing, may provide an alternative mechanism of ERAP1/ERAP2 interaction distinct from heterodimer formation and be more compatible with observations in cellular immunopeptidomes.

7. Relationship between the genetics and the biochemistry of ERAP1/2-MHC-I interaction in disease

As discussed above, the effects of ERAP2 on the HLA-B*27 and A*29:02 peptidomes are different, both on peptide length and P1 residue usage, so that although ERAP2 is a risk factor for AS and BSCR, the basis for its association with both diseases is not necessarily through the same effects on the respective MHC susceptibility allotypes. This difference adds to the fact that the association of ERAP1 with both diseases is also different: whereas in AS low activity ERAP1 variants (Hap10) are protective [21,32], this haplotype favors BSCR [24]. Thus, in AS the high processing activity, including epitope destruction by over-trimming, of both ERAP1 and ERAP2 add to each other in increasing disease risk. Whether this synergistic effect is due to generation of *arthritogenic* epitopes, destruction of *protective* ones or to general effects depending on global alterations in the peptidome remains to be elucidated.

Concerning BSCR, both low activity of ERAP1 and the presence of ERAP2 lead to increased amounts of peptides longer than 9-mers, so that both risk factors add to each other in their modulation of peptide length. However, the effects of low activity ERAP1 variants and ERAP2 presence on P1 residue frequencies and hydrophobicity at this position are different, since hydrophobic P1 residues are favored both by ERAP2 and high activity ERAP1 variants [28,67]. Thus, it is possible that the effects on peptide length, rather than other alterations, may be directly related to the pathogenic effect of both ERAP1 and ERAP2 in BSCR. Whereas this fact might point out to the relevance of specific epitope(s) in this disease, a pathogenetic mechanism based on more general alterations of the peptidome, affecting, for instance, NK recognition and function, cannot be ruled out.

Finally, it should be noted that the alterations induced by ERAP2 both in HLA-B*27 and A*29:02 peptidomes suggest that this enzyme acts separately from ERAP1 in live cells, although the data also suggest a certain degree of functional inter-dependence of both enzymes. These separate roles in the processing of MHC-I peptidomes might somehow be related to the fact that ERAP1, but not ERAP2, is usually in epistasis with the susceptibility MHC-I allele in multiple diseases.

8. Is ERAP2 a tractable target for treatment of disease?

Several accumulating lines of evidence are beginning to solidify the notion that the enzymatic activity of ERAP2 can play significant roles in cytotoxic immune responses and the pathogenesis of disease: i) the association of ERAP2 expression and polymorphism with predisposition to HLA-associated inflammatory diseases [55], ii) the enzyme's effects on the cellular immunopeptidome [64,65,67], iii) the large variability of its expression levels in human cancers – often in imbalanced ways to ERAP1 [53] and iv) the recently demonstrated effects of its polymorphic variation on Natural Killer cell activity towards a cancer cell line [52]. As a result it is conceivable that, similar to ERAP1 [69] ERAP2 can be a tractable pharmacological target for modulating the cellular immunopeptidome with applications for the treatment of cancer or autoimmunity. Interestingly, although the initially described accessory role of ERAP2 apparently reduced interest for any pharmaceutical development for this enzyme, the highly specialized functions of ERAP2, in conjunction with its role in specific diseases, may result in a highly valuable niche for pharmacological interventions. The accessory function of ERAP2 may also present a therapeutic advantage since ERAP2 inhibition may be more tolerable in the context of the general function of adaptive immunity, as suggested by the significant percentage of ERAP2-negative individuals.

In contrast to ERAP1, ERAP2 has already been demonstrated easier to target with small molecule inhibitors and several small molecules have been developed with excellent potency and good selectivity, although plenty of room to improve still exists [12,70]. All published studies regarding the development of ERAP2 inhibitors have targeted the active site of the enzyme either focusing on mechanism-based inhibitors or catalytically relevant features. The most successful approach has involved the phosphinic group that resembles the transition state that is formed during catalysis. A first potent inhibitor using this chemistry was first described in 2013, based on a phosphinic pseudotripeptide scaffold and was very potent (nM range) but lacked selectivity, primarily because it utilized conserved structural features of M1 aminopeptidases (Fig. 1D) [10] Second generation inhibitors however showed enhanced selectivity with one compound showing over 50-fold selectivity versus ERAP1 [71]. Good selectivity with slightly lower potency has also been achieved with dipeptide analogues [72]. Good selectivity with submicromolar potency has also been achieved with a series of 3,4-diaminobenzoic acid derivatives that featured a substrate-like binding configuration [12,73]. Although hydroxamic acid derivatives have been shown to be very effective against other members of this family of aminopeptidases [74], such a derivative co-crystallized with ERAP2 showed the expected binding configuration in the active site but had very poor binding affinity [12].

Unfortunately, very little has been done so far in evaluating ERAP2 inhibitors in the context of cellular or *in vivo* models, in part due to its lack in rodents that are often used in assessing the effects of pharmacological interventions to the immune system function in a preclinical level. Development of different animal models or *ex vivo* systems will be necessary for further evaluation of the therapeutic potential of ERAP2 inhibitors.

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