Molecular pathways for antigenic peptide generation by ER aminopeptidase 1

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ABSTRACT

Endoplasmic Reticulum aminopeptidase 1 (ERAP1) is an intracellular enzyme that can generate or destroy potential peptide ligands for MHC class I molecules. ERAP1 activity influences the cell-surface immunopeptidome and epitope immunodominance patterns but in complex and poorly understood manners. Two main distinct pathways have been proposed to account for ERAP1's effects on the nature and quantity of MHCI-bound peptides: i) ERAP1 trims peptides in solution, generating the correct length for binding to MHCI or overtrimming peptides so that they are too short to bind, and ii) ERAP1 trims peptides while they are partially bound onto MHCI in manner that leaves the peptide amino terminus accessible. For both pathways, once an appropriate length peptide is generated it could bind conventionally to MHCI, competing with further trimming by ERAP1. The two pathways, although not necessarily mutually exclusive, provide distinct vantage points for understanding of the rules behind the generation of the immunopeptidome. Resolution of the mechanistic details of ERAP1-mediated antigenic peptide generation can have important consequences for pharmacological efforts to regulate the immunopeptidome for therapeutic applications, and for understanding association of ERAP1 alleles with susceptibility to autoimmune disease and cancer. We review current evidence in support of these two pathways and discuss their relative importance and potential complementarity.

1. MHCI peptide binding and presentation

The MHCI antigen processing and presentation pathway samples cellular proteins and presents peptide fragments bound to MHCI molecules for scrutiny by CD8^{+} T cells (Rock et al., 2016). Many of these peptides are generated after protein degradation by the proteasome, a multi-subunit enzyme that degrades proteins to peptides 4–20 amino acids in length (Cascio et al., 2001). The majority of these peptides are rapidly destroyed by cytosolic aminopeptidases, but a small fraction escapes to the endoplasmic reticulum (ER) through the action of the transporter associated with antigen processing, TAP (Saunders and van Endert, 2011). Most peptides translocated into the ER contain the same C-terminus as the antigenic epitopes finally presented by MHCI, due to similarities of proteasome cleavage sites and MHC peptide binding preferences, but many contain N-terminal extensions that have to be trimmed by ER resident aminopeptidases (ERAPs), before loading onto MHCI molecules (Blanchard et al., 2010). MHCI molecules assemble in the ER with a polymorphic type I integral membrane glycoprotein heavy chain and an invariant soluble light chain (β2 microglobulin, β2m). The heavy chain forms the peptide binding site, a groove on the upper surface of the MHCI molecule (Hewitt, 2003). MHCI residues within the binding groove interact *via* hydrogen bonds and salt bridges with the free N- and C-termini of peptides, thus limiting binding to (mostly) peptides of 8–9 residues. Side-chain binding pockets within the peptide binding site accommodate two or more residues (anchor residues) of the bound peptide (Neefjes and Ovaa, 2013; Blum et al., 2013). MHCI molecules are highly polymorphic with variation focused in side-chain binding pockets, so that a large variety of different peptides can be bound by various MHCI allelic variants. The loading of the peptide results in stabilization of MHCI-peptide complex, dissociation from the ER peptide loading machinery, and transit through Golgi to the cell surface to become available for immunosurveillance by receptors on CD8^{+} T cells (Yewdell et al., 2003; Neefjes et al., 2011).

2. Biological function of ERAP1

N-terminally extended antigenic peptide precursors that survive cytosolic degradation and enter the ER, need to be further processed by ERAP1 and/or ERAP2, in order to acquire the right length required for MHCI binding. ERAP1 (ERAAP in mouse) is an IFN-γ-inducible,

metalloaminopeptidase that trims N-terminal residues of extended peptide precursors down to 8–9 amino acids (Chang et al., 2005; Serwold et al., 2002; York et al., 2002). ERAP1 can also limit the production of antigenic peptides by overtrimming them (York et al., 2002; Saric et al., 2002). Several independent studies using RNA interference or knock-out animals confirmed this key involvement of ERAP1 in antigen presentation (Chang et al., 2005; Serwold et al., 2002; York et al., 2002; Saric et al., 2002). In addition these studies demonstrated that the absence of ERAP1 activity can lead to reduced number of peptide-MHCI complexes on the cell surface that also have a shorter half-life (Hammer et al., 2007; Yan et al., 2006). In ERAP1-deficient mice the reduction in MHCI expression was proven to be due to faster dissociation of peptide-MHCI complex from the cell surface rather than to a slower rate of complex assembly in the ER (Hammer et al., 2006). Furthermore, ERAP1 plays roles *in vivo* in immune responses to viruses, either enhancing or reducing CTL responses to particular viral epitopes and thereby helping establish immunodominance hierarchy (York et al., 2006). ERAP1 has also been shown to regulate tumor immunogenicity and cytotoxic response to cancer cells (James et al., 2013; Keller et al., 2015; Cifaldi et al., 2011) and to play a role in regulating innate and inflammatory immune responses (Aldhamen et al., 2015; Goto et al., 2014; Cui et al., 2003).

3. Effects of ERAP1 on the immunopeptidome

The collection of peptides that are presented by MHCI molecules on the cell surface define the immunopeptidome (Admon and Bassani-Sternberg, 2011). Although MHCI allelic variation is the most important determinant of the composition of the immunopeptidome, ERAP1 is an additional key editor that affects immunopeptidome quality and constitution (Georgiadou and Stratikos, 2009; Alvarez-Navarro and Lopez de Castro, 2014). As noted above, reduction or loss of ERAP1 expression in cell lines or mouse models, led to presentation of unstable and structurally unique peptide-MHCI complexes to the cell surface. Many conventional complexes were lost, while concomitantly, new, highly immunogenic complexes emerged (Hammer et al., 2007). In other words, in the absence of ERAP1 trimming activity, some endogenous antigens were poorly presented, while presentation of other antigens was enhanced or remained unaffected, suggesting a selective effect on the repertoire of peptide-MHCI complexes (Hammer et al., 2006; York et al., 2006; Nagarajan et al., 2016; Barnea et al., 2017). Additionally, a large fraction of the novel epitopes that arose, were longer in length and carried extended N-termini (Blanchard et al., 2010). Taken together, it appears that ER proteolysis defines the composition and the structure of peptides presented to CD8$^+$ cells.

4. Dimerization and synergism with ERAP2

Although ERAP1 is considered to be the dominant aminopeptidase in the ER for trimming peptide precursors, recent work has highlighted a second trimming enzyme, ERAP2, to be important for correct antigen processing. ERAP2 is highly homologous to ERAP1 (50% sequence identity) and can efficiently generate antigenic peptides *in vitro* (Saveanu et al., 2005; Mpakali et al., 2015a). ERAP2 however, shows distinct trimming patterns compared to ERAP1, has a different specificity for N-terminal residues and appears to select peptides of different lengths (Mpakali et al., 2015a; Zervoudi et al., 2011). It has been proposed that ERAP2 activity is complementary to ERAP1 and several antigenic epitopes with complex or longer extensions seem to require both ERAP1 and ERAP2 for correct processing (Saveanu et al., 2005; Lorente et al., 2013). ERAP1 and ERAP2 co-localize inside the cell, co-elute in chromatographic microsome fractionation and co-immunoprecipitate (Saveanu et al., 2005). This has led to the suggestion that the human ER has a non-redundant system of physically associated ERAP1 and ERAP2 heterodimer that ensures highly efficient trimming of diverse precursor epitopes with complex N terminal extensions. A

direct association of ERAP1 and ERAP2 has not however been demonstrated yet by biophysical methods. Recently, researchers used a leucine-zipper hetero-dimerization domain to construct an ERAP1/ERAP2 heterodimer and demonstrated that dimerization affected their enzymatic properties, suggesting that an ERAP1/ERAP2 dimer may be the biologically relevant antigen trimming entity in humans (Evnouchidou et al., 2014). Structural analysis of ERAP2 by x-ray crystallography indicated a putative homo-dimerization domain in ERAP2 that utilizes residues that are largely conserved in ERAP1 (Birtley et al., 2012). Furthermore, both ERAP1 and ERAP2 are retained in the ER although they do not possess any ER retention signals, suggesting that they are retained there though protein-protein interactions that exist only inside the ER (Hattori and Tsujimoto, 2013). Indeed, disulfide bond formation between exon 10 of ERAP1 and ERp44, a factor involved in disulfide bond formation in the ER, has been suggested to be the main mechanism for ERAP1 retention in the ER and to regulate its release and thus the extracellular degradation of angiotensin II, although other protein-protein interactions with ER chaperones or the Peptide Loading Complex may also be important (Hattori et al., 2012; Hisatsune et al., 2015; Blees et al., 2017). ERAP2 contains an exon of similar length, which has little homology with the ERAP1 exon 10 but still retains the ERp44 interacting cysteine and has been found to fold in an appendix-like structure containing an internal disulfide bond and a helical segment (Mpakali et al., 2015a). It is currently not known if this exon is responsible for ERAP2 retention in the ER, although in an ERAP2 crystal structure it was found to make interactions with an adjacent ERAP2 molecule in the crystal, suggesting that it may constitute a protein-protein interaction structural motif (Mpakali et al., 2015a). IRAP, on the contrary, does not contain a similar exon, but has a transmembrane and a cytosolic domain that target it to endosomal vesicles or the cell membrane and have been suggested to be important for interactions and signaling (Saveanu and van Endert, 2012; Chai et al., 2004; Keller, 2003).

5. Polymorphic variation

ERAP1 is polymorphic and several single nucleotide polymorphisms (SNPs) in its gene that encode amino acid changes have been associated with predisposition to a variety of diseases, ranging from viral infections to cancer and autoimmunity (Alvarez-Navarro and Lopez de Castro, 2014; Stratikos et al., 2014). Functional studies on ERAP1 alleles have suggested that disease-associated SNPs affect enzymatic function and specificity (Evnouchidou et al., 2011; Reeves et al., 2013; Stamogiannos et al., 2015). Indeed, experiments comparing cell lines harboring different ERAP1 allelic forms have revealed distinct effects on the cellular immunopeptidome and provided hints on the pathogenesis of HLA-associated autoimmunity (Martin-Esteban et al., 2017; Guasp et al., 2017; Lopez de Castro et al., 2016). ERAP2 also harbors common coding SNPs although fewer than for ERAP1. The ERAP2 SNP rs2248374 has been found to regulate the expression levels of the enzyme *via* nonsense RNA mediated decay (Andres et al., 2010) and SNP rs2549782 codes for a substitution near the catalytic site of the enzyme, affecting both activity and specificity (Evnouchidou et al., 2012). ERAP2 SNPs have been associated with predisposition to autoimmunity, preeclampsia and resistance to HIV infection, possibly due to changes in antigen processing and presentation (Johnson et al., 2009; Vanhille et al., 2013; Cagliani et al., 2010; Kuiper et al., 2014).

6. Basic and unique enzymatic properties

ERAP1 is a member of the M1 family of aminopeptidases, which utilize a bound zinc ion to hydrolyze the amino terminal peptide bond of a substrate (Hattori et al., 1999; Schomburg et al., 2000). This class of enzyme is found in bacteria and eukaryotes, with myriad substrate specificities and physiological roles, ranging from metabolism to neurotransmitter and hormone degradation, to immune function and

antigen processing (Danziger, 2008; Peer, 2011; Bhosale et al., 2013; Rawlings and Barrett, 1993). This versatility is possible due to the large size of the substrate binding pocket, which extends away from the active site and can accommodate substrates up to 20 amino acids long in the case of ERAP1 (Chang et al., 2005). Different M1 family members have different preferences for substrate N-terminal residues, with ERAP1 exhibiting broad specificity for aliphatic residues (Zervoudi et al., 2011). Along with the length of the substrate, ERAP1 exhibits preferences for particular side chains at particular positions, but in a more degenerate pattern than other more specific proteases. Two notable preferences are for a bulky aliphatic or aromatic group at S$_1$' (amino-terminal to the cleavage site), and for either a large hydrophobic group or a basic group at S$_{omega}$, (carboxy-terminal side chain) (Zervoudi et al., 2011; Evnouchidou et al., 2008).

Unlike other M1 aminopeptidases, ERAP1 has a strong preference for trimming substrates longer than eight or nine amino acids (Chang et al., 2005; York et al., 2002). This activity is well suited for efficient generation of peptides of length suitable for loading onto MHCI proteins. A mechanism for peptide-length dependent regulation of ERAP1 aminopeptidase activity has been proposed, based on cooperativity and allosteric activation observed for short but not full-length peptide substrates (Nguyen et al., 2011). The model postulates a regulatory site distinct from the active site that can be accessed by long substrates, with binding to this site favoring adoption of a closed conformation with increased catalytic activity (Nguyen et al., 2011). Crystal structures of a complex between the C-terminal domain of ERAP1 and a C-terminus of peptides presented in fusion with the same domain suggested a possible location of this regulatory site (Sui et al., 2016).

7. Known crystal structures

Crystal structures have been reported for ERAP1, ERAP2, and IRAP, the members of the oxytocinase subfamily of M1 aminopeptidases, all of which have roles in antigen presentation. The structures confirmed that these enzymes closely resemble the other M1 aminopeptidases structurally. They consist of four domains. Domain I is a mixed all-beta sandwich present in other aminopeptidases that caps off the amino-terminal side of active site. Domain II contains the active-site zinc in a thermolysin-like α/β fold. Domain III is an Ig-like domain unique to the M1 family. Domain IV contains 8 Armadillo-like helix-turn-helix repeats forming a bowl-like structure (Gandhi et al., 2011).

ERAP1 has been crystallized in two different conformations, wherein the C-terminal domain IV is either closely interacting with domains I/II and enclosing the active site (termed 'closed'), or is rotated away in a hinge motion which exposes the active site ('open'). This large motion correlates with a reorganization of the active site, with Tyr438 oriented in a catalytically active orientation only in the closed state. In the open structure, Tyr438 is rotated such that the hydroxyl group is displaced 5.5 angstroms relative to the closed structure, and is no longer oriented to interact with a putative bound substrate (Kochan et al., 2011; Nguyen et al., 2011). This correlation between ERAP1 conformation and active site organization suggests that the enzyme might cycle between these states in order to bind substrate, catalyze hydrolysis, and release product. This is notable within the entire M1 aminopeptidase family, of which several members have been crystallized in the closed conformation, including the homologous ERAP2 and IRAP (Mpakali et al., 2015a; Birtley et al., 2012; Mpakali et al., 2017).

8. Conformational change and conformational dynamics

Conversion between structural conformations is a widely studied feature of many enzymes. Such structural alterations can range from small loop reorganizations to large domain motions (Gutteridge and Thornton, 2004). One longstanding model of enzyme function assumes a structural change upon substrate binding referred to as a 'induced fit' (Koshland, 1958). If these conversions are required for catalysis and

occur slowly relative to the enzyme catalytic rate, small perturbations in conformational dynamics can have notable effects on enzyme's function (Wu and Xing, 2012). The identification of two ERAP1 conformations by crystallography raises the possibility that conformational interconversion may occur for ERAP1 and possibly for other members of the M1 aminopeptidase family. Using molecular dynamics simulations, an energy landscape connecting the open and closed structures has been identified with several local minima. Simulations of polymorphic variants of ERAP1 indicate that Q730E and K528R can each alter the population of conformations (Stamogiannos et al., 2015). The relative stability of the closed conformation requires electrostatic interactions between domains I/II and IV (Stamogiannos et al., 2017). The molecular dynamics model was supported by concordance with small angle X-ray scattering data of mutants designed to disrupt these interdomain interactions (Stamogiannos et al., 2017).

9. Two pathways for peptide processing by ERAP1: solution *versus* on-MHC trimming

Although the importance for ERAP1-mediated peptide generation has been well established in the literature, the physiological substrate for ERAP1 trimming is controversial. As early as 1990, Falk et al. proposed several models to explain MHCI-restricted generation of antigenic peptides from extended precursors (Falk et al., 1990), generally encompassed by two main ideas: that the peptide is generated by proteolysis in solution before binding to MHCI, or it is generated while bound onto MHCI, using the latter as a template. Thus, in one possible pathway ERAP1 trims precursor peptides in solution and relies on its unique enzymatic properties to optimize them for MHCI; mature peptides then can bind onto MHCI at which point they are protected from any further ERAP1 trimming (Fig. 1, left pathway). Alternatively, peptide precursors can bind onto the MHCI with N-terminal extensions such that ERAP1 can trim them while still bound onto the MHCI (Fig. 1, right pathway). Competition between ERAP1 trimming and optimal binding of the peptide onto MHCI is the main driving force behind MHCI cargo optimization in either pathway. Below, we describe each pathway in more detail and outline the main supporting experimental evidence.

9.1. Pathway 1: peptide generation in solution—experimental evidence

Members of the M1 family of aminopeptidases, including ERAP1, trim peptidic substrates in solution (Rawlings et al., 2012). Many studies have demonstrated that ERAP1 has this activity and can efficiently trim peptides in solution, generating appropriate epitopes for MHCI binding (Chang et al., 2005; Lorente et al., 2013; Evnouchidou et al., 2011; Georgiadou et al., 2010; Infantes et al., 2010; Kim et al., 2011). Still, the catalytic efficiency of ERAP1 is not particularly high as suggested by a study comparing trimming of peptides by ERAP1 and Leucine aminopeptidase (LAP), which found that ERAP1 was about 50-fold slower (Stamogiannos et al., 2015). The same study however, showed a lack of length preference by LAP, whereas a strong preference of ERAP1 for longer peptides, consistent with earlier reports (Chang et al., 2005; Stamogiannos et al., 2015; Nguyen et al., 2011). The solution ability of ERAP1 to prefer to trim peptides longer than 9 residues and spare many shorter peptides has been proposed as a mechanism to specifically generate correct-length peptides for binding onto MHCI (Chang et al., 2005; Stratikos and Stern, 2013).

9.2. Pathway 2: peptide trimming while bound onto MHCI—experimental evidence

The unique biological function of ERAP1 and its indispensability for generating MHCI cargo suggests that this aminopeptidase may have specific properties suited for its biological purpose. Although currently there is no direct biophysical evidence that ERAP1 can interact with the
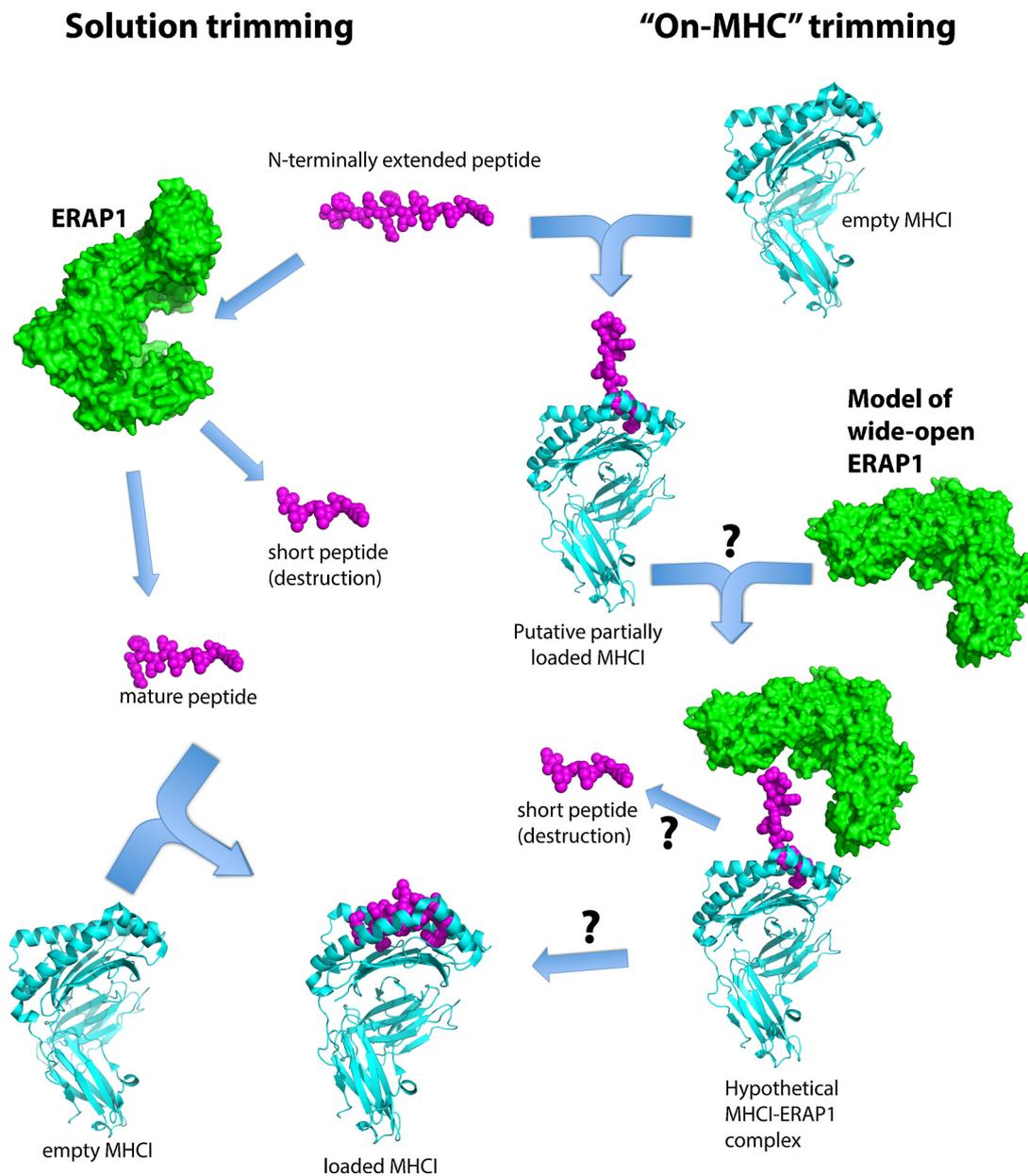
Solution trimming

"On-MHC" trimming

Fig. 1. Pathways for ERAP1-restricted peptide trimming. Left pathway, ERAP1 trims N-terminally extended peptides in solution down to mature, correct-length peptides that can then proceed to bind onto MHCI. Overtrimming to short peptides equates to epitope destruction. Right pathway, N-terminally extended peptides bind onto MHCI in a sub-optimal manner. Formation of a ternary complex with ERAP1 allows N-terminal trimming while the peptide is still bound on the MHCI. Optimal interactions between the generated mature epitope and the MHCI allow for ERAP1 dissociation.

MHCI, some studies have suggested that ERAP1 can trim peptides while they are bound onto MHCI. Specifically, Kanaseki et al. found that ERAAP (the mouse homologue of ERAP1) generated the correct H2-L^d ligand only when both ERAAP and H2-L^d were present in the ER of cells and otherwise overtrimmed the peptide, suggesting the MHC is indispensible for protecting the mature epitope from ERAAP (Kanaseki et al., 2006). In the same study, the authors detected ERAAP-generated, N-terminally extended peptides resistant to further trimming (due to a proline amino acid insertion), bound onto cell-surface MHCI and theorized that ERAAP was trimming the initial precursor while bound onto the MHCI. In a following study however, *Infantes et al.* found that H-2L^d actually protected N-terminally extended peptide precursors from ERAP1 and the 9mer epitope was only generated when the precursor was free in solution, not while bound to MHC (Infantes et al., 2010). In another study supportive of the on-MHC cleavage pathway, Reeves et al. found that ERAP1 was able to trim the N-terminal amino acid

from a precursor inside the ER when the peptide was covalently tethered onto the H2-K^b MHC using a disulfide trap single-chain MHCI construct (Reeves et al., 2013). Finally, recently Chen et al. used a leucine zipper ERAP1-ERAP2 dimer construct to demonstrate *in vitro* trimming of N-terminally elongated epitope precursors bound by MHCI (Chen et al., 2016a). Curiously however, this ERAP1-ERAP2 construct appeared to trim through a Pro residue, in contrast to the inability of ERAAP to trim through Pro in the Kanaseki et al. study and others (Serwold et al., 2002; Kanaseki et al., 2006; Serwold et al., 2001).

10. Interplay of trimming models with crystal structures and conformational dynamics

To date, the known experimental structures of ERAP1 and MHCI are more consistent with pathway #1 than pathway #2. Crystal structures of ERAP1, even in the open conformation, cannot dock onto MHCI such

that a peptide N-terminus could reach into the ERAP1 active site in a canonical configuration for catalysis (Nguyen et al., 2011). The same appears to apply for ERAP2 and IRAP (Mpakali et al., 2015a,b). If ERAP1 has the ability to open further, however, this may allow docking onto MHC to form a ternary structure (Papakyriakou and Stratikos, 2017). Indeed, molecular dynamics simulations have suggested a continuous but rugged energy terrain between different conformations of ERAP1, suggesting that other conformations are possible, albeit of high energy (Stamogiannos et al., 2017). Even if such a "wide-open" structure could form however, then for efficient processing, Tyr438 would have to properly orient for catalysis when ERAP1 is in the open state. Interestingly, in a recent "semi-closed" crystal structure of the homologous IRAP, the catalytic Tyr was found to properly orient towards a transition-state analogue bound in the catalytic site (Mpakali et al., 2015b; Hermans et al., 2015).

11. MHCI peptide editing, N-terminal extensions and the peptide loading complex

Conventionally, peptide-MHCI binding is thought to require both amino- and carboxyl-termini for stable interaction (Madden, 1995; Bouvier and Wiley, 1994). Peptides with longer than optimal length (10–13 residues) have been observed to bind, but in a bulged conformation with buried termini and exposed central regions (Tynan et al., 2005). This might be thought to preclude on-MHC processing by ERAP, which requires a free amino terminus. Recently two groups have reported peptide-MHCI structures with protruding N-termini, for an HIV gag-derived epitope binding/bound to HLA-B57 (Pymm et al., 2017) or HLA-B58 (Li et al., 2017). These add to an earlier report of a LCMV gp33-derived epitope binding to H2-K^b with an exposed N-terminal residue (Achour et al., 2002). In each of these cases the peptide is bound throughout the full length of the peptide binding site, occupying with a small Ser or Ala side chain the "A pocket" that usually accommodates the amino terminus, making a sharp kink stabilized by several hydrogen bonds that orient one or two amino terminal residues outside the groove. Although these structures reveal an exposed amino-terminal residue, trimming by ERAP1 to generate a conventional 9mer-residue peptide would appear to require breaking many peptide-MHC interactions so that the active site could access the scissile bond. Even for the "wide-open" ERAP1 conformation, which is more "open" than currently observed by X-ray crystallography or SAXS, ERAP1 trimming has been estimated to require at least four residues protruding beyond the last MHC interaction sites (Stratikos and Stern, 2013; Papakyriakou and Stratikos, 2017). Thus these N-extended conformations are not likely to represent ERAP1 substrates without considerable additional structural rearrangement or stabilization of the MHC with partially-occupied peptide.

It is possible that cellular antigen presentation machinery can stabilize a complex between MHC and partially-bound peptide in a conformation that is suitable for on-MHC trimming. Nascent MHCI molecules without peptides are kept in a receptive state, through the formation of a complex (peptide loading complex, PLC) with TAP and other ER chaperone proteins, including tapasin, calnexin, calreticulin, ERp57 and protein disulfide isomerase, PDI (Hulpke and Tampe, 2013; Cresswell et al., 1999). In this complex, tapasin interacts directly with MHCI molecules and is required for optimal peptide loading (Williams et al., 2002) *via* a peptide editing process (Thomas and Tampe, 2017), calnexin and calreticulin stabilize the heavy chain before β2-micro-globulin association, and ERp57 is a thioreductase that supports the formation of a disulfide bridge that connects the walls of the MHCI peptide-binding groove with its base (Cresswell et al., 1999). A recently determined structure of the PLC by cryo-EM has shed light on the structural composition of the complex but did not contain ERAP1, although some room for additional interactions is available (Blees et al., 2017). The exact manner in which peptide enters the peptide loading complex is not clear, and it is possible that a complex of stabilized MHC

with partially bound peptide is suitable for ERAP processing. ERAP1 has not been shown to directly interact with this complex, but thiol-dependent interactions similar to those observed for ERp44 could be envisioned that might promote processing of transient states.

12. Role of ERAP1 in antigenic peptide destruction

Whereas the two pathways described above present distinct strategies for generating MHCI peptide ligands, they also may affect the ability of ERAP1 to overtrim peptides to lengths too short for forming stable complexes with MHCI. Several studies have suggested an important role of ERAP1 in destroying peptides by overtrimming, effectively eradicating a pool of peptides from the antigenic peptide repertoire (York et al., 2002; Hammer et al., 2007; Nagarajan et al., 2016; Barnea et al., 2017; Seregin et al., 2013). MHCI have also been demonstrated to be able to protect good peptide ligands from ERAP1 degradation (Infantes et al., 2010; Kanaseki et al., 2006). In Pathway #1, peptide destruction can occur in solution, determined by the affinity of the peptide for ERAP1. Conversely in Pathway #2, destruction can occur on-MHC, determined by a combination of peptide affinity for ERAP1 and MHCI. A combination of both processes however is also possible, with weakly-binding peptides dissociating off the MHCI to be trimmed by ERAP1 in solution. Currently however, the relative importance of destruction *versus* generation for shaping the immunopeptidome is not clear.

13. Functional repercussions of each trimming pathway

A main difference between the two pathways presented in Fig. 1 lies in enzyme kinetics and selectivity. In pathway #1, the substrate for ERAP1 is free peptide, and so both kinetics and selectivity are determined by interactions between the peptide and ERAP1. In pathway #2 however, the ERAP1 substrate is the MHCI-peptide complex, and it is reasonable to argue that in this case both kinetics and selectivity are determined not only by interactions between the peptide and ERAP1, but also by as well as interactions between MHCI and ERAP1. Although a direct ERAP1/MHCI interaction has not been demonstrated up to date, parallels between the formation of a transient MHCI-ERAP1 complex and the formation of Peptide Loading Complex can be easily drawn (Cresswell et al., 1999). Several studies have analyzed the kinetics and specificity of ERAP1 trimming peptides in solution and in general have found good, albeit not absolute, agreement between solution trimming and cell-based experiments (Zervoudi et al., 2011; Reeves et al., 2013; Hearn et al., 2009). On-MHCI ERAP1 trimming kinetic analysis has not been reported, although it may be a powerful tool to compare these two modes of action.

14. Differential effects on generation of immunopeptidome

A key distinction between the two pathways is the shift of the burden from determining selectivity of the antigen generation process from ERAP1 (pathway #1) to MHCI (pathway #2), a distinction that could strongly alter our understanding of how the immunopeptidome is generated. According to the on-MHC trimming pathway, the peptide is still partially bound onto MHC and as a result these peptide-MHCI interactions could guide peptide generation. Such a model reduces the importance of ERAP1 in shaping the immunopeptidome and highlights the importance of peptide-MHCI interactions, which are already well studied (Ayres et al., 2017). The effects of ERAP1 on the immunopeptidome are now well-documented by multiple studies, including specific effects on sequence (Nagarajan et al., 2016; Barnea et al., 2017; Martin-Esteban et al., 2017; Guasp et al., 2016) making it difficult to imagine its role being limited to that of a general amino-peptidase activity. However, solution overtrimming could account for significant effects on the immunopeptidome, even if in the case of an on-MHC trimming pathway. More high-resolution proteomic studies

may be necessary to discern the exact effect of ERAP1 selectivity in shaping the immunopeptidome.

15. Effects of trimming models on inhibitor design

Crystallographic analysis of ERAP1 has shown that the active site is reconfigured between the two known conformations and based on those reconfigurations it has been proposed that the closed conformation is the one that is enzymatically active (Nguyen et al., 2011; Kochan et al., 2011).As noted above, the experimentally determined open conformation has a poorly structured S1 specificity pocket and a catalytic Tyr438 facing away from the Zn(II) atom, casting doubt on its catalytic efficiency. These considerations make structure-based or mechanism-based inhibitor design based on the open conformation difficult and as a result, the closed conformation of ERAP1 has been used for such approaches till now (Zervoudi et al., 2013; Papakyriakou et al., 2015; Kokkala et al., 2016). However, if the on-MHC trimming pathway with a "wide-open" conformation (Papakyriakou and Stratikos, 2017) is dominant in the cell, such design efforts may have to be redirected to using open conformations of ERAP1 for efficient targeting. Indeed, in a recent study, ERAP1 variants with weakened domain-closure interactions were found to be much more weakly inhibited by a mechanism-based transition-state analog inhibitor (Stamogiannos et al., 2017). It should be noted however that the same compound as well as similar structure-inspired inhibitors have been found to be active in cells, indirectly suggesting that the closed ERAP1 conformation is indeed relevant in the cellular context (Aldhamen et al., 2015; Zervoudi et al., 2011; Chen et al., 2016b; Papakyriakou et al., 2015).

16. Possible complementarity of trimming pathways

Determining the exact pathway for the generation or destruction of MHCI peptide ligands by ERAP1 is important for our understanding of the shaping of the immunopeptidome and for designing inhibitors that can manipulate it. It is also possible however that both pathways operate in parallel depending on the peptide and its MHC-binding kinetics and affinity. Slow-binding or weaker binding peptides may spend more time in solution to be trimmed or destroyed by ERAP1, whereas some peptides may associate with MHCI immediately after they are translocated in the ER and can only be trimmed in that context. This notion adds an additional level of complexity in the generation of the immunopeptidome. The effects of ERAP1 deletion on the immunopeptidome are however complex (Nagarajan et al., 2016; Barnea et al., 2017; Martin-Esteban et al., 2017) suggesting that our current understanding of the underlying mechanisms may not yet be sufficient to fully explain the physiological process.

17. On-MHC trimming by ERAP2 and IRAP?

The other oxytocinase subfamily members ERAP2 and IRAP also have been proposed to play important roles in antigen processing in specific different cellular contexts (Tsujimoto and Hattori, 2005). ERAP2 has been proposed to act as an accessory aminopeptidase and supplement ERAP1 specificity while IRAP to operate in a separate pathway of cross-presentation (Saveanu et al., 2005; Saveanu et al., 2009). Interestingly ERAP2 and IRAP do not share the same enzymatic properties with ERAP1 regarding length preference and rather prefer to trim peptides of intermediate length (Mpakali et al., 2015a; Stamogiannos et al., 2015; Mpakali et al., 2015b). Although ERAP2 was proposed to trim on-MHC bound peptide precursors in the context of an ERAP1/ERAP2 dimer, neither ERAP2 nor IRAP alone is known if they can perform the same function. Still, multiple conformations have been suggested to be accessible for these two enzymes also, opening up the possibility that they also employ similar mechanisms to peptide trimming as ERAP1 (Mpakali et al., 2015a; Birtley et al., 2012; Mpakali et al., 2015b; Papakyriakou and Stratikos, 2017).

18. Future directions—insights

It has been 15 years since the first description of the role of ERAP1 in antigenic peptide generation (Serwold et al., 2002; York et al., 2002; Saric et al., 2002) and although many studies have demonstrated a key role in immune homeostasis and in immune dysfunction, significant controversy remains regarding its molecular mechanism and physiological substrate. The two pathways described in this review can serve as a distillation of current literature and as a starting point to design experiments to discern the relative importance and contributions of each proposed pathway *in vivo*. Given the importance of ERAP1 in shaping immune responses, a deeper understanding of the relative importance of solution *versus* on-MHC processing is necessary to allow both prediction of the repercussions of ERAP1 activity modulation on immune function and also the optimization of small molecule inhibitors of ERAP1 for pharmacological applications.

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