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The role of Endoplasmic Reticulum Aminopeptidases in type 1 Diabetes Mellitus.

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Abstract

Type-I Diabetes Mellitus (T1DM) is generally considered as a chronic, T-cell mediated autoimmune disease. This notwithstanding, both the endogenous characteristics of β-cells, and their response to environmental factors and exogenous inflammatory stimuli are key events in disease progression and exacerbation. As such, T1DM is now recognized as a multifactorial condition, with its onset being influenced by both genetic predisposition and environmental factors, among which, viral infections represent major triggers. In this frame, Endoplasmic Reticulum Aminopeptidase 1 (ERAP1) and 2 (ERAP2) hold center stage. ERAPs represent the main hydrolytic enzymes specialized in trimming of N-terminal antigen peptides to be bound by MHC class I molecules and presented to CD8+ T cells. Thus, abnormalities in ERAPs expression alter the peptide-MHC-I repertoire both quantitatively and qualitatively, fostering both autoimmune and infectious diseases. Although only a few studies succeeded in determining direct associations between ERAPs variants and T1DM susceptibility/outbreak, alterations of ERAPs do impinge on a plethora of biological events which might indeed contribute to the disease development/exacerbation. Beyond abnormal self-antigen peptide trimming, these include preproinsulin processing, nitric oxide (NO) production, ER stress, cytokine responsiveness, and immune cell recruitment/activity. The present review brings together direct and indirect evidence focused on the immunobiological role of ERAPs in T1DM onset and progression, covering both genetic and environmental aspects.

Keywords: ERAP1; ERAP2; MHC-I; Pancreatic β-cell; ER stress; Preproinsulin; Viral Infections

1.Introduction.

Diabetes mellitus (DM) is one of the most common and fastest growing diseases worldwide, with about 693 million individuals being estimated to suffer from by 2045[1]. Diabetes includes a group of heterogeneous metabolic disorders featuring chronic yet variably severe hyperglicaemia. Besides specific and relatively uncommon subtypes, DM can be broadly classified into type 1 (T1DM) and 2 diabetes (T2DM); the pathogenesis of these two forms of DM is based on the destruction or the failure of pancreatic β-cells, respectively [2]. These cells are located in the Langerhans islets within the endocrine pancreas and are specialized in the production of insulin, which grants glucose homeostasis by lowering blood glucose levels. The development of DM is influenced to varying degrees by both genetic and environmental factors, though the former are key in promoting T1DM while the latter play a critical role in T2DM onset[3],[4]. In detail, T1DM is generally considered as a chronic, T-cell mediated autoimmune disease which leads to pancreatic β-cells' destruction and progressive depletion of endogenous insulin secretion. Several genetic factors have been identified within the human leukocyte antigen (HLA) region on chromosome 6, and nearly other 60 loci, mostly involved in immune system regulation, are suggested to affect disease susceptibility [5]. These results, together with the presence of multiple islet-specific autoantibodies in patients with T1DM, corroborate the key role played by the immune response in disease development [5–9]. Studies on both mouse models and diabetic patients have shown that CD8+ T cells, in particular, are involved in T1DM onset and progression [10–13,14–17,18–21]. CD8+ T cells response is directly dependent on the recognition of antigens presented on cells' surface by MHC class I molecules, suggesting that abnormalities in their cytotoxic activity may be also related to alterations along the antigen presentation machinery. In this frame, Endoplasmic Reticulum Aminopeptidase 1 (ERAP1) and 2 (ERAP2), which perform antigen peptide trimming within the ER [22,23], are likely to play a role. Although evidence about the role of ERAPs in T1DM is scarce, it is tempting to assume that abnormalities in their expression play a role in T1DM pathogenesis by altering the antigen presentation pathway and the efficacy of the immune response. This has been broadly demonstrated in the context of autoimmunity and infectious diseases [22–24], which in turn, represent major environmental triggers for T1DM pathogenesis [3,4]. Indeed, both the endogenous characteristics of β-cells, as well as their response to environmental factors and exogenous inflammatory stimuli are key events in T1DM disease progression and exacerbation. Pointing to a role of β-cells that goes beyond being a non-provoking victim of an autoimmune attack [25–28], innumerous dysregulations which contribute to the development/exacerbation of T1DM do occur at the cross-road of β-cell metabolism,

innate and adaptive immune pathways. Beyond abnormal self-antigen peptide trimming, these include a plethora of biological events whereby ERAPs hold center stage, namely preproinsulin processing, Nitric Oxide (NO) production, ER stress, cytokine responsiveness, and T regulatory (Treg) cell recruitment.

After providing a brief overview on the immunobiological mechanisms behind T1DM pathogenesis, the present review aims at bringing together direct and indirect evidence focused on the role of ERAPs in T1DM onset and progression.

2. Immunologic mechanisms involved in T1DM pathogenesis: genetic and environmental factors.

The synergic action displayed by environmental factors on a genetically susceptible background leads to the autoimmune destruction of the pancreatic islets over a period of years. In the first phases of this process, individuals remain asymptomatic and euglycemic, despite already presenting a positive serology for relevant autoantibodies [29]. Only after a latency period, patients develop overt hyperglycemia and frank diabetes as a consequence of the observation that progressively greater amounts of β cells are affected and destroyed. CD68+ macrophages and CD8+ cytotoxic cells may contribute to β cell death during early insulitis, while CD20+ B cells are recruited in great numbers during late insulitis, underscoring the critical role of immune dysregulations in the pathogenesis of T1DM [30]. As recently reported, another factor that could potentially favor the onset of autoimmune dysfunction in T1DM patients is quantitative and/or qualitative abnormalities in regulatory T cell population (Tregs) [31–33]. This cellular subpopulation plays a pivotal role in the maintenance of peripheral tolerance and any alterations of their balance may cause the onset of autoimmune responses. In fact, under physiological conditions, during the selection process of T lymphocytes in the thymus, and of B lymphocytes in the bone marrow, central tolerance regulates the elimination of potentially dangerous cells [34]. When this selective mechanism is faulty, the process of peripheral tolerance allows the neutralization or suppression of self-reactive lymphocytes that have ended up in the periphery. Some viral infections have been hypothesized to affect human thymus, thus disrupting this immunologic balance. Indeed, thymic epithelial cells and thymocytes can be infected by a variety of viruses that represent environmental triggers for T1DM. As detailed in section 2.2, these include coxsackievirus B4 and other enteroviruses, which may be responsible for an altered maturation and differentiation of T cells [35,36]

Apoptosis, a process mediated by the caspases cascade, was shown to be the most likely mechanism mediating β-cells death in experimental animal models as well as in humans [37–40]. The caspase cascade

would be activated following the massive production of proinflammatory cytokines (IL-1β, TNF-α and IFN-γ) triggered by the immune reaction, sustained in turn, by the autoreactive T lymphocytes within the islets of the endocrine pancreas. Another stream of evidence suggests that apoptosis-mediated β-cell destruction is induced by direct contact of autoreactive T cells with the insulin-producing ones, via the release of granzyme, perforin, or Fas/Fas ligand interaction. In both cases, abnormalities in cytokines production occur as a common disease signature [31,37–39,41].

Abnormalities in immunity concern the humoral arm as well, leading to the production of autoantibodies that can be observed several years before the clinical onset of T1DM. Clinical studies revealed that the most frequently detected autoantibodies are those against the glutamic acid decarboxylase 65-kilodalton isoform (GAD65), islet-specific glucose-6-phosphatase catalytic subunit–related protein (IGRP), tyrosyl phosphatase (IA-2), insulin (IAA), and zinc transporter (ZnT8) [31]. Notably, studies performed in non-obese diabetic (NOD) mouse models point out at proinsulin as the primary target. Deletion of this gene has been shown to markedly modify disease progression by abrogating the T-cell stimulation of insulin autoreactive NOD T-cell clones; conversely, during the pre-diabetic period, the clinical manifestation of T1DM may be delayed or even prevented by proinsulin administration [42,43]. Thus, one of the most reliable markers of progression to clinical T1DM is the concomitant presence of two or three of the abovementioned autoantibodies [44].

In summary, T1DM pathogenesis features a plethora of immune alterations which, as detailed in the following sections, are bound to both genetic and environmental factors.

2.1 Genetic factors.

In T1DM, genetic and familiar factors play a decisive role by conferring 40-50% of disease susceptibility, as demonstrated by twin and family studies [45]. Since some early investigations established a correlation between HLA variants and T1DM risk, a total of nearly 60 loci has been associated with T1DM onset by genome-wide association and meta-analysis studies [5,6]. As for HLA, correlations with specific genetic variants mainly refer to HLA class II region, which encodes molecules crucial for the recognition of antigens by CD4+ T lymphocytes [46]. Specifically, the HLA-DR3, DQB1*0201 (also referred to as DR3-DQ2), HLA-DR4, and DQB1*0302 (also referred to as DR4-DQ8) haplotypes have been identified in more than 90% of the T1DM patients, while only 40% of the healthy population carries them. Of note, up to 30% of T1DM patients present both haplotypes (DR3/4 heterozygotes) [47,48]. Conversely, a negative correlation between some DR4 alleles – namely DRB1*0403 and DPB1*0402 – and T1DM susceptibility was reported, even in the presence of the

DQB1*0302 risk allele [49]. Moreover, the DQB1*0602 allele was found in approximately 20% of the general US population and only in 1% of the pediatric T1DM patients, suggesting a protective role for this variant against T1DM [31,49–51].

As mentioned above, CD8+ cytotoxic T cells are the most conspicuous cell population observed in pancreas in T1DM-associated insulitis [30]. Their activity is directly dependent on HLA class I molecules, which are responsible for antigen exposition on cell's surface. In line with this, some class I alleles do affect T1DM susceptibility [52–56]. Specifically, HLA-A*24, B*18, and B*39 promote a faster progression from the prediabetic state to overt T1DM after the appearance of autoantibodies. Notably, since the HLA region presents some of the strongest linkage disequilibrium in the genome, the association of these HLA-I alleles with T1DM is restricted to specific HLA class II haplotypes: thus, the B*18 effect occurs in the DR3-DQ2 haplotype, A*24 and B*39:01 effects occur in the DRB1*04:04-DQA1*03-DQB1*03:02 haplotype, and the B*39:06 effect occurs in the DRB1*08-DQB1*04 haplotype[56–58]. Another study performing linkage disequilibrium adjustment indicates that the two, most strongly, T1DM–associated alleles are HLA-B*5701 – playing a protective role – and HLA-B*3906 – having a predisposing role[55]; other alleles associated to T1DM to a lesser extent include A*2402, A*0201, B*1801, and C*0501 (predisposing) and A*1101, A*3201, A*6601, B*0702, B*4403, B*3502, C*1601, and C*0401 (protective)[55].

Besides HLA genes polymorphisms, genetic variants of the *insulin* (*INS*) and *PTPN22* (encoding a lymphocyte protein tyrosine phosphatase) genes have also been correlated to T1DM susceptibility by affecting systemic tolerance and/or T-cell activation [59,60]. Again, as identified by genome wide association studies (GWAS), T1DM-associated polymorphisms in *FOXP3* and *CLTA-4* genes foster uncontrolled autoimmune reactions [61,62,63,64], while *IFIH1 and TYK2* polymorphisms affect type I interferon (IFN) production within β-cells, suggesting that the nonfunctional variants of these genes might play a protective role in T1DM [65,66].

2.2 Environmental factors: from diet to viral infections.

Even though the role of environmental factors in the pathogenesis of T1DM needs to be further ascertained, their key role is once again evincible from monozygotic twin studies: the disease occurrence in both siblings, indeed, tends to vary around 50%, without ever reaching 100% [67,68]. It is now increasingly accepted that environmental factors may affect the incidence of T1DM [69]. These include inadequate diet, (such as vitamin D deficiency[70–74], nitrate exposure derived from water [75], inadequate intake of omega-3 fatty acids [76], early integration of cereals [77], precocious administration of

cow's milk to newborns [78–80], and altered gut microbiota [81,82]), as well colder climate, environmental pollution, and viral, bacterial, and yeast-like fungi infections.

A variety of epidemiological observations indicate that viruses represent major environmental triggers for T1DM pathogenesis. Plenty of viruses have indeed been associated to T1DM development, including enteroviruses, such as Coxsackievirus B (CVB) [83], rotavirus [84,85], mumps virus [86], and cytomegalovirus [87]. Rubella virus also seemed to be correlated to T1DM onset, but further studies revealed that only congenital rubella syndrome is associated with the disease [88–90]. Enteroviruses are considered the major viral candidates associated with the development of T1DM in humans. Infections with this virus genus are more frequent in siblings who develop T1DM than in nondiabetic ones and antibodies against enteroviruses have been found to be increased in pregnant mothers whose children would later develop the disease [91]. Of note, a study conducted among Finnish individuals showed that the emergence of autoantibodies goes along with the seasonal pattern of enteroviruses infections[92]. A temporal correlation has indeed emerged between the appearance of autoantibodies and the first signs of infection in both siblings of affected children, and children with a greater disease susceptibility conferred by specific HLA genes [93]. In particular, CVB4 is the most common enteroviral strain among pre-diabetic and diabetic individuals. CVB RNA has been found in blood samples collected from patients at the onset or during the progression of T1DM [94,95]. In addition, cellular immune responses against CVB antigens were increased in T1DM patients after the clinical onset of the disease [96]. Again, a CVB4 strain, which was isolated from the pancreas of a deceased pediatric diabetic patient, was documented to cause diabetes in mice [97]. In line with this, CVB4 was also found in pancreatic tissue specimens of T1DM patients[98]. Elshebani et al. recently showed that human islet cells could be destroyed following *in vitro* enterovirus infection with isolates obtained from newly diagnosed T1DM patients [99]. Oikarinen and colleagues succeeded in isolating enteroviruses from intestinal bioptic samples of 75% of T1DM patients but from only 10% control individuals [100]. This finding suggests a persistent gut mucosa enterovirus infection in T1DM cases.

To sum up, the isolation of enterovirus antigens from diabetic patients seems to be an increasingly common feature, suggesting a key role for this particular virus genus in the disease pathogenesis. However, further research is necessary to confirm whether this infection is directly responsible for the majority of diabetes cases, or if it rather concerns a small percentage of T1DM-diagnosed patients who may be genetically more susceptible to virus infections.

Still in the frame of viral infections related to T1DM, a worldwide increase in cases of diabetic ketoacidosis, a life-threatening complication associated with T1DM, has been documented in both

adults and children during the coronavirus 19 disease (COVID-19) pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[101]. In fact, recent evidence suggests that SARS-CoV-2 could trigger diabetic ketoacidosis in T1DM susceptibility and previous insulitis[102].

Some case reports also document an association between SARS-CoV-2 infection, long-lasting aberrant glycometabolic control[103], and also new-onset T1DM in individuals with no previous history of diabetes, with the incidence of diabetes steadily rising during the pandemic, especially in the pediatric population[101]. Again, COVID-19 progression and severity are enhanced in T1DM patients, especially those with comorbidities and vitamin D deficiency[104]. However, the role of SARS-CoV-2 as a trigger for both new-onset diabetes and related complications is still under investigation.

Likewise, many aspects of the mechanisms through which viruses modulate T1DM development are still unknown. On the one hand, virus infections may activate a strong immune response. For instance, in pancreatic islets, CVB4 infection was specifically shown to induce a powerful inflammatory reaction mediated by NK cells infiltrating the area[98]. Such a strong inflammatory reaction within the endocrine pancreas may cause autoimmunity through potentiation of IFN pathways and upregulation of MHC class I molecules on β-cells' surface, thus promoting detection of endogenous antigens, including insulin, by CD8+ T cells. On the other hand, viral infections may evade host immune responses by antagonizing IFN pathways while inhibiting viral antigen processing and presentation machinery, thus hampering the susceptibility of infected cells to viral antigen-specific cytotoxic T lymphocytes. This might in turn contribute to promoting an excess of pro-inflammatory cytokines favoring pyroptotic cascades, while qualitatively altering the antigen peptide repertoire to be presented via MHC molecules [105]. In either case, alterations of ERAPs, which hold center stage in a variety of cell pathways beyond antigen processing and presentation, are likely to play a critical role, as summarized in Figure 1 and detailed in section 3.

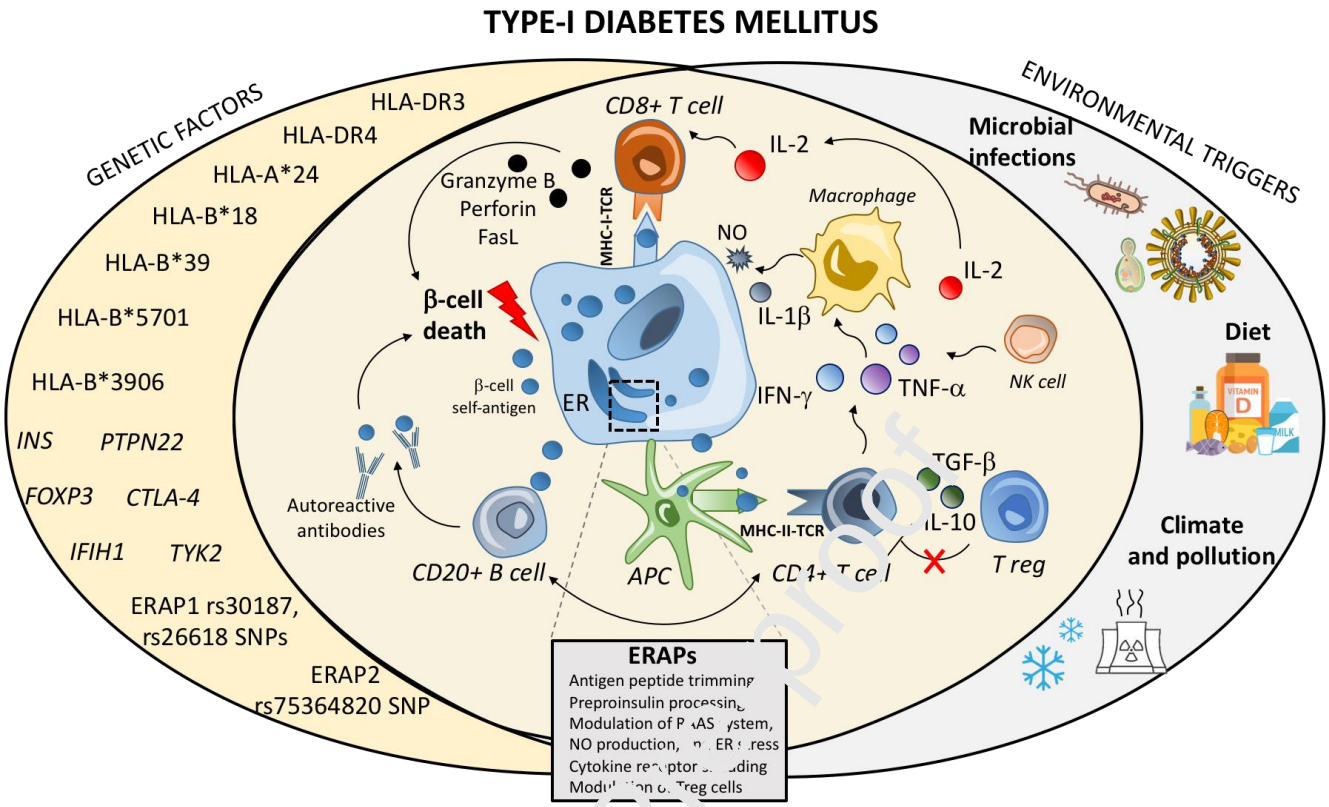

Figure 1. ERAPs at the crossroad of genetic and environmental determinants of Type-I Diabetes. The synergic action displayed by environmental factors on a genetically susceptible background leads to the autoimmune destruction of β-cells within the pancreatic islets. Such a final event results as a consequence of innumerous dysregulations at the cross-road of β-cell metabolism and immune pathways, whereby ERAPs hold center stage. In fact, alterations of ERAPs are expected to impinge on a plethora of biological events which might contribute to the development/exacerbation of T1DM, including abnormal self-antigen peptide trimming, preproinsulin processing, NO production, ER stress, cytokine responsiveness, and Treg cell recruitment.

3.An overview on ERAP1 and ERAP2.

ERAP1 (formerly called LRAP) and ERAP2 are two IFNγ- and TNFα-inducible enzymes ubiquitously expressed in human tissues, which belong to the M1 family of zinc aminopeptidases. Collectively, ERAPs represent the main hydrolytic enzymes specialized in N-terminal peptide trimming in humans. The shaping of endogenous and exogenous peptides begins in the cytoplasm, where the proteasome cleaves proteins into fragments of variable length; the latter are then transported by TAP (transporter

associated with antigen processing) into the endoplasmic reticulum (ER). Within the walls of the ER, these peptides are further trimmed by ERAPs at the N-terminus to generate optimal length peptides to be bound by MHC class I molecules and presented to CD8+ T cells, which initiate the immune response [106]. In order to fulfill their function, ERAP1 and ERAP2 act concertedly while maintaining significative differences in enzymatic specificity. ERAP1 cleaves peptides with large hydrophobic C-terminal amino acids and has a strong tendency to cut 9–16 amino acids peptides into shorter pieces 8–9 amino acids long, which corresponds to the suitable length for binding to MHC I molecules[23,107–110]. On the other hand, ERAP2 shows a pronounced preference for the positively charged arginine and lysine residues located at the N-terminal and presents a greater efficiency toward shorter peptides compared to ERAP1 [107,111]. Of note, ERAPs have also been shown to physically interact and form heterodimers [23], which changes their enzymatic features and leads to increased peptide-trimming efficacy, thus allowing the presentation of a more variegated and immunogenic antigen repertoire [110]. These processes may indeed be altered in individuals who lack one of the two proteins, since the structural changes made by ERAP1 and ERAP2 are essential for antigenic epitopes maturation [112]. Interestingly, even if ERAPs share about 50% sequence homology and their function can be carried out via heterodimer formation, the evolution of these two genes seems to be different [107]. Indeed, while ERAP1 has a homolog in rodents, namely ERAAP (i.e. ER aminopeptidase associated with antigen processing), ERAP2 has no homolog in mice and it may derive from a relatively recent duplication of the ERAP1 gene [113].

ERAP1-mediated proteolysis is key in determining both the characteristic length, and the composition of MHC class I-binding peptides. Thus, abnormalities in ERAP1 expression alter the peptide-MHC-I repertoire both quantitatively and qualitatively[114]. Analysis of the individual peptides displayed on the cell surface showed that ERAP1 deficiency dramatically alters peptide repertoire, with some peptides being unaffected, and others being either absent or even up-regulated [111,114–117]. Again, ERAP1 deficiency causes a marked increase in the length of peptides normally presented by MHC class I molecules, as shown by an analysis of natural and viral peptides processed in mice lacking ERAP1 [118].

Of note, ERAP1 plays a role in innate immunity as well: during the first phases of pathogen recognition, it regulates and participates to cytokine receptors shedding, NK cells development and function [119,120], nitric oxide formation[121], and triggering of phagocytic activity of splenic DCs and macrophages [122]. Besides their intracellular functions, ERAP1 and ERAP2 are also involved in various non-immunological activities, namely angiogenesis and blood pressure regulation. The latter is bound to ERAP1's and ERAP2's abilities to cleave angiotensin II into angiotensin III and IV, and angiotensin

III to angiotensin IV, respectively [123–125]. Considering the extracellular localization of angiotensins, ERAPs must necessarily be released in the extracellular milieu in order to fulfill these tasks. In line with this, the ability to secrete ERAP1 in either soluble or extracellular vesicle forms has been observed in immune cells and murine macrophage cell lines [126]. Likewise, recent research documented that ERAP2 can be also released by human macrophages following inflammatory stimuli [127]. In the extracellular milieu, beyond blood pressure control and RAS regulation, both aminopeptidases are involved in the activation of immune system. Indeed, ERAP1 modulates cytokine receptor shedding, phagocytosis, NO synthesis, inflammasome activation, and TNF-α and IL-1β production[121,122,126,128,129], while ERAP2 fosters the monocyte/macrophage lineage activation, by increasing inflammasome assembly and phagocytosis[130]. These findings have indicated ERAPs as likely innate and adaptive immune targets for a myriad of human diseases [22,131,132].

3.1 ERAPs polymorphisms in diabetes

Early studies of linkage analysis of genome-wide gene-expression patterns in lymphoblastoid lines showed ERAP1's gene expression to be modulated by one of the most pronounced effects *in cis*, mapping to a single-nucleotide polymorphism, i.e. rs2762 [133]. Based on these premises, in 2006, Qu and his research group designed a study in order to investigate whether its expression in β-cell derived lines was controlled by a haplotype marked by rs2762 and whether this would form the basis of a correlation with T1DM. However no association of this SNP with T1DM was found, as this variant, which significantly affects its expression levels, did not seem to modify the risk of autoimmunity [134].

Three years later, Fung and colleagues focused on SNPs which were convincingly associated with at least one other autoimmune condition [135]. Considering the autoimmune nature of T1DM, their aim was to verify if any of these SNPs could increase the disease susceptibility. Among other genes, 5q15/ERAP1 was taken into account, since the rs30187 SNP had been previously found to be correlated to ankylosing spondylitis [53,136]. Notably, a correlation with T1DM was observed. Moreover, considering ERAP1's key role in antigen presentation, ERAP1 polymorphisms were also tested for interaction with HLA I, specifically HLA-A and HLA-B, though no evidence for an interaction emerged.

A subsequent case-control study investigated the association between the rs30187 polymorphism and T1DM onset in 234 patients and 314 controls from continental Italy [137]. Specifically, genotyping of the rs30187 SNP of ERAP1 was performed by the allelic discrimination assay on DNA extracted from

whole blood. Interestingly, in contrast to the results obtained by the previously mentioned research, Gianchecchi and her group did not find a statistically significant prevalence of the rs30187 polymorphism of ERAP1 in the cohort of patients compared to controls, thus observing a probably minor contribution of this gene to the pathogenesis of T1DM in Italian affected individuals [137]. In a recent study carried out in 120 Egyptian patients with T1DM, positive correlations with T1DM were identified for both ERAP1 (rs26618) and IFN-λ-4(rs73555604) SNPs [138]. Furthermore, T allele frequency in comparison to C allele frequency was significantly increased in T1DM patients when compared to control group, indicating that patients with CC genotype were less likely to develop T1DM than those with TC and TT genotypes for both genes. Very recently, a whole-exome sequencing (WES) study in large cohorts well-phenotyped for chronic kidney disease (CKD) and diabetes (DKD), identified ERAP2 locus, as well as an ERAP2 variant (rs75364820) as promising genes candidates in DKD, suggesting that ERAP2 might play an important role in the disease [139]. This does not come as a surprise considering the key role of ERAP2 in RAAS regulation and its implication in a number of autoimmune conditions. Intriguingly, evidence from a recent preprint suggests that two SNPs of ERAP2 (rs2549794 and rs2248374) are strongly associated with severe respiratory infection, while opposing effects are identified for T1DM and Crohn's disease[140]. Thus, increased ERAP2 transcription or protein levels might confer protection against respiratory infections while increasing the risk for T1DM or other autoimmune conditions. These pieces of evidence strengthen the need for further studies uncovering the biological effects of specific ERAP polymorphisms and their association with T1DM development.

Similar contradictory results have been obtained in the frame of T2DM. In detail, an analysis on the potential associations between 33 ERAP1 tag-SNPs, and T2D incidence in 22,718 Caucasian females failed to provide any correlations with the disease[141]. Contrariwise, by integrating GWAS data with human islet eQTLs and *in vitro* models, a recent study disclosed causal risk genes, networks, and pathways that are potentially shared by T1DM and T2DM [142]. The authors identified 9 shared genes within the "T1D-T2D islet eQTL interaction network" (*HEMK1, GSDMB, ERAP1, PPIP5K2, TMEM69, DNLZ, SDCCAG3, CARD9, and PLEKHA1*), among which ERAP1 was included. An extension of the network surrounding these shared genes also revealed highly interconnected nodes which are putatively involved in regulating common processes that could lead to either type of disease [142]. Although these findings suggested a possible role played by ERAP1 in the pathogenesis of both T1DM and T2DM, further confirmatory research is needed.

3.2 ERAP and insulin metabolism: preproinsulin

Various proteins have been proposed as possible autoreactive T cell targets in the context of T1DM; however, murine and human studies suggest that insulin itself may be the primary autoantigen eliciting T cells reaction [42,143,144]. Insulin derives from its precursor preproinsulin (PPI) via a posttranslational processing pathway. Briefly, the signal peptidase Sec11 cuts off the signal peptide (SP) co-translationally upon translocation of the protein into the ER via the translocon Sec61 [145]. Following protein folding and disulfide bonds formation, proinsulin is transferred by the Golgi system into immature secretory vesicles. Within their walls, mature insulin is produced through the concerted action of prohormone convertases and the release of the C-peptide central region [145,146]. The 24 amino acids–long SP insulin sequence is primarily involved in insulin-derived class I epitope generation [147]. By means of cell-free translocation assay and CRISPR/Cas technology, a study carried out in the 2018 pointed out the relevance of the concerted action of the SP peptidase and ERAP1 in the trimming of the PPI SP and in the production of PPI SP–derived epitopes [148]. Indeed, ERAP1 trimming activity is required for PPI signal peptide loading onto nascent HLA class I molecules. This phenomenon is either direct or follows cytoplasmic translocation via TAP, with the selected pathway being determined by the HLA allele. The presence of ERAP1 and TAP in insulin-containing islets of T1DM patients studied post mortem configures them as critical mediators in the CTL–β-cell dialogue in the disease setting [148]. More recently, Thoumaidou and colleagues disclosed a role for inflammation and ER stress on ERAP1 gene expression in human β-cells [149]. The authors demonstrated that within β-cells, proinflammatory cytokines regulate ERAP1 gene expression both at transcriptional and posttranscriptional levels. While the transcriptional regulatory mechanism was IFN-γ-dependent, the posttranscriptional control was related to the expression of specific miRNAs belonging to the miR-17 family, which negatively affect ERAP1 expression via direct interaction with its 3'-UTR region [149]. This suggests that the inflammatory milieu within and surrounding β-cells may be critical to determining the effects of ERAP1 in the frame of PP1 processing and consequent metabolic and immunologic alterations. In fact, based on the role of miR-17 as a bridge linking the endoribonuclease IRE1α and β-cells' destruction, the same study demonstrated that specific inhibition of IRE1α partially reduces cytokine-induced ERAP1 expression. As a result, SP-derived epitopes presentation and their immune recognition by PPI autoreactive CTLs was reduced [149]. These findings also establish a direct correlation between ER stress and β-cell death, highlighting the primary role of ER modulators, and potentially ERAP1, in shaping antigenic insulin peptide presentation to autoimmune diabetogenic CTLs (Figure 2).

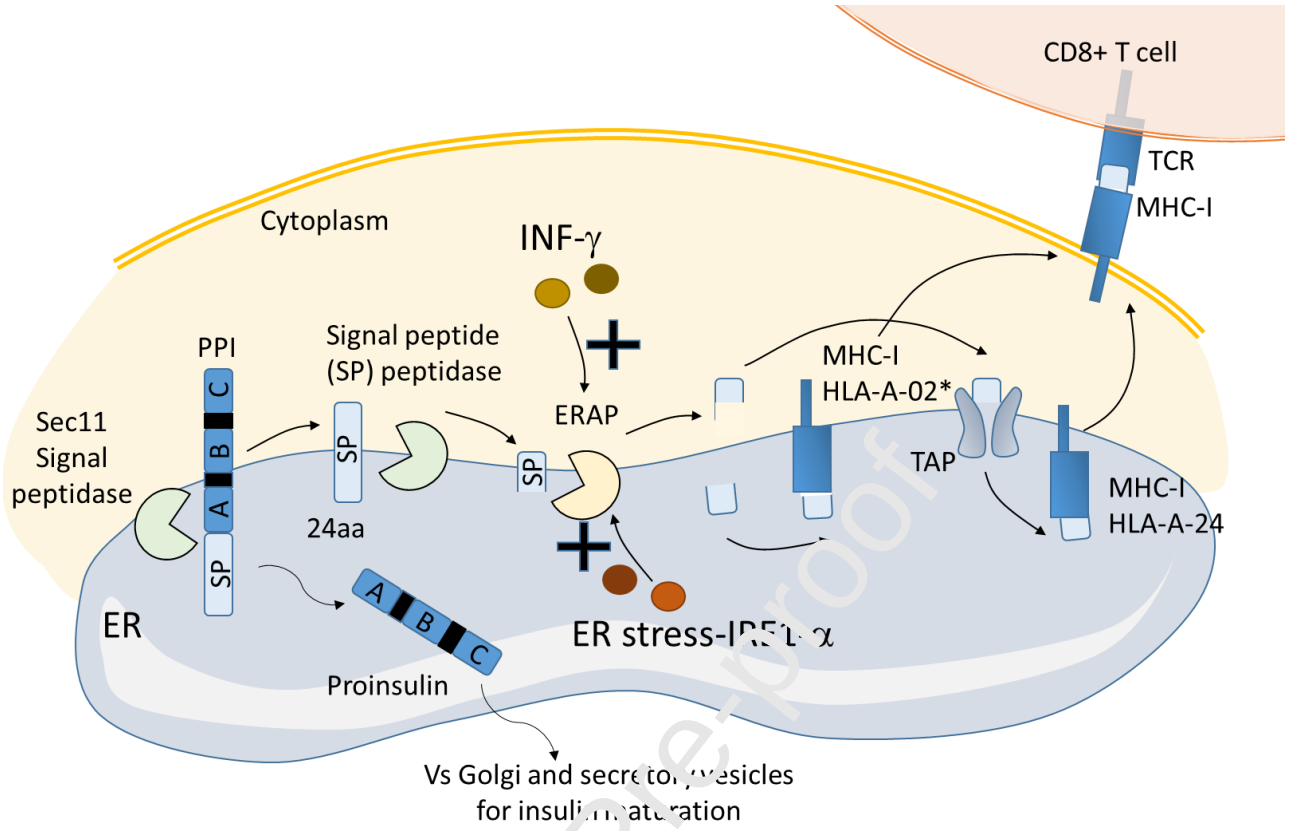

Figure 2. **Role of ERAP1 in preproinsulin processing**. Insulin derives from its precursor preproinsulin (PPI) via a posttranslational processing pathway. The signal peptidase Sec11 cuts off the signal peptide (SP) to form proinsulin. Following protein folding and disulfide bonds formation, proinsulin is transferred by the Golgi system into immature secretory vesicles, where mature insulin is produced. The SP peptidase and ERAP1 act in concert to trim the 24 amino acids–long SP insulin sequence for the production of PPI SP–derived class I epitopes. In particular, ERAP1 trimming activity is required for loading of the PPI signal peptide onto nascent HLA class I molecules either directly or following cytoplasmic translocation via TAP, with the selected pathway being determined by the HLA allele (HLA-A-02*, and HLA-A-24*, respectively). Pro-inflammatory cytokines (IFN-γ), and well as ER stress conditions through recruitment of the endoribonuclease IRE1-α boost ERAP1 trimming activity, and consequently, the antigenic insulin peptide presentation to autoimmune diabetogenic CD8+ CTLs.

3.3 ERAPs in T1DM-related viral infections.

ERAP1 is inhibited by human CMV, a major player in the pathogenesis of T1DM[105,150,151]. This is related to the expression of viral microRNAs (miRNAs), especially miR-UL112-5p and miR-US4-1, which have been found to contribute to immune evasion by targeting ERAP1 [105]. In this frame, naturally occurring SNPs within the miRNA binding sites of target genes, including ERAP1, were found to affect miRNA-target interactions. In particular, the rs17481334 G variant naturally occurring in the ERAP1 3' UTR, preserves ERAP1 from miR-UL112-5p-mediated degradation. Specifically, HCMV miR-UL112-5p binds the 3' UTR of ERAP1 A variant, but not the 3' UTR of ERAP1 G variant, and, accordingly, ERAP1 expression is reduced both at RNA and protein levels only in human fibroblasts homozygous for the A variant. Consistently, HCMV-infected GG fibroblasts are more efficient in trimming viral antigens and being targeted by HCMV-peptide-specific CTLs. Notably, GG individuals suffering from Multiple Sclerosis display significantly decreased HCMV seropositivity, with HCMV infection being negatively associated with adult onset disorder [105]. Such a resistance mechanism to HCMV through miR-UL112-5p-based immune evasion strategy, suggests a potential role for ERAP-related individual susceptibility to both infectious and non-infectious diseases. Yet, how the immune system detects ERAP dysfunction remains unknown. It has been previously documented in WT mice that ERAAP-deficient cells present an immunogenic pMHC I repertoire that elicits CD8+ T cell response [152]. Remarkably, the WT CD8+ T cells are capable of recognizing novel peptides on ERAAP-deficient cells, that are presented by non-classical, or MHC class Ib, molecules. Thus, ERAAP-deficient cells are targeted by MHC Ib restricted WT CD8+ T cells, suggesting that ERAP dysfunctions may elicit an abnormal autoimmune reaction aimed at eliminating ERAP-deficient cells[152,153]. This evidence helps understanding how *ERAP1* and its variants may alter the mechanism of action of HCMV and viral infections in general. Indeed, common ERAP1 allotypes can be a major source of heterogeneity in antigen processing, thus contributing to variable immune responses, including those observed in COVID-19 [154,155]. In fact, while all ERAP1 allotypes are capable of producing optimal peptides for MHC I molecules, including known SARS-CoV-2 epitopes, they present significant differences in peptide sequences produced, suggesting allotype-dependent sequence biases[154]. This is key since SARS-CoV-2 alters ERAPs expression in both human epithelial lung cells and PBMCs[156].

Again, ERAPs display *in vitro* antiviral activity against Human Immunodeficienv Virus (HIV), which is associated with innate immune activation besides their canonical role in antigen presentation and $CD8^+$ T cell activation[130]. This is key since T2DM, and to a lesser extent, T1DM may develop in (HIV)-infected patients under antiretroviral therapy[157]. In this frame, a DM-predisposing role is played

by concurrent infections with hepatitis C (HCV), whereby ERAPs alterations have been widely reported[22].

Thus, it is not surprising that an altered functioning of ERAPs, due to either specific mutations or transcriptional/post-translational alterations, may significantly impact the susceptibility/progression of infectious diseases, which might in turn, contribute to the onset/exacerbation of T1DM. This brings up the possibility that modulating ERAPs efficacy represents a potential strategy to improve and boost the effectiveness of antiviral responses. These data show that, through their trimming activity, aminopeptidases can markedly filter and determine which antigens can be presented by MHC I molecules [158]. Thus, ERAPs genes and their allelic variants establish a bottleneck for the fitting of processed antigens into the binding pocket of MHC I molecules, representing a potential bridge between viral infections and metabolic complications, which remains to be explored in the frame of T1DM specifically.

4. Conclusions and future perspectives.

While the role of ERAPs in autoimmunity has been widely documented, only a few T1DM-related studies succeeded in determining direct associations between ERAPs variants and disease susceptibility/outbreak.

Considering the multifaceted role of ERAPs, our hypothesis is that these proteins modulate T1DM susceptibility in an indirect and subtle way, mainly through their intervention in antigen presentation during viral infections which can favor T1DM development, and/or by influencing insulin-sensitivity at different levels. As discussed herein and elsewhere[22,132], ERAPs' loss of function or alteration can significantly modify the repertoire of antigens presented by MHC I molecules, thus critically affecting the activation of NK and $CD8^+$ T cells. This in turn, might contribute to the maintenance of chronic infection.

Although direct evidence bridging ERAPs and viral infections in the frame of T1DM specifically is still limited and controversial, it is conceivable that any variation affecting the presentation of pathogen-derived peptides might lead to an inadequate immune response favoring disease onset/progression.

ERAPs alterations are also linked to a variety of pathological outcomes which may occur in chronic inflammatory diseases, including diabetes; these encompass chronic kidney disease[139], spontaneous

intestinal dysbiosis and increased susceptibility to colitis, which is due to reduced numbers of both "Tr1-like" regulatory T cells and tolerogenic dendritic cells [159].

Regulation of insulin sensitivity represents an additional potential mechanism through which ERAPs might influence T1DM pathogenesis. As a support to this hypothesis, a study published in 2014 identified an association between the eQTL rs1019503 SNP for ERAP2 and 2-h glucose levels in skeletal muscle, and potentially, in other tissues where such a SNP influences ERAP2 expression [160]. Coupled with evidence on the activity of ERAP1 as an inflammation-induced hepatokine [161], these studies support a role of ERAPs in regulating insulin sensitivity and whole-body glucose metabolism.

To date, only a limited number of studies investigated potential therapeutic strategies targeting ERAPs and/or related functions to prevent/revert T1DM, showing that reduced ERAP1 expression limits SP-derived epitope presentation to PPI autoreactive CD8+ T cells [149]. However, drawing sound conclusions on the therapeutic potential of ERAPs' modulators in the frame of diabetes appears still premature, as the role of ERAPs and related polymorphisms deserves to be investigated more in depth, especially in relation with both insulin metabolism and viral infections. This might also contribute to shed light on the biological and molecular bases through which viral infections, including COVID-19, influence the pathogenesis/complications of diabetes.

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Graphical abstract