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Proteasome isoforms in human thymi and mouse models

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ABSTRACT

The thymus is the organ where functional and self-tolerant T cells are selected through processes of positive and negative selection before migrating to the periphery. The antigenic peptides presented on MHC class I molecules of thymic epithelial cells (TECs) in the cortex and medulla of the thymus are key players in these processes. It has been theorized that these cells express different proteasome isoforms, which generate MHC class I immunopeptidomes with features that differentiate cortex and medulla, and hence positive and negative $CD8^+$ T cell selection. This theory is largely based on mouse models and does not consider the large variety of noncanonical antigenic peptides that could be produced by proteasomes and presented on MHC class I molecules. Here, we review the multi-omics, biochemical and cellular studies carried out on mouse models and human thymi to investigate their content of proteasome isoforms, briefly summarize the implication that noncanonical antigenic peptide presentation in the thymus could have on $CD8^+$ T cell repertoire and put these aspects in the larger framework of anatomical and immunological differences between these two species.

1. Thymus as primary lymphoid organ

The thymus is an immune organ, whose primary purpose is considered to be the providing of a diverse environment for successful thymocyte maturation and expression of a functional and comprehensive T cell receptor αβ (TCRαβ) repertoire of peripheral $CD4^+$ and $CD8^+$ T cells. Lymphoid progenitors from bone marrow travel to the thymus for cell lineage refinement and control of self-tolerance, and, consequently, can serve as a functioning part of the adaptive immune system in recognizing and neutralizing aberrant or infected cells. The thymic stroma, compartmentalized into multi-lobular cortex and medulla, consists of heterogeneous cell populations to support thymocyte maturation and to provide them with a selective environment, thereby establishing the cornerstone of the central tolerance process. Maturing thymocytes travel through the compartments of the thymus where they interact with diverse populations of stromal cells, including thymic epithelial cells (TECs), fibroblasts, dendritic cells (DC), and other immune and non-immune cells [1-4]. Cortical thymic epithelial cells (cTECs) interact with developing double positive $CD8^+$ $CD4^+$ (DP) thymocyte progenitors and are needed for successful T cell development during a process called positive selection (Fig. 1). If we focused on $CD8^+$ T cell's selection, according to the most accepted model, the positive selection process aims to eliminate DP T cell clones expressing TCRs with no affinity or very low affinity for a pool of self-peptides presented by the major histocompatibility complex class I (MHC-I) molecules of cTECs, *i.e.* a cTEC's self-MHC-I immunopeptidome. Elimination at the positive selection stage is thought to be the fate of most thymocytes. If a TCRαβ had a low affinity for cTECs' self-MHC-I immunopeptidomes, the DP T cell clone survives ('positive selection', indeed) and migrates to the medulla where it can develop into a single positive (SP) T cell with specialized $CD8^+$ T cell phenotype [5,6]. In this compartment, SP thymocytes interact with medullary thymic epithelial cells (mTECs) and DCs, thereby probing the affinity of their TCRαβ for the self-MHC-I immunopeptidomes of these professional antigen-presenting cells (APCs). If this affinity is too strong, the SP $CD8^+$ T cell clones can be eliminated in a process called negative selection, through clonal deletion, receptor editing or anergy. The aim of this step is the elimination of potentially autoreactive cytotoxic $CD8^+$ T cell clones. To expand the presented antigenic repertoire for representation of any self-peptide of the periphery, a subpopulation of mTECs expresses the Aire transcription factor which stimulates promiscuous gene expression and the expression of tissue-restricted antigens [7,8]. Additionally, studies on

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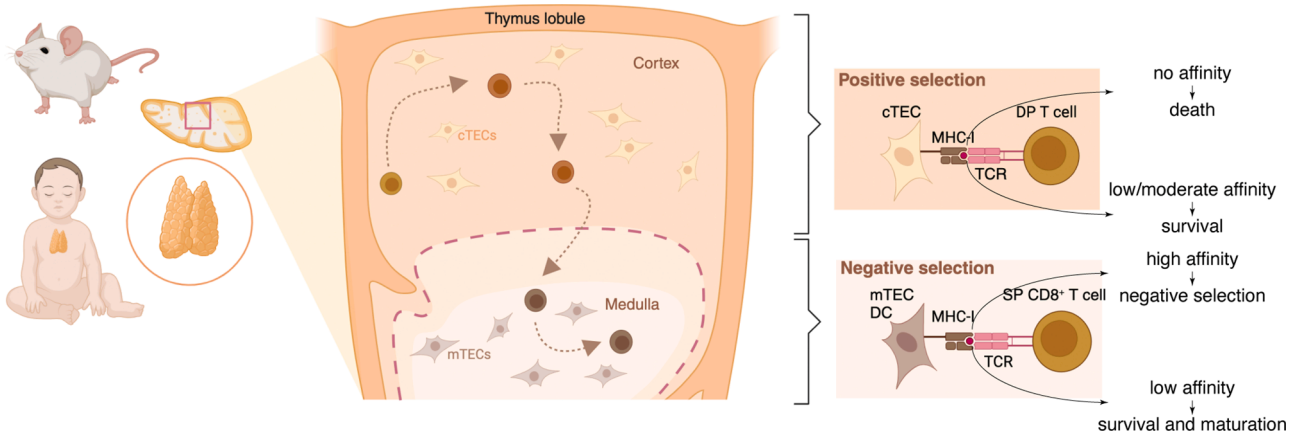

Fig. 1. Central tolerance theory for CD8+ T cells.

mouse models have suggested mTEC heterogeneity to be increased via transcription factors' control, thereby generating mimetic cells mirroring the peripheral self [9-11]. The heterogenic populations of mTECs have been identified both in humans and mice, although not exact counterparts of each other, and have been described by cell type and organism-specific gene expression patterns [1,3,4,9,11-13].

A barrage of evidence suggests that the thymic deletion of self-reactive CD8$^+$ T cells is not perfect. Many potentially autoreactive CD8$^+$ T cells, which can recognize both canonical and noncanonical antigenic peptides presented by MHC-I molecules, are present in periphery [14-18]. There, they can be controlled by peripheral tolerance mechanisms such as quiescence, clonal ignorance, anergy and tolerance-induced cell death [16], although seldom autoimmune responses can occur when both central and peripheral tolerance failed.

2. Thymoproteasomes and other proteasome isoforms: differences in antigen presentation and beyond

For the development of a functional CD8$^+$ T cell repertoire, both cTECs and mTECs are needed in mice [19-22]. As with any other nucleated cell, mouse TECs present mainly 8 – 11 residue long peptides on their MHC-I molecules, whereas human TECs present slightly longer peptides on their MHC-I molecules, ranging from 8 to 15 residues (and even longer, for some MHC-I allotypes) [23,24]. In both mammals, these antigenic peptides are produced by the antigen processing and presentation (APP) pathway (Fig. 2). It is widely accepted that proteasomes are the main proteases producing the MHC-I-presented peptides [24,25], despite few studies stating otherwise [26]. Proteins are directed to degradation by proteasomes through ubiquitin-dependent or -independent pathways, and processed into peptides in the proteasomal catalytic chamber. The 20S proteasomal core complex is comprised of four consecutive heteroheptameric rings stacked over each other. The outer rings consist of seven α subunits (α1-α7; and their cognate genes *PSMA1–7*), and stack with two inner rings containing seven β subunits (β1-β7; and their cognate genes *PSMB1–11*). Three of the proteasome β subunits directly catalyze peptide hydrolysis and splicing through their threonine 1 [27,28]. There are several 20S proteasome isoforms depending on the catalytic subunit composition in the β rings. Standard proteasomes are present in most cells and contain the catalytic β1, β2 and β5 subunits, which are translated from the human *PSMB6, PSMB7,*

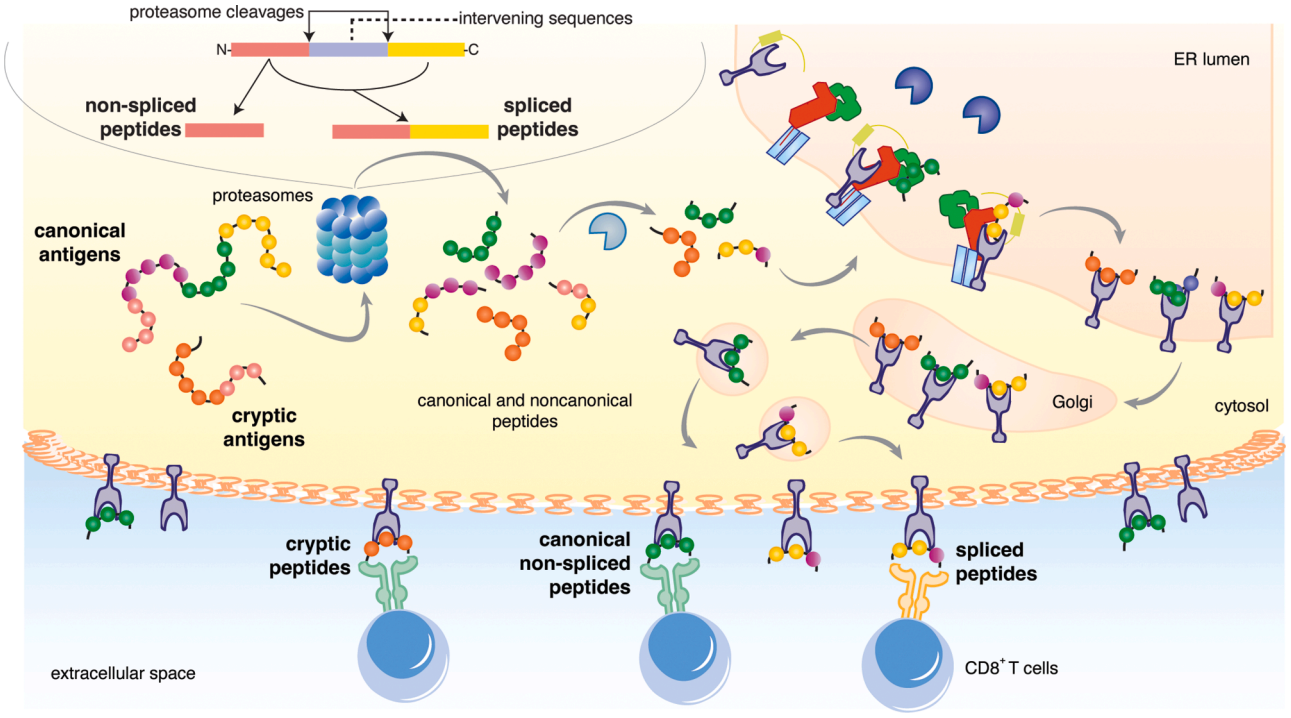

Fig. 2. MHC-I antigen processing and presentation pathway.

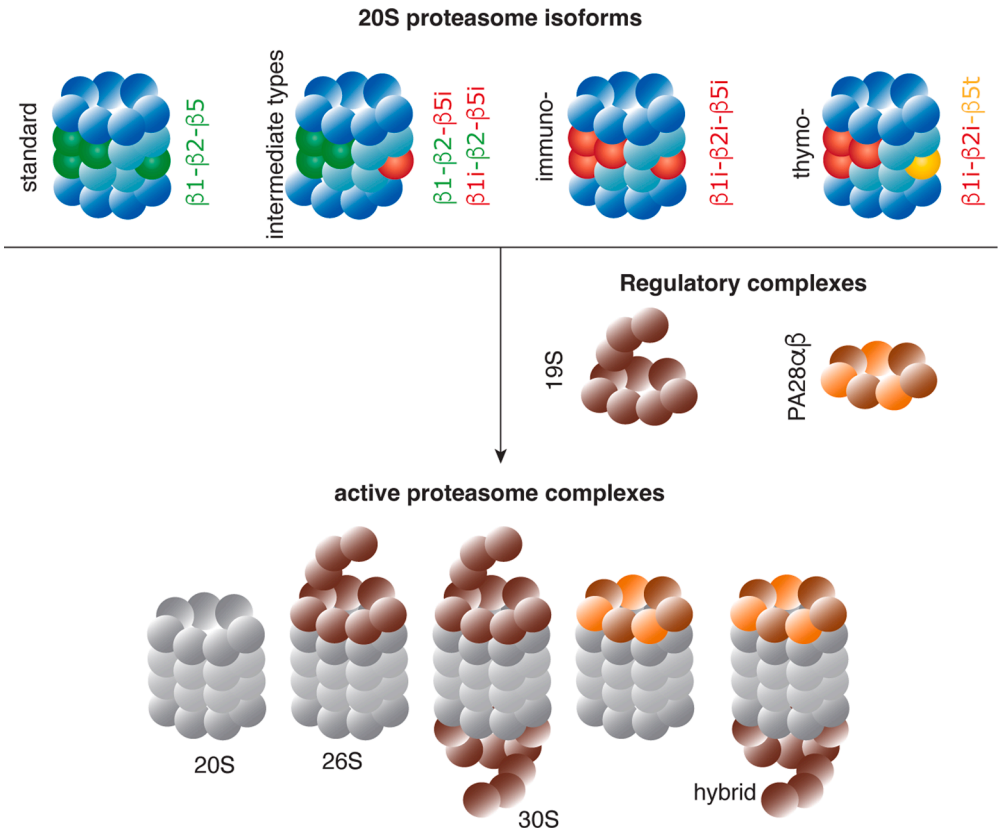

Fig. 3. Main proteasome isoforms.

Table 1
PA28αβ and 20S proteasome genes and subunits in mice and humans.

Human gene	Mouse gene	Subunit	Subunit type	-Proteasome isoform
PSMA6	*Psma6*	α1	non-catalytic	all
PSMA2	*Psma2*	α2	non-catalytic	all
PSMA4	*Psma4*	α3	non-catalytic	all
PSMA7	*Psma7*	α4	non-catalytic	all
PSMA5	*Psma5*	α5	non-catalytic	all
PSMA1	*Psma1*	α6	non-catalytic	all
PSMA3	*Psma3*	α7	non-catalytic	all
PSMB3	*Psmb3*	β3	non-catalytic	all
PSMB2	*Psmb2*	β4	non-catalytic	all
PSMB1	*Psmb1*	β6	non-catalytic	all
PSMB4	*Psmb4*	β7	non-catalytic	all
PSMB6	*Psmb6*	β1	catalytic	standard
PSMB7	*Psmb7*	β2	catalytic	standard and intermediate
PSMB5	*Psmb5*	β5	catalytic	standard
PSMB9	*Psmb9*	β1i	catalytic	immuno, thymo and intermediate
PSMB10	*Psmb10*	β2i	catalytic	Immuno and thymo
PSMB8	*Psmb8*	β5i	catalytic	immuno and intermediate
PSMB11	*Psmb11*	β5t	catalytic	thymo
PSME1	*Psme1*	PA28α	regulator	n.a.
PSME2	*Psme2*	PA28β	regulator	n.a.

PSMB5 genes, respectively (Fig. 3 and Table 1). Immunoproteasomes contain the β1i, β2i, and β5i subunits (*PSMB9, PSMB10, PSMB8* genes, respectively) in place of the standard subunits, and are present in most immune cells (*e.g.*, DCs), cells exposed to an inflammatory milieu (they are IFN-γ-inducible, *e.g.*) and, at least in mice, in mTECs [29–31]. We are not aware of any study showing an immunoproteasome expression in human mTECs (Table 2). Intermediate proteasomes containing either β1-β2-β5i or β1i-β2-β5i subunits have been observed in several human organs, in tumor cells and DCs [32,33].

All three proteasome catalytic subunits have a significant impact on protein degradation [34,35]. Standard- and immuno-proteasomes have different sequence preference and regulation of their catalytic activities, although it is still a matter of debate if they generate distinct pools of peptide products, how that could impinge upon MHC-I immunopeptidomes, and whether the functional differences between mouse proteasome isoforms are reflected in humans [27,35-48]. The specific involvement of immunoproteasomes has been extensively documented in immune-related neurological diseases, autoimmunity and infections [49-61]. In 2007, Murata and colleagues [20] discovered that mouse cTECs expressed a specific proteasome catalytic subunit, *i.e.* β5t (from the human *PSMB11* and the mouse *Psmb11* genes; see Table 1), which is assembled with β1i and β2i subunits in the thymoproteasome (Fig. 3). In mouse models, thymoproteasomes are essential for the positive selection (but not the negative selection) of functional $CD8^+$ T cells [20-22] and responsiveness of peripheral $CD8^+$ T cells [62]. The substitution of the β5t subunit with the β5i subunit does not generate a normal $CD8^+$ T cell repertoire in a C57BL6/J mouse background [19]. Based on mouse models, it has been hypothesized that thymoproteasomes generate a specific pool of peptides that are presented in MHC-I immunopeptidomes to DP thymocytes (also defined as private MHC-I immunopeptidomes), thereby promoting the positive selection [20,21,63-65]. A handful of studies showed that thymoproteasomes differ from immunoproteasomes in terms of cleavage preferences both in mouse [64,66,

Table 2
What we know about the expression of proteasome catalytic subunits, PA28αβ and cognate genes in TECs in mice and humans. Supporting references are reported.

mRNA	**Expression of proteasome catalytic subunit genes and PA28αβ genes**								
		PSMB6 **(β1)**	*PSMB7* **(β2)**	*PSMB5* **(β5)**	*PSMB9* **(β1i)**	*PSMB10* **(β2i)**	*PSMB8* **(β5i)**	*PSMB11* **(β5t)**	*PSME1* **(PA28α)**	*PSME2* **(PA28β)**
Mouse	cTECs	High [30]	High [30]	High [30]	None [30]	None [30]	None [30]	High [117]	High [30]	High [30]
	mTECs	High [30]	High [30]	High [30]	High [30]	High [30]	High [30]	None [117]	High [30]	High [30]
Human	cTECs	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
	mTECs	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Proteins	**Expression of proteasome catalytic subunits and PA28αβ**									
		β1	**β2**	**β5**	**β1i**	**β2i**	**β5i**	**β5t**	**PA28α**	**PA28β**
Mouse	cTECs	Low [20,30,71]	Low [20,30,71]	Low [20,30] None [71]	High [20] None [30]	High [20] None [30]	Low [20] None [30]	High [20,117] Yes [71]	Unknown	Unknown
	mTECs	Low [30,71]	Low [30,71]	None [30] Low [71]	High [30] Yes [71]	High [30] Yes [71]	High [30] Yes [71]	None [20,71,117]	Unknown	Unknown
Human	cTECs	Unknown	Unknown	Unknown	High [119]	High [119]	None [119]	High [119]	Unknown	Unknown
	mTECs	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown	None [119]	Unknown	Unknown

67] and human [68] systems. The only attempt of comparing the MHC-I immunopeptidomes of cells expressing either thymo- or immuno-proteasomes used immortalized murine embryonic fibroblasts transduced with either murine β5i or β5t subunits in a common proteasome background expressing β1i and β2i subunits [64]. Mass spectrometry-based analysis of the MHC-I immunopeptidomes identified a hundred peptides, with more than a half of them not shared between cells expressing either one or the other proteasome isoform [64]. However, commonly conducted MHC-I immunopeptidomics typically identifies thousands of peptides in mouse cells and organs [23], indicating a shallow depth in the immunopeptidomics study of Sasaki and colleague [64] that might have limited the information that could be gathered from that study.

The initial hypothesis that this putative private MHC-I immunopeptidome produced by thymoproteasomes is characterized by a general low binding affinity for MHC-I molecules [20,21,67] has been dismissed, at least in mouse models [19,64], and has not been investigated in human cells, yet. Takahama [65] hypothesized that the thymoproteasome-dependent private immunopeptidomes might consist of a dozen antigenic peptides, which would drive the positive selection by cTECs in mouse models. A comparison of thymoproteasomes with other proteasome isoforms both in human [68] and mouse [19,64] systems showed that thymo- and immuno-proteasomes can produce specific canonical epitopes with different efficiency.

Because proteasomes are involved in the degradation of most of the cytoplasmic proteins, thymo- and immuno-proteasomes might differ in many other aspects of cell metabolism and regulation in TECs rather than the mere MHC-I immunopeptidomes. Contradictory results obtained on few mouse models left this hypothesis still unresolved [69-72].

In summary, we do not know yet how thymoproteasomes regulate the $CD8^+$ T cell repertoire development in mice and whether this phenomenon is also occurring in human thymi.

3. Proteasome regulators are key players in antigen presentation

So far, we focused on the diversity of the 20S proteasome isoforms and their implication in protein degradation and antigen presentation. However, 20S proteasomes are often associated to complexes that regulate the proteolytic activity of these proteases. Briefly, 20S proteasomes preferentially process intrinsically disordered proteins, which contain large unstructured segments or even completely lack a defined tertiary structure in their native state, and have been estimated to represent up to 30 % of the intracellular proteome [73-76]. The specific targeting of 20S proteasome degradation could be controlled by the catalytic core regulators (CCRs) family [77]. The most know configuration of proteasomes is, however, 20S proteasome bound to the 19S regulator – thereby forming 26S/30S proteasomes - that degrade poly-ubiquitylated proteins. This is considered the main degradation pathway of the ubiquitin proteasome system (UPS) [78]. Among the other complexes that bind proteasome gates, the heptameric proteasome activator (PA28)αβ is particularly relevant for MHC-I immunopeptidomes, modifies proteasome conformation and regulates the degradation of non-polyubiquitinated proteins [79] (Fig. 3). The preferential binding of a proteasome isoform by PA28αβ complexes is a matter of debate [80,81]. PA28-α and -β are translated from the *PSME1* and *PSME2* genes (Table 1), and can be combined as either $PA28\alpha_4\beta_3$ or $PA28\alpha_7$ in mouse, with the former structure assumed to be the most stable, active and hence physiological [82]. In both mouse and human cellular systems, PA28αβ activity has been shown to impact proteasome proteolysis, MHC-I antigen presentation and $CD8^+$ T cell-mediated response to infections [24,83-87].

4. MHC-I molecules present canonical and noncanonical peptides to T cells: potential implication in central tolerance

As for several other proteases, proteasomes and cathepsins can catalyze peptide-bond break by peptide hydrolysis as well as ligation of two noncontiguous fragments by peptide splicing [88-91]. The immunological relevance of epitopes generated by the latter mechanism, known as post-translationally spliced epitopes, has been demonstrated for both MHC-I and -II antigen presentation and linked to cancer, autoimmunity and infections as well as TCR-T cell therapies and vaccine development [36,43,90,92-101]. Post-translationally spliced epitopes are only a sub-group of the dark immunopeptidome, a term coined by Purcell and Ternette [102] and used to define noncanonical antigenic peptides that derive from cryptic antigens (such as putative non-coding regions of the genome) and are produced by canonical peptide-bond cleavage (*i.e.*, by peptide hydrolysis), as well as from canonical antigens modified after their translation [24,103-107] (Fig. 2). Both human standard- and immuno-proteasomes have been proved to catalyze peptide splicing, which is a catalytic activity common to proteasome derived from several other species [27,36,40,108]. A formal proof that thymoproteasomes could also produce post-translationally spliced peptides is still missing.

Bearing in mind that the theoretical number of post-translationally spliced peptides that could be generated by proteasomes is huge [109, 110], one could be concerned about the potential impact that a large number of 'reshuffled' peptide sequences could have on the positive and negative selection of thymocytes. Indeed, if an unlimited barrage of post-translationally spliced peptides derived from self-antigens were presented by mTECs and thymic DCs to SP thymocytes in the medulla, the TCRαβ repertoire of the latter could be dramatically reduced, thereby creating immunological holes in the ability of $CD8^+$ T cells to

recognize foreign epitopes [18]. To address this issue, it is pivotal to know that both peptide hydrolysis and peptide splicing are regulated processes, with driving forces and sequence preferences, which avoid the random formation of any peptide sequence [27,75,111-114]. In fact, in the first study on proteasome-catalyzed peptide splicing of whole proteins (rather than synthetic protein fragments, as largely done in the past) it has been estimated that human 20S proteasomes produce 1 out of 6 of the potential peptides via peptide hydrolysis and 1 out of over 200,000 of the potential peptides via peptide splicing [75]. Because of this, post-translationally spliced peptides seem to be a minority of the pool of peptides produced by proteasomes [75] and presented by MHC-I molecules [24,115]. Few years ago, we applied this concept to the theoretical problem of an impact of post-translational peptide splicing on central tolerance, and particularly negative selection, and the generation of a functional TCRαβ repertoire of $CD8^+$ T cells against non-self. Through our computational model, we estimated that the problem did not really exist. Indeed, the theoretical frequency of MHC-I-restricted antigenic peptides that could be derived from human proteome as well as from the proteome of a hundred viruses with human tropism was very small for canonical non-spliced peptides, whereas reached a 4 % considering post-translationally spliced peptides, even neglecting any TCRαβ degeneracy [18]. When we considered the actual number of post-translationally spliced peptides so far measured in human MHC-I immunopeptidomes, that frequency drastically dropped to a barely negligible level [18]. This suggested that although post-translationally spliced epitopes derived from viruses could theoretically mimic human self-antigenic peptides, and therefore impinge upon TCRαβ repertoire, central and peripheral tolerance, and could have an autoimmune potential [49,116], their frequency in the MHC-I immunopeptidome should be extremely low [18].

5. What do we exactly know about the expression of proteasome isoforms and regulators in mouse and human thymi?

The theory of a specific pool of peptides produced by thymoproteasomes that results in a private MHC-I immunopeptidome promoting the thymocytes' positive selection requires a specific expression of thymoproteasomes in the cTECs. Indeed, if thymoproteasomes were also expressed in mTECs and other thymic APCs, the theory could be challenged. Similarly, the presence (or even the quantitative prevalence) of other proteasome isoforms in the same cTECs might render less convincing this theory because of the potential prevalence of antigenic peptides mainly generated by other proteasome isoforms inside cTECs.

Another factor to consider is the expression of proteasome regulators in cTECs and mTECs. For instance, proteasome activity is frequently regulated by PA28αβ in the context of MHC-I antigen presentation, and if the expression of this regulator differed between cTECs and mTECs, we would also expect an impact on the hypothetical diversity of MHC-I immunopeptidomes of the two TEC types.

The thymoproteasome catalytic β5t subunit and the cognate *Psmb11* transcript have been shown to be expressed in mouse cTECs [20,71,117], and transiently in β5t-expressing TEC progenitors [118]. The subunits β1i and β2i are also expressed in mouse cTECs, whereas the remaining catalytic subunits β1, β2, β5 and β5i have been reported to display only low or non-detectable protein expression in mouse cTECs by [20,71] but not by [30]. The PA28αβ genes *Psme1* and *Psme2* were detected in mouse cTECs by [30] (Table 2).

The murine mTECs have been shown to display strong expression of β1i, β2i and β5i subunits and cognate mRNAs [30]. The standard proteasome subunits β1, β2 and β5 were reported to have appreciable mRNA expression in murine mTECs [30], where they seemed to be translated to low or non-detectable protein expression [30,71] (Table 2). Additionally, the PA28αβ genes *Psme1* and *Psme2* were reported to be expressed in murine mTECs [30], although their protein expression remains to be clarified.

In the human thymus, the β5t subunit has been detected in cTECs and some cortical DCs, and proteasomes carrying the β5t subunit seem to contain β1i and β2i subunits [31]. Also, an absence of β5i subunit in human cTECs was reported in the same study [119]. No information on the other catalytic subunits, the PA28αβ subunits, or the gene expression of these components has been discussed so far in human cTECs (Table 2).

No information about the expression of proteasome catalytic subunits and cognate genes has been reported so far for human mTECs (Table 2), and we do not know whether an important regulator of proteasome activity such as PA28αβ is expressed in these cells.

In addition, the potential heterogeneity of proteasome isoforms' gene expression in TECs of both mice and humans has not been addressed yet, and hence it is unknown whether and how much TECs differ among each other, either in cortex and medulla, in terms of proteasome isoforms expression.

6. How could potential immunological diversities between mouse and human thymi influence the central tolerance theory?

A major challenge to the study of the human immune system – with exception of blood – is the restricted access to donor tissues. Only through the increasing multi-disciplinary nature of life science research in recent years, new studies succeeded in investigating also human bone marrow, thymus, and other immune organs. Most of these studies took advantage of next generation sequencing as well as of the most recent multi-omics technologies, which could allow a systematic comparison of human and mouse thymi. In the coming years, we expect to learn more and more about the expression of proteasome subunits and regulators in TECs as part of a systematic exploration of mice as representative models of human TECs. Such studies should carefully assess if the theory of a private MHC-I immunopeptidome generated by thymoproteasomes driving positive selection (preliminarily tested only in mice) holds true in human thymi. This specific aspect should also be considered bearing in mind the increasing evidence of differences between mouse and human thymic biology and anatomy, which open the possibility that various aspects of thymocyte development and mechanisms establishing central tolerance could also differ between those species. For instance, tolerance in humans is established in the foetus but can be reversed in mice by neonatal thymectomy, highlighting a differential developmental timeframe across species [120]. Failures in Aire-dependent central tolerance provoke much more severe pathology in humans than in mice [121,122] and the presence of anti-type I interferons (IFNs) could not be recapitulated in mouse models of Aire-KO [123]. Additionally, there may be underlying subtle but functionally relevant species differences in the role of cytokines influencing T cell function or development. For example, human but not mouse thymocytes have a constitutive expression of CXCL8, and CXCL8-producing human T cells have a protective role in defence against infections in newborn; in contrast even the murine ortholog could not be used to assess CXCL8 production *in vivo* [124]. Thymic regulation of γδT cell development is species-specific [125]. Human thymus structure is more complex than the mouse one, including Hassall's Bodies (HB) implicated in thymic DC and regulatory (Treg) T cell development that are not present in mouse thymi. Single cell transcriptomics have highlighted a higher complexity of human versus mouse stroma during development and postnatally [3,4,13,126]. As last example, Notch ligands (*e.g.*, DLL4), whose expression in mouse cTECs is crucial in inducing lymphoid fate of haematopoietic stem cells [127], are not recapitulated in humans, where DLL4 is mainly expressed by non-epithelial stroma and endothelial cells [128].

In summary, it remains an open question as to whether and how the observed differences between mouse and human thymi may influence the fundamental mechanisms that establish central tolerance. Should further comparative multi-omics analyses reveal analogous disparities between these species in terms of proteasome machinery in cTECs and mTECs, we might need to perform functional assays on human thymi (perhaps developing better *in vitro* systems) to potentially refine the central tolerance theory of $CD8^+$ T cells, and the role (still largely

unknown) that proteasome isoforms play in it.

CRediT authorship contribution statement

Michele Mishto: Writing – original draft, Conceptualization, Visualization. **Iina Takala:** Data curation, Investigation, Visualization, Writing – original draft. **Paola Bonfanti:** Writing – original draft. **Juliane Liepe:** Writing – original draft.

Declaration of competing interest

The authors have no conflicts of interest.

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