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DOI:

[10.1016/j.it.2017.07.011](https://doi.org/10.1016/j.it.2017.07.011)

Document Version

Peer reviewed version

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Citation for published version (APA):

Mishto, M., & Liepe, J. (2017). Post-Translational Peptide Splicing and T Cell Responses. *TRENDS IN IMMUNOLOGY*. <https://doi.org/10.1016/j.it.2017.07.011>

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Post-translational peptide splicing and T cell response

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Running title: antigenic spliced peptides

Abstract

CD8⁺ T cell-mediated specificity depends on the recognition of the MHC class I – epitope complexes at the cell surface. These epitopes are mainly produced upon the degradation of proteins by the proteasome, generating fragments of the original sequence. However, it is now clear that proteasome can produce a significant portion of epitopes by reshuffling the antigen sequence, thus expanding the potential antigenic repertoire. MHC class I-restricted spliced epitopes have been described in tumors and infections, suggesting an unpredicted relevance of these peculiar peptides. Here, we review current knowledge about proteasome-catalyzed peptide splicing, the emerging rules of this process, and the potential implications for our understanding and therapeutic use of CD8⁺ T cells, as well as mechanisms generating other non-canonical antigenic epitopes targeted by T cell response.

The unpredicted frequency of proteasome-generated antigenic spliced peptides

CD8⁺ T cell specificity is achieved through the recognition by the T cell receptor (TcR) of peptides presented in the cleft of **major histocompatibility complex class I** (MHC-I; see Glossary) molecules. The process of antigen presentation, from the original protein to the surface display of MHC-I molecule, has been the focus of intense studies. Since more than two decades, the proteasome is known to be the main protease responsible for the generation of the vast majority of peptides that are bound to the MHC-I cleft at the cell surface (**Box 1**). This macromolecular enzyme plays a critical role in protein homeostasis in all cells. However, each cell type can express different proteasome isoforms, which preferentially regulate specific pathways. For instance, the proteasome isoform expressed in immune cells or upon inflammation, *i.e.* the immunoproteasome, was initially thought to be specialized only in epitope generation, although later it became evident that this proteasome isoform is involved in several other inflammatory processes [1]. Another example is represented by the thymoproteasome, which impinges upon the mouse CD8⁺ T cell repertoire by generating the immunopeptidome of the **cortical thymic epithelial cells** (cTECs), thereby regulating the thymocytes' positive selection, [2-5] (**Box 2**). Until 2004, all MHC-I-restricted epitopes known to the scientific community were directly derived from their corresponding antigen sequences, and, when being generated by proteasome, they were products of canonical peptide-bond hydrolysis. In 2004 however, two groups identified two epitopes, whose sequence was the fusion of two non-continuous segments of the tumor-associated **glycoprotein-100** (gp100) or the **fibroblast grow factor-5** (FGF-5) antigens carried out through **proteasome-catalyzed peptide splicing** (PCPS) [6-8]. In the following decade, the pioneering work of Vigneron, Van den Eynde and colleagues [9-11] led to the identification of only a few other proteasome-generated spliced epitopes (**Table 1**). Therefore, immunologists tagged PCPS as a rare event of marginal immunological relevance. The identification of this handful of spliced epitopes was possible because of the availability of **tumor-infiltrating CD8⁺ T lymphocytes** (TILs) specific for the spliced epitopes. Conversely, other researchers developed reverse-immunology and bioinformatics approaches combined with mass spectrometry and database search strategies for the systematic identification of spliced peptides generated by proteasome. Novel methods were mandatory since the sequences of the spliced peptides were not included in any known proteome database, and the enormous number of theoretically possible combinations of peptide fragments that proteasome could ligate rendered the known proteomic approaches almost useless. These novel strategies allowed the

identification of several spliced peptides produced by proteasome during *in vitro* digestions of synthetic polypeptides [12-16], and the identification of the unforeseen dark side of the MHC-I immunopeptidome, *i.e.* thousands spliced peptides presented at the cells surface by MHC-I molecules of human B lymphoblastoid cell lines as well as primary fibroblasts [17].

The fact that almost one third of the antigenic peptides presented by different cells onto the MHC-I molecules are generated by PCPS was certainly surprising and might throw a spanner in the works that has important theoretical and therapeutic implications. Can this knowledge perturb our notion of self and non-self? How shall we reconsider the models of central and peripheral tolerance? Shall we reassess our most innovative strategies for epitope identification and validation used, for instance, for anti-cancer immunotherapies?

Here, we review first the mechanisms and regulation of PCPS, before focusing on its important ramifications.

Mechanisms and driving forces of PCPS

Proteasome-generated spliced peptides can be formed in different ways (**Fig. 1A, B**). The two splice-reactants are generated inside the proteasome chamber by cleavage of a single peptide sequence (*cis* PCPS), the excision of the sequence between the two splice-reactants (namely the intervening sequence) followed by the ligation of the splice-reactants in normal order, *i.e.* following the orientation from N- to C-terminus of the parental protein (normal *cis* PCPS), or in the reverse order (reverse *cis* PCPS). Furthermore, PCPS can also occur between two splice-reactants originating from two distinct proteins (*trans* PCPS). Although this occurs *in vitro* [8, 13, 15], its occurrence in cells is disputable [8]. It would however be difficult to reckon an immunological relevance of *trans* spliced peptides. Indeed, two specific antigens should repeatedly enter the proteasome in the same order and the putative formation of the *trans* spliced peptide should occur frequently enough to survive the reduction steps in the downstream MHC-I antigen presentation pathway and to trigger an efficient T cell response (**Box 1**). This could likely happen only in specific conditions (*e.g.* between viral antigens during viral infection) or in specific cellular compartments and/or macro-complexes, which would favor the reaction by molecular crowding of a limited variety of peptides. A similar speculation has been proposed to explain the production of hybrid epitopes able to trigger the CD4⁺ T cell response in **type 1 diabetes mellitus** (T1DM) and generated by the fusion of fragments derived from distinct antigens by an unknown enzyme [18-20] (see below).

Despite the recent observation that PCPS can be carried out by a **condensation reaction** both *in vitro* (either in a cell-free system or in live cells) [16], the most studied and likely the most predominant PCPS mechanism is transpeptidation. According to the model originally proposed by Stroobant and colleagues [6] and later confirmed with diverse techniques [9, 13], the reaction starts with the break of the peptide-bond between the residue of the N-terminal splice reactant (*i.e.* the residue P₁) and the following intervening sequence, and the formation of an acyl-enzyme intermediate between the residue P₁ and the proteasome's catalytic Thr₁. During the canonical peptide hydrolysis, which produces all non-spliced peptides, the acyl-enzyme intermediate would react with a molecule of water leading to the release of the N-terminal (non-spliced) peptide fragment. In PCPS, on the contrary, the

nucleophilic attack to the acyl-enzyme intermediate is mediated by a C-terminal splice-reactant, thereby leading to the ligation between the residue P₁ of the N-terminal splice-reactant and the residue P₁' of the C-terminal splice-reactant (**Fig. 1C**). Since during the canonical peptide hydrolysis the substrate-binding sites of proteasome catalytic pockets are mandatory requirements for the efficient reaction catalyzed by the proteasome Thr₁, it has been hypothesized that also in PCPS specific substrate binding sites exist [10, 13, 21]. Whether these are the same binding sites used for the peptide hydrolysis still needs to be proven.

The existence of PCPS specific binding sites is, however, supported by preliminary evidences that PCPS is a finely tuned mechanism rather than a random process. In fact, the formation of given spliced peptides is more efficient when the splice-reactants have specific sequence characteristics [15] and the substrate's amino acid residues frequently used by proteasome for peptide hydrolysis are not those most frequently used for PCPS [13]. For one specific spliced epitope, the minimal length of the splice-reactant was shown to be 3 residues [10], although for other spliced peptides a single residue could be ligated in a transpeptidation reaction [22]. Furthermore, it has been shown for a specific spliced epitope that the longer is the intervening sequence, the less efficiently the spliced epitope is generated [8]. In the much larger pool of spliced peptides of the MHC-I immunopeptidomes studied so far, however, we do not see a correlation between the intervening sequence length and the antigenic spliced peptide frequency, although the method applied did not allow the identification of spliced peptides with intervening sequences longer than 20 amino acid residues [17].

Regarding the factors ruling PCPS, standard proteasome and immunoproteasome differ in the generation of specific spliced peptides [12, 13, 16, 23, 24]. This is not surprising since they have distinctive dynamics of the different aspects of their activities [25] (**Box 2**), although overall qualitative and/or quantitative differences of the PCPS carried out by the two isoforms have not been demonstrated so far [13]. How proteasome regulators such as 19S and PA28 $\alpha\beta$ affect PCPS is still unknown.

Not just few molecules of each proteasome-generated spliced peptide

For immunologists, PCPS represents a spanner in the works not because of its existence, but because of its high frequency. Although the handful of spliced epitopes discovered in the last decade was an excellent pioneering work, it had no general consequences in translational medicine. On the contrary, the unexpected large variety and the abundance of the self spliced peptides eluted from the MHC-I molecules and identified by mass spectrometry - around one third of the immunopeptidome variety and one fourth of its abundance [17] – have some practical and theoretical implications. Regarding the practical implications, for instance, it will be now harder to annotate peptide sequences derived from MHC-I immunopeptidomes without considering spliced peptides. The latter would lead to the wrong assignment of 5-10% sequences, depending on the mass spectrometry method used [17]. Considering that the MHC-I immunopeptidome analysis is emerging as an extremely helpful tool for the identification of epitope targets for anti-cancer immunotherapy [26-28], the implications of PCPS in translational research are clear.

Regarding the theoretical implications, we have to consider that in immunobiology, quantity matters. Quantitative alterations of epitope production dynamics by the proteasome can lead to the complete abrogation of the CD8⁺ T cell response in mouse models [29-32]. Therefore, determining the quantity of antigenic spliced peptides exposed to CD8⁺ T cells by MHC-I molecules is a key element. Our quantification of the spliced and non-spliced peptides identified in three MHC-I immunopeptidomes was carried out by a label-free strategy based on the intensity of the mass spectrometry ion peak area [17]. Although this method is not directly suitable for quantitative comparison of single peptides [13, 33], it has become well accepted when analyzing large proteomics datasets to compare two groups of peptides making use of the law of large numbers [33-35]. Other quantitative methods [36] could be used to corroborate our recent results on three MHC-I immunopeptidomes, where spliced peptides showed an abundance distribution comparable to non-spliced peptides, although significantly lower [17]. A comparable abundance of spliced epitopes vs non-spliced epitopes at the cell surface has also been suggested by assays carried out with CD8⁺ T cell clones specific for either spliced or non-spliced epitopes derived from melanoma-associated antigens [16]. In our experience, the latter strategy is more sensitive than modern mass spectrometry approaches - despite their sensational progresses in terms of accuracy and sensitivity obtained in the last years [37] – and could provide direct indications about the spliced peptides' immunogenicity. Nonetheless, the isolation of specific CD8⁺ T cell clones is laborious and thus impractical for a system-wide analysis.

An alternative strategy could be the investigation of PCPS *in vitro* using purified proteasome and synthetic polypeptide substrates, which would in principle allow the quantification of a large number of spliced and non-spliced peptide products. Absolute quantification of the peptide products can be done through different mass spectrometry-based strategies [13, 37] and a correlation of quantitative analyses of proteasome-mediated digestions in cell-free systems with data obtained in cell culture and *in vivo* has been extensively reported [16, 23, 24, 31, 38-43]. The few *in vitro* studies on PCPS conducted so far were carried out with less informative mass spectrometry methods [13, 15] and we reckon that the use of more sensitive and accurate mass spectrometers will significantly enlarge our horizon about the frequency, relevance and characteristics of PCPS.

Proteasome-generated spliced epitopes derived from tumor-associated antigens

All six spliced epitopes validated so far in a human system derive from tumor-associated antigens (**Table 1**). For all of these spliced epitopes, human TILs or specific CD8⁺ T cell clones have been isolated. Furthermore, a natural response of memory T cells derived from the peripheral blood of melanoma patients against autologous cells presenting spliced epitopes derived from the well-known melanoma-associated antigen gp100 – namely gp100_{47-52/40-42} or gp100_{195-202/192} – has been described [16].

In addition, the adoptive transfer of a TIL clone specific for the spliced epitope tyrosinase_{368-373/336-340} into the autologous melanoma patient was followed by tumor regression [44]. In nonobese diabetic/severe combined immunodeficient mice (NOD/SCID), a CD8⁺ T cell clone specific for SP110_{296-301/286-289} inhibited engraftment of human acute myelogenous leukemia cells [45].

Although these studies all indicate that spliced epitopes could be useful targets for anti-cancer immunotherapies, the dimension of the MHC-I spliced immunopeptidome of tumor cells still needs to be determined. This is a critical aspect, which might have a significant impact on the most innovative anti-cancer immunotherapies such as **adoptive T cell therapies** (ATTs). The latter can use TILs, or autologous CD8⁺ T cells expressing specific TCRs, as effectors for the recognition and elimination of cancer cells [46]. A significant determinant of the efficacy of ATTs resides in the identification of the “best” tumor antigens. Ideal targets are epitopes carrying driver somatic mutations, both because they represent tumor-specific neoantigens and because they are not easily lost by the tumor, since those mutations are involved in the process of malignant transformation [47]. The frequency of these tumor-specific mutated epitopes is, however, limited by the mutation frequency and by the fact that epitopes need to have not only specific motifs for being presented onto MHC-I molecules, but also for passing all steps of the antigen presentation pathway (**Box 1**). As a consequence, the majority of the TILs used in clinical trials target tumor-associated rather than tumor-specific antigens, which could in consequence trigger undesirable autoimmune reactions [48].

In these therapeutic frameworks, the inherent ability of spliced epitopes to extend the antigenic landscape and to have potentially extremely large sequence variety could be useful to target tumor-specific driver mutations that would not be efficiently exposed at the cell surface through non-spliced peptides because of incompatibility of the mutated protein sequence with the most common MHC-I variants. For instance, the HLA-A*02:01 complex, which is the predominant MHC-I variant in the Caucasian population, cannot efficiently present any of the non-spliced peptides that carry some of the most common cancer mutations, such as **BRAF** V600E or **KRAS** G12D. The latter, on the contrary, can be efficiently presented by a non-spliced epitope on the HLA-C*08:02 complex, which can be targeted by ATT thereby leading to a complete remission of metastatic colorectal cancer [49]. ATT strategies could also target a pool of spliced and non-spliced epitopes, which carry the same driver mutation and are presented onto the different MHC-I variants of the patient. This approach would prevent immune-escaping phenomena like the selective deletion of specific MHC-I-expressing genes [49].

Impact of PCPS during infections and implications for autoimmunity

CD8⁺ T cell responses are often mandatory for the clearance of viruses and some other pathogens. Furthermore, vaccination trials for Dengue virus, Ebola virus or HIV showed how CD8⁺ T cell activation can play a key role in vaccine development [50-52]. The evidences of the immunogenicity of pathogen-derived spliced epitopes during infection are preliminary, and limited so far to the intracellular pathogen *Listeria monocytogenes* [24, 53]. Although the immunological relevance of PCPS still needs to be demonstrated on a large scale, some evolutionary advantages of the human immune system to target pathogen-derived spliced epitopes can be easily estimated. For instance, PCPS significantly enlarges the number of self antigens represented onto the MHC-I molecules at the surface of human B lymphoblastoid cell lines or primary fibroblasts [17]. Similarly, during *Listeria monocytogenes* infection, the **phosphatidylcholine-preferring phospholipase C** (PlcB) antigen elicits a CD8⁺ T cell response in the C57/BL6 mice only through the presentation of PlcB-derived spliced epitopes [54]. The potentially very large variety of the spliced epitopes could also be a useful strategy of the immune system to counter-attack mutation-based escape mechanisms of viruses.

Indeed, although a mutation can wipe out a single epitope [39], it is less likely to hamper the productions of an entire pool of spliced and non-spliced epitopes derived from that portion of the antigen.

However, it is important to consider that the same potentially very large variety of the spliced epitopes could influence autoimmune disease etiology. Calis and colleagues [55] estimated that the complete sequence overlap between *in silico* predicted antigenic peptides derived from self (human) or from a thousand different viruses is 0.15%, which raises to 0.7% considering the TCR recognition degeneracy. Of course, at the time that study did not include spliced peptides. It is easy to predict that the overlap between the self and non-self immunopeptidome will dramatically increase by encompassing PCPS in the frame. Therefore, theoretically, and depending on how PCPS is carried out in the thymus during T cell selection and maturation, we might have two extreme scenarios: either a scenario where autoimmunity is epidemic (due to a high overlap between self and non-self) or one where many viral antigens are not recognized by peripheral CD8⁺ T cells, since the corresponding TCRs were deleted because of self-recognition, thereby further increasing the so-called holes in the T-cell repertoire [55]. Understanding why this is not the case requires investigating the link between PCPS and antigen presentation in thymus and in periphery.

At the moment, we can only speculate that PCPS could be involved in the CD8⁺ T cell-mediated immune response against self antigens in those diseases (e.g. myelin in multiple sclerosis or insulin in T1DM), where infections have been hypothesized to be involved in the autoimmune pathogenesis [56, 57].

Hybrid insulin peptides in T1DM

In multiple sclerosis and T1DM, both CD4⁺ and CD8⁺ T cells can target their response toward self epitopes presented onto either MHC-I or MHC-II molecules [20, 57-60]. In particular, in T1DM, MHC-II-restricted and post-translationally spliced epitopes have been recently described. Indeed, two independent groups identified hybrid epitopes – *i.e. trans* spliced epitopes according to our nomenclature - that are specifically recognized by CD4⁺ T cells derived from NOD mice or pancreatic islets of patients affected T1DM [18-20]. These hybrid peptides derive from the ligation of few sequences of proinsulin to fragments of antigens immunologically relevant in T1DM and packed in the secretory vesicles [19, 20]. One CD4⁺ T cell clone, grown directly from an islet of a T1DM patient, recognizes a hybrid peptide derived from the ligation of the insulin C peptide and the insulin A chain. This specific epitope could thus be either a *trans* or a *cis* spliced peptide, depending on whether it is generated after or before the processing of the proinsulin that lead to the formation of the mature insulin form [20].

Because of the targeted-sequence strategy applied in both studies, it is still unclear which is the general prevalence of antigenic spliced (both *cis* and *trans*) peptides bound to the MHC-II molecules, and whether the phenomenon is relevant only for T1DM. Furthermore, it is still unknown which enzyme catalyzes their production. Beside the proteasome, other proteases such as Trypsin, Lys-C and plant-derived Asparaginyl endoprotease are known to catalyze peptide splicing [61]. In particular, the human homologous of the latter protease is also involved in the generation of MHC-II-restricted

epitopes [62], and thus it could be a validate candidate for the production of hybrid insulin peptides as well as other spliced epitopes presented onto MHC-II complexes.

Concluding remarks

Bearing in mind the enormous theoretical number of potential combination of splice-reactants, we reckon that algorithms estimating the likelihood of a spliced peptide to be generated and to bind to the MHC-I molecules will soon be mandatory to investigate the therapeutic applications of PCPS (see Outstanding Questions). Although preliminary evidences that PCPS is not a random process are encouraging, the pioneering studies on PCPS should be validated on much larger spliced peptide pools than the few peptides used as preliminary models. Furthermore, the present MHC-I-peptide binding affinity prediction algorithms should be applied carefully to spliced peptides, because they have mainly been trained using non-spliced peptide databases, and therefore their use could lead to a large number of false negatives [17]. The prediction of immunologically relevant spliced epitopes will likely be aided by the analysis of the corresponding antigen characteristics, abundance, turnover and translation rate, as these parameters have been correlated with non-spliced immunopeptidomes and intracellular proteomes in recent brilliant studies [28, 33, 63-66].

Despite the large number of spliced peptides identified in MHC-I immunopeptidomes, and the current focus on PCPS, we shall not forget that spliced peptides are only one part of the “non-canonical” peptides that are presented onto MHC molecules. In addition to the recent identification of the MHC-II-restricted hybrid insulin epitopes, which has been here reviewed, there are studies suggesting that a sizeable number of MHC-I-restricted antigenic peptides can derive from alternative open reading frames [67]. For instance, in T1DM patients, Kracht and colleagues described cytotoxic CD8⁺ T cells capable of killing human β cells by targeting an epitope generated by the usage of an alternative open reading frame in the insulin gene [68]. Furthermore, MHC-I-restricted epitopes can originate from non-coding sequences of the genome [67, 69], and, in principle, also from intergenically spliced chimeric RNAs [70].

Understanding the extension and the immunological relevance of the non-canonical “dark side” of the immunopeptidome will be a great challenge with potentially strong translational implications.

Acknowledgment

This work was supported in part by Berlin Institute of Health (BIH, CRG1-TP1).

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Figure 1. Proteasome-catalyzed peptide splicing. Spliced peptides can be formed by: **(A)** *cis* PCPS, *i.e.* when the two splice-reactants derive from the same polypeptide molecule. The ligation of the splice-reactants can occur in normal order, *i.e.* following the orientation from N- to C-terminus of the parental protein (normal *cis* PCPS), or in the reverse order (reverse *cis* PCPS); **(B)** *trans* PCPS, *i.e.* when the two splice-reactants originate from two distinct protein molecules or two distinct proteins [8, 13]. **(C)** The most defined PCPS mechanism is the transpeptidation. According to this model, the proteasome's catalytic Thr₁ breaks the peptide bond of the residue (P1) of the protein - thereby forming an acyl-enzyme intermediate with the N-terminal splice-reactant, coupled to the release of the intervening sequence – and, instead of catalyzing the canonical peptide hydrolysis, it catalyzes the ligation between the P1 residue of the N-terminal splice-reactant with the residue P1' of the C-terminal splice-reactant [6, 13].

Table 1. Spliced epitopes derived from tumor-associated antigens

epitope	antigen	identification method	notes	Reference
[RTK][QLYPEW]	gp100 _{40-42/47-52}	CD8 ⁺ T cell clone		[6]
[NTYAS][PRFK]	FGF-5 _{172-176/217-220}	isolated TIL		[7]
[SLPRGT][STPK]	SP110 _{296-301/286-289}	CD8 ⁺ T cell clone	specific CD8 ⁺ T cell clone inhibited engraftment of human acute myelogeneous leukemia cells in mouse model	[9]
[IYMDGT][ADFSF]	tyrosinase _{368-373/336-340}	isolated TIL	tumor regression in melanoma patient after ATT with the TIL	[11]
[RSYVPLAH][R]	gp100 _{195-202/192}	isolated TIL	natural response of memory CD8 ⁺ T cells from peripheral blood of melanoma patients	[10, 16]
[QLYPEW][RTK]	gp100 _{47-52/40-42}	<i>in vitro</i> digestions	natural response of memory CD8 ⁺ T cells from peripheral blood of melanoma patients	[16]

Text box 1

Proteasome-mediated MHC-I-restricted antigen presentation pathway.

The peptides presented at the cell surface on MHC-I molecules represent the immunopeptidome of the cell, which is specific to a given cell and it is influenced by several factors such as its MHC-I haplotype (each diploid human cell has six usually distinct MHC-I isoforms), and its intracellular proteome, *i.e.* the proteins expressed in the cell [71]. If an antigenic peptide bound to the MHC-I complex is recognized by TCRs of CD8⁺ T cells, it is defined as epitope. The large majority of the MHC-I-restricted antigenic peptides are produced in the proteasome chamber by canonical peptide-bond hydrolysis or, as discussed here, by PCPS. These peptides can derive from rapidly degraded proteins (RDPs), which are supposed to include defective ribosomal products (DRiPs) and non-exponentially degraded proteins (NEDPs), although the quantitative analysis of the immunopeptidome's source is still a matter of debate [33, 64, 65, 71-73]. In the cytosol, the peptide fragments released from the proteasome are further degraded by aminopeptidases, thereby regenerating the cellular amino acids pool. Few of these peptides survive this step and are transported into the endoplasmic reticulum (ER) through a channel constituted by the transporters associated with antigen processing (TAPs). TAP-independent pathways, which are only partially proteasome-dependent, have also been described [74, 75]. In the ER, the peptides are bound to the peptide-loading complex, can be further trimmed by ER-resident aminopeptidases (ERAPs) and can finally bind the MHC-I complexes. The MHC-I-peptide complex undergoes modifications, and is transported through the Golgi to the cell surface. There, it is presented for a variable period of time, which depends in part on the affinity between the MHC-I complex and the peptide, as empty MHC-I molecules are rapidly recycled. At the cell surface, the MHC-I-peptide complex can be recognized by the TCR and CD8 of T cells, and, in presence of co-stimulatory factors (*e.g.* CD28, CD80, CD86), the binding induces the priming/activation of T cells [76].

Text box 2

Proteasome isoforms and functions

The 20S proteasome is the core of the ubiquitin-proteasome system (UPS), which is responsible for the degradation of the large majority of the cytosolic proteins. In eukaryotic cell, the complete inhibition of proteasome activity induces cell death through necrosis and apoptosis. Although there are evidences of the function of the 20S proteasome alone [77], this protease is often associated to regulatory particles such as 19S, PA28 $\alpha\beta$, PA28 γ , PA200, which affect proteasome conformation and activity [78-81]. Different 20S proteasome isoforms exist. The most common is the standard proteasome, whose catalytic activity is displayed by the Thr₁ of the β 1, β 2 and β 5 subunits. The proteasome in which these subunits are replaced by the β 1i/LMP2, β 2i/MECL1 and β 5i/LMP7 subunits is named immunoproteasome and it is present in immune cells and when cells are exposed to an inflammatory milieu. Between the standard proteasome and immunoproteasome, there are also intermediate-type 20S proteasomes, which carry a different combination of standard- and immuno-subunits [80]. Of note, cells generally contain a mixture of different 20S proteasome isoforms. In 2007,

a novel proteasome isoform has been identified, which is specific for the thymic cortex, *i.e.* the thymoproteasome, which carries the $\beta 1i$, $\beta 2i$ and $\beta 5t$ subunits [3].

The effects of the changes in the catalytic subunit composition of standard proteasome and immunoproteasome have been studied by applying a large variety of methods, whereas for the thymoproteasome the research mainly focused so far on its role to determine the T cell repertoire in mice.

The modifications of the catalytic subunits alter, of course, the catalytic pocket and thus the cleavage preferences but also they induce subtle modifications in the proteasome conformation, which affect proteasome proteolytic dynamics with consequences on protein turnover and on which and/or in which amount peptide products are produced [25, 29, 40, 41, 82-85].

For an overview of proteasome isoform functions, we suggest a few outstanding reviews [1, 80, 86].

Glossary

adoptive T cell therapies (ATTs): is a highly personalized cancer therapy that involves administration of T cells with direct anti-cancer activity to the cancer-bearing host [46].

BRAF: an isoform of RAF. RAF proteins are intermediate to Ras and MAPK in the cellular proliferative pathway. The BRAF V600E is the most common oncogenic mutation of BRAF in cancer. The mutation results in constitutive activation of the BRAF kinase and promotes cell transformation [87].

condensation reaction: in this review's context, is a peptide splicing reaction catalyzed by proteasome, which does not need the initial peptide-bond cleavage (*i.e.* the step 1 in **Fig. 1C**).

cortical thymic epithelial cells (cTECs): antigen presenting cells involved in the positive selection of the thymocytes in the cortical portion of the thymus. In mouse, they express mainly thymoproteasome, which has $\beta 1i$, $\beta 2i$, $\beta 5t$ as catalytic subunits [3].

fibroblast grow factor-5 (FGF-5): a secreted protein that is member of a family of heparin-binding growth factors. It regulates cell proliferation and it is overexpressed by multiple adenocarcinomas, thereby representing a tumor-associated antigen and a potential immunotherapy target.

glycoprotein-100 (gp100): gp100, also known as melanocyte protein PMEL, is a tumor-associated antigen expressed by the majority of malignant melanomas. It has been shown to be immunogenic and elicits an antigen-specific CD8⁺ T cell response. It is currently used in anti-melanoma immunotherapy trials.

KRAS: one of the Ras proteins, which are proto-oncogenes that are frequently mutated in human cancers. HRas, KRas, and NRas are GTPase that function as molecular switches in regulating pathways that are responsible for proliferation and cell survival. The KRAS G12D is a very frequent mutation in colon carcinomas and other cancers; it favors GTP binding and produces constitutive activation of Ras [88].

major histocompatibility complex class I (MHC-I): is the complex that presents epitopes to the TcR of CD8⁺ T cells the epitopes. The recognition of the MHC-I-epitope complex by the TcR of CD8⁺ T cells is the pre-requisite for the priming and/or activation of the CD8⁺ T cells (in presence of other co-factors). The MHC-I complex is constituted of a molecule of $\beta 2$ -microglobulin and the MHC-I heavy chain, and is expressed by almost all cells but erythrocytes.

phosphatidylcholine-preferring phospholipase C (PlcB): *Listeria monocytogenes* is a Gram-positive bacterium that primarily infects phagocytes and then mobilizes the host cell cytoskeleton to spread to neighboring cells. To enter the cytosol of infected cells, the bacteria secrete LLO, the phospholipases PlcA/phosphatidylinositol-specific phospholipase C (PI-PLC) and

PlcB/phosphatidylcholine-preferring phospholipase C (PC-PLC). Bacterial clearance during *L. monocytogenes* infection is mediated by CD8⁺ T cells specific for the secreted bacterial proteins.

tumor-infiltrating CD8⁺ T lymphocytes (TILs): are CD8⁺ T cells present in the tumor. TILs are one of the main source of T cells for ATTs. They can specifically recognize tumor-associated antigens and tumor-specific epitopes.

proteasome-catalyzed peptide splicing (PCPS): the activity of proteasomes whereby two non-contiguous fragments of a polypeptide substrate or a protein are ligated, thereby generating a spliced peptide with a sequence not present in the original protein.

type 1 diabetes mellitus (T1DM): an autoimmune disease mediated by T cells responding to self antigens (e.g. proinsulin and islet amyloid polypeptide) in the pancreatic β cells of islets of Langerhans. Both CD4⁺ and CD8⁺ T cell responses play a central role in the β cell destruction, which leads to insulin deficiency and hyperglycemia.

Outstanding questions

Which biochemical characteristics of proteasome catalytic sites favor PCPS efficiency so much more than expected? How are they altered by proteasome regulators such as 19S or PA28 $\alpha\beta$?

Do tumor cells carry out PCPS as efficiently as lymphoblastoid B cells and primary human fibroblasts?

Does cell infection affect proteasome dynamics and regulate PCPS efficiency? Can proteasome-generated spliced epitopes derived from pathogens be as immunodominant as non-spliced epitopes?

What is the frequency and abundance of proteasome-generated spliced epitopes at the cell surface of cTECs, mTECs and other professional antigen presenting cells involved in the positive/negative selection of thymocytes? How does the immune system cope with the larger antigen landscape generated by PCPS? Is the latter somehow related to the onset / development of any autoimmune response?

Is it worth investing time and resources to identify spliced epitopes and target them through immunotherapies?

Is PCPS relevant only for the MHC-I-restricted antigen presentation or does it have other functions either inside or outside the cell?

Which are the real dimension and immunological relevance of the non-canonical MHC-I and -II immunopeptidomes?

Trends Box

The proteasome does not only cut proteins into fragments through canonical peptide-bond hydrolysis, but also ligates them through proteasome-catalyzed peptide splicing (PCPS).

Through PCPS, the proteasome can significantly shape antigen presentation. This process is estimated to produce about one fourth of the antigenic peptide molecules and to enlarge the antigenic landscape - in term of antigenic peptide variety and antigens represented at the cell surface - by around 30%.

PCPS is not a random process that ligates any fragment produced by proteasome. On the contrary, it seems to be finely tuned by driving forces that have been only preliminary investigated.

Proteasome-generated spliced epitopes derived from tumor-associated antigens activate TILs, which in turn can reduce the tumor mass in patients and animal models.

MHC-I and MHC-II immunopeptidomes contain other non-canonical peptides, which can play a special role in autoimmune diseases such as T1DM

A **B** **C****step 1:** formation of acyl-enzyme intermediate **step 2:** attack by water molecule and release from Thr₁ **step 3:** formation of acyl-enzyme intermediate between Thr₁ and a new substrate residue **step 4:** attack by C-terminal splice-reactant **step 5:** peptide bond formation and release of spliced peptide from Thr₁

20S proteasome subtypes

Active proteasome isoforms