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Review

In search of the cell biology for self- versus non-self- recognition

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Several of today's cancer treatments are based on the immune system's capacity to detect and destroy cells expressing neoantigens on major histocompatibility class-I molecules (MHC-I). Despite this, we still do not know the cell biology behind how antigenic peptide substrates (APSs) for the MHC-I pathway are produced. Indeed, there are few research fields with so many divergent views as the one concerning the source of APSs. This is quite remarkable considering their fundamental role in the immune systems' capacity to detect and destroy virus-infected or transformed cells. A better understanding of the processes generating APSs and how these are regulated will shed light on the evolution of self-recognition and provide new targets for therapeutic intervention. We discuss the search for the elusive source of MHC-I peptides and highlight the cell biology that is still missing to explain how they are synthesised and where they come from.

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Major histocompatibility class-I peptide presentation

Self- versus non-self-recognition is a fundamental aspect of biology and even viruses have mechanisms to select self from non-self. The biology involves many different features and here we will focus on one essential part of self-recognition, which is the presentation of 8–10 amino acid-long peptides on major histocompatibility class-I (MHC-I) molecules and on the cellular mechanisms underlying their production. We will highlight the search for the missing cell biology to explain the origin and selection of peptide substrates for the MHC-I pathway and why this is important, not only to understand the evolutionary origin of this process, but also to improve therapeutic strategies aimed at increasing recognition of harmful cells or the suppression of autoimmune disorders.

The MHC-I coupled with peptides serves to display cell status and to allow the immune system to eliminate infected or transformed cells by circulating cytotoxic T cells [1]. Elegant studies have unravelled how peptide substrates are transported into the endoplasmic reticulum (ER) via the TAP1–2 peptide transporter to be further trimmed by ER aminopeptidases (1/2) to their final size [2–4]. On the ER lumen side, attached to the TAP, is the peptide-loading complex (PLC) that consists of ERp57, tapasin and calreticulin that assist the assembly of MHC class-I molecules to beta(2)-microglobulin for further transport via Golgi to the cell surface [5,6]. While there is little controversy regarding the role of the PLC, there are reports showing alternative TAP-independent mechanisms of peptide entry to the ER [7]. But what about peptide exit from the ER? The ER is highly sensitive to the presence of unfolded or misfolded proteins that can trigger the unfolded protein response. The absolute majority of peptides imported to the ER will not end up being loaded onto MHC molecules and must be rapidly eliminated in order not to destabilise the ER homoeostasis and to allow the continued influx of new peptides. However, if this vast pool of peptides that are not loaded on MHC molecules is exported to the cytoplasm via a retrograde transport system, or if they are further processed to shorter peptides within the ER lumen, is not well known. The TAP transporter has a one-way direction and other peptide transporters with similar properties of channelling shorter peptides with low selectivity out of the ER have not been reported. Unfolded proteins are actively exported out of the ER for cytosolic degradation via the ERAD/Hrd1 pathway that involves ubiquitination and de-ubiquitination [8,9], but if short peptides can use this pathway without the ubiquitination steps is not known. Koopmann et al.

addressed this question over two decades ago and suggested Sec61 as putative candidate, but if this sophisticated import channel indeed is bidirectional and open for shorter peptide of mixed character is yet unclear [10]. Another possibility is COPII vesicles that export secretory proteins and, perhaps, also redundant peptides for the lysosomal pathway and further degradation [11]. However, these putative peptide export pathways have in common a selective process for their respective cargos and one could expect that if TAP is the front door into the ER, the back door should have the characteristics of a similar low selective peptide transporter.

The odyssey of the source of peptides for the major histocompatibility class-I pathway

While unravelling the mechanisms of peptide transport into the ER and the loading onto MHC molecules was taking place, less emphasis was given to the peptide source. It was simply assumed this constituted degradation of 'old' proteins by the proteasomes [12]. This assumption went as far as claiming that antigenic peptide substrates (APSs) were ubiquitinated before degraded and presented to the MHC-I pathway [13]. There were, however, no data supporting this statement and one might have thought that ubiquitination would be a sitting duck for viral immune evasion, simply by avoiding lysine residues in the viral proteins. Indeed, later works showed that ubiquitination and 26S-mediated proteasome degradation of full-length proteins does not provide APSs [14]. Furthermore, deletion of every lysine residue in the chicken ovalbumin (Ova), including the lysine (K > R) in the MHC-I epitope (SIINFE**K**L), had no significant effect on antigen presentation (unpublished) [15]. The vast number of peptides derived from proteasomal degradation of full-length proteins was something of an 'elephant in the room'. How does the immune system stay sensitive to a virus producing small amounts of proteins, or to transformed cells expressing few neoantigens in this vast sea of peptides with a restricted number of class-I molecules? This and the poor correlation between protein amounts, turnover rate and class-I presentation indicated something was not quite right about the assumption that processing of full-length proteins was the source of APSs. This notion was further highlighted by the observation that cytolytic T cells recognise peptides that correspond to an exon–intron sequence [16–18]. There was however, no explanation at hand for the presentation of non-exon-derived peptides and this observation initially sunk without much of a splash. Nevertheless, these different observations prompted the idea that APSs might originate from alternative peptide sources, such as defective ribosomal products (DRiPs) [19]. The DRiPs versus full-length protein discussion livened up antigen presentation meetings for several years [20]. The DRiP model postulated what the source of antigenic peptides *was not* but did not offer a clear idea of what it actually *is*. Defective translation products giving rise to misfolded proteins that constitute up to 30% of all translation events [21]. But a third of translation initiation events not giving rise to the expected full-length protein posed a problem that required an explanation, for which there was none. Nevertheless, the dam was broken and the idea that APSs might not be derived from degradation of full-length proteins was becoming accepted, at least in some quarters. The lively discussions on the source of APSs illustrated that the cell biology that could explain the different observations was missing and thus, making it difficult to test the different models. It should be kept in mind that the requirement for proteasome-mediated processing as an early step in peptide processing was agreed by both camps, but neither model offered an explanation for how proteasome processing of one class of peptide substrates, and not the other, generates antigenic peptides for the MHC-I pathway [22]. This paradox has not been solved by any proposed model before, or since, and remains an interesting issue.

A step forward in understanding the source of peptide substrates came from the observation that a leucine codon (CUG) can be used to initiate translation of an APS from within the 3′ untranslated region of an mRNA [23]. Until then, it was assumed that translation initiation takes place on an AUG codon by a methionine-carrying tRNA. Thus, here was another surprising observation pointing at cell biology for self- versus non-self-recognition for which there was no known underlying molecular mechanism.

With the arrival of immunepeptidome proteomics, it was possible to analyse peptides presented on MHC-I on a larger scale [24]. One early outcome of this was the suggestion that a major source of antigenic peptides is derived from peptide splicing. It had been observed in vitro that during proteasome processing, peptides can reanneal within the proteasome and it was suggested that this process made up for as much as 30% of peptides presented on MHC-I [25]. This was another complete surprise and questions were asked, such as why would the immune system have evolved to use spliced peptide products as a source for fighting viral infection and detect transformed cell? And could it really be so that thymic and peripheral tissues would all process proteins in the same way to generate the same spliced peptides? Do all proteins undergo this process, or is it restricted to some, but not others? This observation raised more questions than it answered and later works showed that the high number was likely based on incomplete bioinformatics analyses that mistook peptides derived from non-coding regions for spliced peptides (see further below) [26].

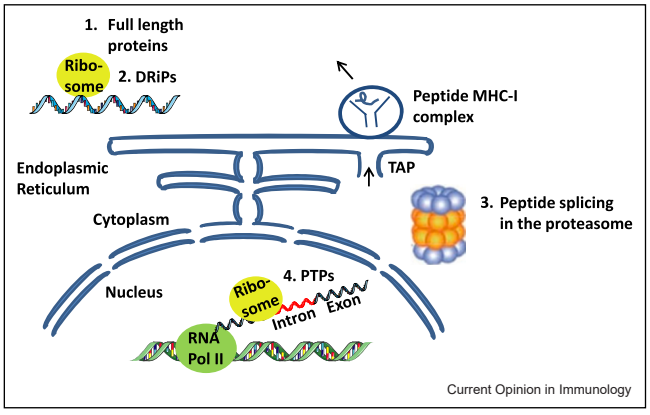
Intron-derived peptides on the immunopeptidome

Apart from the question of numbers, a second issue that models on the origin of antigenic peptide have to address is that the same peptides that are presented for the education of T cells in the thymus must also be presented on cells in the periphery, including professional antigen-presenting cells that prime the CD8+ T cells [27]. Hence, the same mechanism producing APSs in the thymus should also take place in the periphery. This is interesting with regard to the question of how the immune system tolerates alternative tissue-specific mRNA splicing. This question has not been sufficiently addressed but is important as for each gene, there are several splice variants and some of them are expressed in tissue-specific fashion [28]. It should be noted that it is far from clear that all reported splice variants detected by PCR-based techniques are actually translated. Nevertheless, many are and alternative splicing gives rise to mRNAs encoding different proteins and, thus, potentially different APSs. The question is if the thymic cells express all possible splice variants and their respective encoded proteins, or can there be another explanation? Different observations suggested that there indeed might be another possibility (Figure 1).

Inspired by the observation that alternative codons, such as CUGs, can be used to initiate translation of APSs, synonymous mutations were introduced in leucine codons upstream of the SL8 MHC-I epitope in the chicken Ova, or upstream of the MHC-I epitope in the myelin basic protein. Interestingly, mutations in some, but not other leucine codons, had an important effect on the presentation of the respective antigen without affecting the expression of the corresponding full-length proteins [14]. This was seen as a confirmation of the importance of leucine codons in generating antigenic peptides but with the important difference that these leucine codons were within the open-reading frame and not at the +1 initiation site. These observations provided a further indication that an alternative mRNA translation event plays a role in producing APSs. This notion was underlined by the observation that APSs were generated within the first two hours following transfection of the chicken Ova mRNA, while synthesis of the full-length Ova protein continued for at least eight hours. The difference in kinetics of antigenic peptide and full-length protein synthesis implicated a fundamental difference in the mechanism producing peptides for the self-recognition and for full-length proteins [14].

Protein synthesis takes place by a canonical mRNA translation event in the cytoplasm. But it is known that alternative mRNA translation events exist and, for example, that mRNAs are scanned by ribosomes in a quality control process to detect premature termination codons (PTCs) on spliced mRNAs [29]. RNAs found to have PTC are prevented from being translated and are targeted for nonsense-mediated degradation (NMD) [30]. But no peptide products have been attributed to this quality control event under physiological conditions. To test if peptide substrates are derived during the RNA quality control scanning, an antigenic peptide sequence was inserted in different sites in the β-globin gene in the presence, or not, of PTCs. It was quite unexpected that all constructs gave rise to a similar production of APSs, irrespectively, if the antigenic peptide was inserted in, exons, in introns 1 or 2 or in the presence of a PTC [31]. These peptide products were called pioneer translation products (PTPs). Since NMD scanning takes place on spliced mRNAs, these observations indicated that translation also takes place on pre-mRNAs before splicing, implicating that additional mRNA translation events take place before the canonical translation of mature mRNAs. To test the physiological relevance of intron-derived peptides, an animal model was created in which the antigenic peptide sequence was knocked into an intron of the β-Globin gene of the mouse genome. The animals were happy and the splicing of the β-Globin pre-mRNA was not affected by the presence of this extra sequence. Introducing OT-1 CD8+ T cells that are specific for the SL8 epitope showed that the

Figure 1



Proposed sources of peptide substrates for the MHC class-I pathway. All models put forward so far suggest that peptide substrates are processed by proteasomes. Peptides are delivered into the ER via the TAP peptide transporter for further processing and loading on MHC class-I molecules and transport to the cell membrane. It should be noted that TAP-independent antigen presentation is reported. It was originally thought that degradation of full-length proteins (**1**) was the source of antigenic peptides. Later studies showed that 26S proteasome-mediated degradation does not significantly contribute with APSs. As an alternative to full-length proteins, DRiPs (**2**) were suggested to be a major source. Peptides generated by proteasome splicing (**3**) were also proposed to contribute. PTPs derived from translation of pre-mRNAs (**4**) have more recently been demonstrated to generate immune tolerance. PTPs explain the vast amount of intron-derived peptides presented on MHC-I molecules. Since PTPs include both intron- and exon-encoded peptides, the relative contribution of other sources is difficult to estimate.

antigenic peptide was indeed expressed in the animals. Importantly, the animals were tolerant towards this epitope. So, here was an animal model demonstrating that translation of pre-spliced mRNAs produces PTPs that generate immune tolerance [32]. These observations raised the possibility that the reason the immune system tolerates alternative tissue-specific splicing is because the peptide substrates for immune tolerance are derived from translation of pre-spliced mRNAs. Another interesting aspect of these results is that thymic cells do not need to produce all different proteins in order to generate T-cell education, the RNAs are sufficient. This also indicates that the first peptide products derived from newly synthesised mRNAs are destined for the MHC-I pathway, which would make early detection of virus more efficient. On the other hand, one would expect that viruses would have found ways to overcome this early translation event and, indeed, both the Epstein–Barr virus-encoded EBNA1 and the Kaposi sarcoma virus-encoded LANA1 are using cis-acting mechanisms to suppress translation of their own mRNAs in order to evade the immune system [33–35]. It will be interesting to see if other viruses also use a similar strategy.

The uptake of peptides by professional antigen-presenting cells, such as dendritic cells (DCs), and the loading of these peptides on their MHC-I molecules, is critical for T-cell activation and is referred to as cross-presentation [36]. As long as it was thought that antigenic peptides for the MHC-I pathway were derived from the processing of full-length proteins, the issue of substrates for cross-presentation did not pose a problem. But if antigenic peptides for the direct pathway are from another source, including introns, cross-presentation becomes an issue. Hence, the same peptides that are presented via the direct pathway in the infected or transformed cell have to be cross-presented by DCs in order to generate a correct T-cell response. Indeed, PTPs from pre-spliced mRNAs are cross- presented via extracellular vesicles [37]. It is interesting to note that transfection of the chicken Ova mRNA resulted in the same rapid increase in peptides for cross- presentation as it does for the direct pathway but with the important difference that cross-presentation of the APS did not decline after two hours, but remained steady for up to eight hours post transfection [37]. This indicates that the pool of peptides for the direct and for the cross-presentation pathways are separate and that once the peptides are committed to the compartment for cross-presentation, there is no way over to the direct pathway. How this is possible is just another outstanding question that implicates peptide channelling as an important factor in presenting PTPs to the immune system.

What made the observation of intron-derived peptides further interesting was that translation initiation of the SL8 epitope in the β-Globin intron is localised to a short sequence about 270 nts upstream of the peptide sequence that does not include an AUG codon. Ribosome profiling has shown a high frequency of global non-AUG initiation of short reading frames [38]. But there was no physiology role linked to these initiation events or the encoded products and it is possible that it represents the synthesis of APSs.

If peptide splicing raised a few eyebrows, it is nothing compared with translation of pre-spliced mRNAs. And it is not just that there is no cell biology to explain this early translation event, the dogma was that it simply does not happen. It changes a lot, in particular for the splicing and the mRNA translation communities, and is worth a closer look. The β-Globin gene is used as a model for co-transcriptional splicing [39] and little wonder that the observation of translation of the β-Globin pre-mRNA roughened a few feathers. There are reports of intron retention, in particular following treatment with drugs hampering splicing, but this is a rare event under normal conditions and it is unlikely that the large number of intron-derived peptides detected on MHC-I molecules would all come from retained introns [40]. What further speaks against intron retention is the fact that placing the SL8 epitope in either intron 1 or 2 of the β-Globin gene had no significant effect on antigen presentation [31]. Nevertheless, inhibition of the splicing machinery induces a modification of the immunopeptidome and increases the immune recognition of cancer cells in vivo, making it an interesting target for immune therapies [41,42]. To make matters worse is the question of where translation of pre-mRNA takes place. Nuclear translation is considered an absolute heresy, but since pre-spliced mRNAs are not found in the cytoplasm and splicing takes place co-transcriptionally, it has to be considered. The arguments used to support the dogma against nuclear translation include i) maturation of the ribosomal large subunit takes place in the cytoplasm, ii) translation of pre-spliced mRNAs in the nucleus would generate dangerous peptides or iii) translation factors are not found in the nucleus and iv) it is not believed to take place due to lack of data showing what these nuclear translation products are, or their role [43–45]. The latter is a fair argument. In support of nuclear translation are instead experimental data. Labelling the nascent peptide chain with puromycin, followed by translation elongation inhibition, the proximity ligation assay showed a nuclear signal between puromycin and an intron-derived HA tag [31]. Puromycin labelling was also used to visualise nuclear peptides under normal and stress conditions [46,47]. Interestingly, a co-transcription/translation event taking place in eukaryotic cells has been suggested [48]. It should be added that it has been suggested that nuclear peptides have the ability to enter the

cytoplasm and it has been suggested that both chromatin and chaperones can protect peptides from degradation [49,50].

The missing cell biology: questions begging for answers

The odyssey of the origin of antigenic peptides started with the degradation of ubiquitinated full-length proteins to be followed with DRiPs before the discovery that peptide synthesis can be initiated from CUG codons, to later include spliced peptides and more recently PTPs derived from pre-mRNAs. Certainly, the field of antigen presentation does not lack imagination, but every model proposed has its problems. For example, it is important to ask 'why'. Why, for example, would self- versus non-self-recognition evolve around a specific mRNA translation event taking place on pre-mRNAs in the nuclear compartment? One idea is that a basic and primitive translation event based on the ribosome entering directly on the mRNA in a structure-dependent fashion would make it difficult for virus to evade the immune system and would ensure that the regulation of protein expression would not interfere with the immune system. We know that direct internal ribosome entry exists [51] and we will see how/if this is utilised for the production of antigenic peptides from non-AUG codons. Another aspect reflects the 'elephant in the room', that is, the number of peptide substrates. If the synthesis and processing of APSs are kept in a different compartment from that of full-length proteins, the number of potential substrates that can enter the MHC-I pathway could be lower. And then, there is the issue of translation of pre-mRNAs themselves. Is it possible that the splicing community had it wrong and that a translation event takes place before or during splicing? Or is there some other explanation? Are some pre-mRNAs selected for producing APS while others are spliced to produce proteins? There are, however, no reports to suggest the existence of such a selective process.

The production and presentation of APSs for self- versus non-self- recognition are fundamental for our survival and the idea with this short overview is to illustrate that studying how this takes place will continue turning up more surprises and interesting cell biology. One question is what happens with peptides that are not loaded onto MHC-I molecules in the ER. How are they disposed of? Another major question is how is it possible that one pool of proteasome-mediated degradation products ends up in the MHC-I pathway and not the other [52]? Is it possible that synthesis of proteins and APSs taking place in two separate compartments can help answer this question? And does the endogenous MHC-I immunopeptidome correspond to the whole genome? To determine self, only a fraction would be required, as long as they are also presented during T-cell education. Not all thymic cells express the whole self-peptide repertoire suggesting that mechanisms are in place to select certain regions of the genome for presentation to the MHC-I pathway [53]. If so, would cancer cells have a tissue-specific selection for mutations in genes that do not provide peptides? Moreover, what are the underlying molecular mechanisms for translating pre-mRNAs? Are some mRNAs selected for producing APS, whereas others for producing proteins? Does the ribosome translating pre-mRNAs differ from the one producing proteins? As soon as one scratches the surface, one finds questions begging for answers.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

The authors have no conflict of interest.

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