Estimating the Contribution of Proteasomal Spliced Peptides to the HLA-I Ligandome

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Abbreviations: \textit{APPM}: Antigen processing and presentation machinery; \textit{HLA}: Human Leukocyte Antigen; \textit{HLA-Ip}: HLA class I binding peptides; \textit{FDR}: false discovery rate; \textit{FP}: false positive; \textit{AA}: amino acid; \textit{LC}: liquid chromatography; \textit{MS}: mass spectrometry; \textit{MS/MS}: tandem mass spectrometry; \textit{PSPs}: proteasome-spliced peptides; \textit{DeNovo_spliced}: spliced peptides identified by de-novo; \textit{DeNovo_non-spliced}: non-spliced peptides not in UniProt identified by de-novo; \textit{LM_spliced}: spliced peptides identified by Liepe et al.; \textit{LM_UniProt}: UniProt peptides identified by Liepe et al.; \textit{PSM}: peptide spectrum match.

Keywords: de-novo sequencing, HLA, Immunopeptidomics, proteasomal splicing.
Abstract

Spliced peptides are short protein fragments spliced together in the proteasome by peptide bond formation. True estimation of the contribution of proteasome-spliced peptides (PSPs) to the global Human Leukocyte Antigen (HLA) ligandome is critical. A recent study suggested that PSPs contribute up to 30% of the HLA ligandome. We performed a thorough reanalysis of the reported results using multiple computational tools and various validation steps and concluded that only a fraction of the proposed PSPs passes the quality filters. To better estimate the actual number of PSPs, we present an alternative workflow. We performed de-novo sequencing of the HLA-peptide spectra and discarded all de-novo sequences found in the UniProt database. We checked whether the remaining de-novo sequences could match spliced peptides from human proteins. The spliced sequences were appended to the UniProt fasta file, which was searched by two search tools at a FDR of 1%. We find that maximally 2-4% of the HLA ligandome could be explained as spliced protein fragments. The majority of these potential PSPs have good peptide-spectrum match properties and are predicted to bind the respective HLA molecules. However, it remains to be shown how many of these potential PSPs actually originate from proteasomal splicing events.
Introduction

The antigen processing and presentation machinery (APPM) is responsible for the cell surface display of thousands of peptides in the context of the HLA class I (HLA-I) molecules. The proteasome is considered as the main protease that cleaves endogenous proteins. However, in addition to the proteasome, the APPM comprises several other proteases, transporters and chaperones that cooperatively digest the proteins in the cytoplasm, funnel the peptides into the ER, further trim and edit them, load them on newly synthesized HLA-I, and finally direct the stable complexes to the cells' surface [1]. The selective interaction between the HLA-I complex and the peptides is the major factor that defines the presented repertoire and is often represented with binding motifs.

Currently, the only unbiased methodology to comprehensively interrogate the repertoire of the HLA-I binding peptides (HLA-Ip) is based on mass spectrometry (MS). HLA complexes are immunoaffinity-purified from cells in culture or from tissues; the peptides are extracted and subjected to reverse-phase liquid chromatography (LC) coupled online to sensitive MS instruments. The acquired tandem mass spectrometry (MS/MS) data is normally searched against a database of protein sequences. Applying a stringent FDR of 1% using a comparable decoy database leads to the accurate identification of thousands of HLA-Ip. HLA-Ip are mainly 9-11 amino acids (AA) long and usually about 95% of the peptides fit the consensus binding motifs of the HLA expressed in the samples [2].

In a recent MS-based HLA-I ligandomics study a novel computational algorithm has predicted that a surprisingly large fraction, up to 30%, of the ligands may be derived from transpeptidation of two noncontiguous fragments of a parental protein that are spliced together within the proteasome [3]. Earlier work showed several cases of such proteasomal spliced HLA-I peptides that were naturally presented and recognized by cytotoxic T cells [4-9]. Hence, these may be highly interesting therapeutic targets. However, the authors of [3] noticed that unlike the non-spliced peptides, proteasome-spliced peptides (PSPs) had
low HLA binding affinities and produced ambiguous binding motifs compared to normal HLA-Ip. HLA loading takes place after the peptides have exited the proteasome and entered the ER, and hence lost the identity of their creation mechanism. Currently, there is no mechanism or biological process that could explain how the APPM can prioritize loading of HLA-I molecules with low affinity PSPs over high-affinity non-spliced peptides.

Understanding the contribution of PSPs to the HLA ligandome is crucial, especially as they may indeed be highly interesting therapeutic targets in many diseases. Here we critically investigated PSPs reported in Liepe et al. [3] and found that most of spectra attributed to them could be assigned with higher scores to normal peptide sequences within the UniProt database of human proteins. We further describe an alternative computational pipeline to estimate the contribution of spliced peptides to the immunopeptidome. Our results suggest that less than 2-4% of the HLA-Ip may be spliced. As opposed to the spliced peptides reported in [3], these peptides fit well to the relevant HLA binding motifs.

Materials and Methods

HLA ligandomic data

We selected previously published MS HLA-Ip datasets of exceptionally high coverage representing a variety of binding specificities (Supplementary Table 1). MS raw files of HLA-Ip isolated from two melanoma tissues, Mel15 and Mel16 [10], RA957 B cell line [11] and Fibroblast (Fib) cells [2] were downloaded from the PRIDE repository [12] dataset PXD004894, PXD005231 and PXD000394, respectively. One of the four raw MS files of the Fib cells (20130504_EXQ3_MiBa_SA_Fib-2.raw) was also used by Liepe et al. More details about these datasets can be found on PRIDE and the respective manuscripts.

Data processing
If not otherwise mentioned, data were processed with the R statistical scripting language (version 3.3.2) ([https://www.r-project.org/](https://www.r-project.org/)).

**Identification of HLA-ip using PEAKS**

Raw files were analyzed with the de-novo sequencing software PEAKS Studio 8.0 [13]. General parameters were set to “Ion Source”: ESI (nano-spray), “Fragmentation Mode”: high energy CID (y and b ions), “MS Scan Mode” and “MS/MS Scan Mode”: FT-ICR/Orbitrap. The different PEAKS modules were used in the following order with their default parameters while special parameters are indicated in parenthesis: 1. DATA REFINE, 2. DENOVO (“Parent Mass Error Tolerance”: 10 ppm, “Fragment Mass Error Tolerance”: 0.02 Da, “Enzyme”: None), 3. PEAKS (“Parent Mass Error Tolerance”: 10 ppm, “Fragment Mass Error Tolerance”: 0.02 Da, “Variable Modifications”: Oxidation (M) 15.99, “Database”: Homo_sapiens_UP000005640_9606), 4. PEAKS PTM (“Parent Mass Error Tolerance”: 10 ppm, “Fragment Mass Error Tolerance”: 0.02 Da, “Enzyme”: None, “Database”: Homo_sapiens_UP000005640_9606), 5. SPIDER (“Variable Modifications”: Oxidation (M) 15.99, “Fragment ion tolerance”: 0.02). All peptides with \(-10\log P > 15\) were considered having a match with a human protein from UniProt (Homo_sapiens_UP000005640_9606). Peptides with a \(-10\log P \leq 15\) (FDR around 1.5%) and an ALC% $\geq 15$ were considered as “de novo only peptides”. Peptides with mutations and modifications were ignored.

For the subsequent analysis we only kept peptides with a length between 8 and 25 AA and a local confidence score over 80 for every amino acid position. In order to simplify association of peptides with their corresponding HLA alleles, peptides containing PTMs were removed.

**Identification of possible spliced-peptides using TagPep**

We checked whether the filtered list of sequences from the “de-novo only peptides”, which did not match any UniProt sequence, could be spliced fragments from UniProt (Homo_sapiens_UP000005640_9606).
proteins. We applied an in-house software tool TagPep, which uses the index strategy described for fetchGWI [14] adapted for AAs instead of nucleotides. TagPep first matches the whole peptide sequence to the database. If there is no complete hit, it looks for hits allowing for one splicing event, where both spliced fragments are from the same protein (Supplementary Data 1-4). We excluded trans-spliced peptides where the fragments stem from two different proteins for three reasons. First, all known spliced peptides are concatenated fragments from the same protein [15]. Second, the huge number of trans-spliced may lead to strongly increased false positive rates in subsequent bioinformatics analysis, and third, for trans-splicing to happen the two source proteins need to be present in the same proteasome at the same time, which is unlikely to happen on a large scale. The spliced fragments can lie anywhere in the protein, but their sequences cannot overlap. We only considered TagPep matches if the splice gap, i.e. the minimal distance between the two spliced fragments, was less than 20 AA. It has been argued that the splice gap in truly existing spliced peptides is usually lower than 20 AAs [16]. This distance threshold was used in Liepe et al. [3]. We named the resulting set of spliced peptides as DeNovo_spliced. The remaining peptides in the PEAKS “de-novo only” list that are not present in DeNovo_spliced were named DeNovo_non-spliced.

Confirmation of identification of spliced peptides using MaxQuant and Comet

We employed the MaxQuant platform [17] version 1.5.5.1 and the Comet software release 2016.01 [18] to search the peak lists against the UniProt database (Homo_sapiens_UP000005640_9606) and a fasta file containing 247 frequently observed contaminants. For each sample, we added to the fasta file the list of DeNovo_spliced and the DeNovo_non-spliced peptide sequences. For Fib, we also added the 1,154 spliced peptides identified by Liepe et al. [3], which we named LM_spliced. The list of spliced peptides was kindly provided to us by the authors of [3] (Supplementary Data 5, type = “psp”). Peptides with a length between 8 and 25 AA were allowed. MaxQuant parameters: The second peptide identification option in Andromeda
was enabled. The enzyme specificity was set as unspecific. A FDR of 1% was required for peptides and no protein FDR was set. The initial allowed mass deviation of the precursor ion was set to 6 ppm and the maximum fragment mass deviation was set to 20 ppm. Only peptides identified with delta score larger than zero were considered. For the modification search methionine oxidation (15.994915 Da), N-terminal acetylation (42.010565 Da), and glutamine/asparagine deamidation (+0.98402 Da) were set as variable modifications. Comet parameters: activation method: HCD, peptide mass tolerance: 0.02 Da, fragment mass tolerance: 0.02, fragments: b- and y-ions, precursor tolerance, modifications: for the modification search same as MaxQuant plus methylation (14.01565 Da) on K,R,D,E.

**LC-MS/MS analyses and identification of selected synthetic HLA-Ip**

Synthetic peptides (PEPotech Heavy grade 3, Thermo Fisher Scientific) (listed in Supplementary Table 2) corresponding to peptides identified from Fib were mixed and desalted on a C-18 spin column (Harvard Apparatus, 74-4101) and measured at a total amount of 10 and 20 pmol. Synthetic peptides were separated by a nanoflow HPLC (Proxeon Biosystems, Thermo Fisher Scientific, Odense) and coupled on-line to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific, Bremen) with a nanoelectrospray ion source (Proxeon Biosystems). We packed a 20 cm long, 75 μm inner diameter column with ReproSil-Pur C18-AQ 1.9 μm resin (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) in buffer A (0.5% acetic acid). Peptides were eluted with a linear gradient of 2–30% buffer B (80% ACN and 0.5% acetic acid) at a flow rate of 250 nl/min over 90 min. Data was acquired using a data-dependent ‘top 10’ method. We acquired full scan MS spectra at a resolution of 70,000 at 200 m/z with an Auto gain control (AGC) target value of 3e6 ions. Ten most abundant ions were sequentially isolated, activated by Higher-energy collisional dissociation and accumulated to an AGC target value of 1e5 with a maximum injection time of 120 ms. In case of unassigned precursor ion charge states, or charge states of four and above, no fragmentation was performed. The peptide match option was disabled. MS/MS resolution was set to 17,500 at 200 m/z.
Selected ions form fragmentation were dynamically excluded from further selection for 20 seconds. We employed the MaxQuant settings mentioned above for synthetic peptides identification.

**Comparison of MS/MS annotations of endogenous HLA-Ip and their synthetic counterparts**

To investigate if spliced peptides match the MS/MS spectra better than possible alternative sequences we first compared the MS/MS scans identified by Liepe et al. as spliced peptides and those identified by MaxQuant. The mapping to the relevant MS scans and their Mascot ion scores were kindly provided to us by the authors of [3] (Supplementary Data 5). For the Fib data, we selected three MS/MS scans of *LM_spliced* peptides identified both by MaxQuant and by Liepe et al., and 21 MS/MS scans corresponding to *LM_spliced* peptides that MaxQuant identified instead as UniProt peptides. This selection was not biased and was not based on prior knowledge, which would favor MaxQuant. We synthesized the 21 pairs of peptide sequences and the three *LM_spliced* peptide sequences and analyzed them by MS as mentioned above. For visual inspection we printed the endogenous and synthetic spectra to pdf files. For each of the 21 pairs, we calculated the similarity between the spectrum of the eluted peptide from Fib, annotated as *LM_spliced* and the spectrum of the synthetic *LM_spliced* peptide. We also calculated the similarity between the spectrum of the eluted peptide from Fib annotated as a UniProt peptide, and the spectrum of the synthetic Uniprot peptide. Similarly, we calculated the similarity between the three spectra of the identically identified spliced sequences and the spectra of their synthetic counterparts. The similarity was computed by the cosine score (correlation coefficient; value between 0 and 1, where a value of 0 corresponds to spectra with no peaks in common and a value of 1 to identical spectra) [19]. The MzJava class library [20] was used to read the .mgf spectrum files and to calculate the similarity.

**Binding affinity prediction and clustering of peptides**

We used the NetMHCpan [21] to predict binding affinity of 8-14 mer peptides to the respective HLA alleles expressed in the sample and assigned them based on maximum affinity. Hits with a rank <2% were
considered as binders. Gibbscluster-1.1 [22] was run independently for each list of peptides identified from the different samples, with the default settings except that the number of clusters was tested between 1 and 6, a trash cluster was enabled and alignment was disabled [23]. The MixMHCp tool (http://mixmhcp.org/) was used to cluster the peptides with default settings [11, 24].

Results

Predicted spliced peptides from Liepe et al. do not fit well to the consensus binding motifs

Spliced HLA-Ip identified by Liepe et al. in the Fib sample (LM_spliced) were reported to be barely compatible with the corresponding HLA-I binding motifs [3]. First, we tested to what extent the reported 1,154 LM_spliced peptides LM_spliced follow the same binding specificity as the other 2,882 identified HLA-Ip, which matched proteins in UniProt (LM_UniProt, type = “pcp” in Supplementary Data 5). 90% of the LM_UniProt peptides were predicted binders by NetMHCpan compared to only 33% of the LM_spliced peptides (Supplementary Figure 1 A and B). Second, we used the computational tools MixMHCp [11, 24] and GibbsCluster [22] to identify the consensus binding motifs within the lists of 9-mer HLA-Ip in a fully unsupervised way (i.e., without predicting their binding affinity). Four dominant motifs corresponding to the HLA-A and HLA-B allotypes expressed in the Fib cells were identified for the LM_UniProt peptides with both methods, whereas the motifs found in the LM_spliced peptides were much less specific and did not match the known alleles (Figure 1 A). Furthermore, we observed a different length distribution of the LM_spliced and LM_UniProt peptides (Figure 1 B). LM_Uniprot peptides followed the expected peptide length distributions of HLA-I alleles with the majority of peptides of length 9, while LM_spliced peptides unexpectedly displayed the same amount of 9- and 10-mers. Altogether, our results show that expected HLA-Ip characteristics can be clearly recovered from the list of UniProt peptides, but cannot be observed in the spliced peptides.

Spliced peptides from Liepe et al. produce more ambiguous peptide spectrum matches (PSMs)
We checked whether the spliced PSMs reported by Liepe et al. could be explained as matches to UniProt peptides. We added the list of 1,154 \textit{LM\_spliced} peptide sequences to the UniProt fasta file and re-searched the MS/MS data of the Fib raw file used by Liepe et al. using MaxQuant and Comet firstly without variable modifications. Out of the 6,358 (7,523) MS/MS scans identified by MaxQuant (Comet) at an FDR of 1%, 3,300 (3,540) MS/MS scans were also matched formerly by Liepe et al. (Supplementary Table 3). Regarding these common MS/MS, we found very good agreement between MaxQuant and Comet in terms of matches of MS/MS scans to \textit{LM\_UniProt}. Only 1.7% (0.5%) of these scans matched a different peptide sequence in the MaxQuant (Comet) search (Figure 1 C). Since all searches were performed at a spectrum level FDR of 1%, the differences should not be larger than 2% and these values are within this range. However, for the group of common MS/MS scans, which matched to \textit{LM\_spliced} peptides in [3], the disagreement was 76.3% and 63.2% for MaxQuant and Comet, respectively. MaxQuant or Comet matched a UniProt peptide for the majority of these conflicts (98.2% for MaxQuant and 98.3% for Comet) and only in a few cases a different spliced peptide sequences. Overall, MaxQuant could confirm identification of 142 out of the 1,154 (12.3%) spliced peptides found by Liepe et al., whereas Comet could confirm 179 (15.5%). Therefore, the results obtained by Liepe et al., where the spliced peptides scored higher than the competing UniProt peptides, could not be confirmed by this reanalysis. More details about these comparisons can be found in Supplementary Table 3. Interestingly, if we compare results between MaxQuant and Comet, the group of MS/MS scans matched to a peptide in \textit{LM\_spliced} show a highly inflated disagreement (44.4%) compared to MS/MS scans matched to UniProt (1.1%) (Figure 1 C). Therefore, these results do not depend on the particular choice of search tool, but it seems that the PSMs assigned as \textit{LM\_spliced} peptides were more ambiguous compared to UniProt peptides.

To increase the coverage of possible spliced peptides, we searched three additional raw files of HLA-Ip derived from the same Fib cells. In total, MaxQuant identified 202 (17.5 %) and Comet 213 (18.5%) peptides as \textit{LM\_spliced} sequences (Supplementary Table 4). Compared to PSMs assigned as UniProt,
peptides assigned as *LM_spliced* were characterized by lower Andromeda score for the best MS/MS spectrum (Supplementary Figure 2 A), lower Andromeda score difference to the second best identified peptide (Supplementary Figure 2 B), higher absolute precursor mass deviation (Supplementary Figure 2 C), fewer peaks matching to the predicted fragmentation spectrum (Supplementary Figure 2 D), lower fraction of total MS/MS peak intensity matched (Supplementary Figure 2 E) and a larger fraction of singly charged MS/MS spectra matched (Supplementary Figure 2 F). In agreement to our previous analysis employing a single Fib raw file, the combined analysis of four files showed similar results; the group of MS/MS scans matched to a spliced peptide from Liepe et al. showed a highly inflated disagreement (43.1%) compared to the UniProt scans (0.9%) (Supplementary Figure 2 G). Altogether, these results indicate the matches to most *LM_spliced* peptides are of lower quality.

**Spliced peptides from Liepe et al. conflict with modified UniProt peptides**

Next, we tested how the inclusion of variable modifications in the searches influences the identification rates in the different groups. When adding variable modifications to the MaxQuant (deamidation of asparagine and glutamine, oxidation of methionine, acetylation of protein N-term) and Comet (same as MaxQuant plus methylation on lysine, arginine, aspartic and glutamic acid), we observed that the percentage of spliced peptides decreased (Supplementary Figure 3 A, Supplementary Tables 3 and 4). The MS/MS scans previously matched to spliced peptides were now matched to modified peptides in UniProt, mainly oxidations (64.7%) and deamidations (28.5%). Again, more conflicting sequences were found in the spliced peptide group (data not shown). If we restricted the analysis to the high quality PSMs, where both MaxQuant and Comet found the same peptide, the overall contribution of *LM_spliced* peptides shrinks further (Supplementary Figure 3 A) to about 2% of the UniProt peptides.

**Synthetic Peptide Searches**

We selected 21 MS/MS scans from the Fib sample, where Liepe et al. matched a spliced peptide and MaxQuant matched a UniProt peptide. This selection was not biased to favor MaxQuant results, but we
chose spectra that appeared to be typical for the group of spectra that produced conflicting results. For each of the 21 scans we synthesized two peptides: one according to the LM-spliced identification and one for the UniProt alternative. We compared the MS/MS spectra of the synthetic peptides to the spectra of the endogenous peptides. As an example, we show the endogenous and synthetic spectra of the *LM_spliced* peptide DHAQQPYSM (Figure 2 A) and its UniProt competitor peptide DHRSEQSSM (Figure 2 B). All the 21 comparisons can be found in Supplementary Figure 4. Some *LM_spliced* peptides (for examples LENKKGKAL, RVTGALQKK) differ in only two positions from the alternative UniProt peptides (EINKKGKAL, RLSGALQKK), reflecting how similar spliced and UniProt matches can be. We computed the cosine-similarity score between synthetic and endogenous (i.e. original eluted HLA-Ip) spectra. For almost all the 21 cases, synthetic spectra from UniProt peptides fit better to the original MS/MS spectra compared to their *LM_spliced* peptides counterparts (Figure 2 C).

**Alternative pipeline to estimate the contribution of spliced peptides to the HLA-peptidome**

In order to shed more light on the detection of PSPs by MS, we implemented a different computational pipeline (Figure 3 A), which is based on de-novo sequencing [25]. This pipeline proceeds in three steps: 1) de-novo sequencing of MS/MS spectra and removal of de-novo sequences found in UniProt. 2) listing possible spliced and non-spliced sequences computationally identified by an alignment tool (*DeNovo_spliced* and *DeNovo_non-spliced* peptides). 3) adding possible spliced sequences into fasta protein/peptide reference database files and searching them with MaxQuant and Comet at FDR of 1% with variable modifications. The idea behind the last step is to match each MS/MS spectrum to both *DeNovo_spliced* and Uniprot. Importantly, this computational pipeline bypasses the step of matching MS/MS spectra to a huge database of potential spliced peptides. We used the PEAKS tool [13] for de-novo sequencing of the MS/MS spectra from four different immunopeptidomics samples (see Methods for more details). De-novo sequences, that did not match a UniProt sequence and had a local confidence score higher than 80 were further examined. We estimated that this list of *DeNovo_spliced* sequences contains
about 20% incorrect entries (Supplementary Figure 5 A). We can accept this fairly high error rate, since in our computational approach the proposed peptides are subsequently filtered by the consecutive MaxQuant or Comet analysis at FDR of 1%. Comparison for the UniProt sequences showed that PEAKS could identify about half the peptides as compared to MaxQuant at the given thresholds (Supplementary Figure 5 B). This is expected since de-novo sequencing requires searching a large search space of all AA combinations. Furthermore, we observed that PEAKS is not optimized for non-tryptic peptides (see below).

In order to check whether the identified de-novo sequences could be PSPs, we employed an in-house alignment tool called TagPep [14]. TagPep is a very fast alignment tool employing efficient indexing. TagPep first matches the whole de-novo peptide sequence to the protein database and if there is no hit it tries to match it with one splicing event (Supplementary Data 1-4). The number of TagPep matches per de-novo sequence depends on the allowed splice gap between the spliced protein fragments. Having no restrictions on the splice gap, 20% of random amino acid sequences of length 8-11 could be matched as a spliced peptide to the human proteome database. Figure 3 B shows that more than half of the de-novo sequences produce a unique TagPep match with a splice gap of less than 20 AA, whereas 90% of the sequences have three matches or less. Only about 10% of the sequences have more than 3 matches and have many possible explanations.

Next, for each biological sample we created a separate fasta file by adding the list of DeNovo_spliced and the DeNovo_non-spliced peptide sequences to the UniProt fasta files. All MS/MS spectra from an immunopeptidomics sample are subsequently matched against these fasta databases using the sequence search tools MaxQuant and Comet. To test the performance of our pipeline we used previously published immunopeptidomics high quality datasets (Mel15, Mel16, Fib and RA957 samples), which represent a variety of HLA binding specificities (Supplementary Table 1). For the Fib, RA957, Mel15 and Mel16 samples we identified 105 (135,84), 140 (137,83), 546 (550,359) and 99 (146,99) spliced peptides with MaxQuant (Comet, consensus of MaxQuant and Comet), respectively. The MaxQuant-Comet consensus
identifications constitutes 1.7%, 1.1%, 2.1% and 0.7% of the identified UniProt immunopeptidome (Supplementary Table 5). Most of the PSPs found by our pipeline were predicted to bind to the HLA-molecules (Figure 3 C). Moreover, in both the PSPs found by our pipeline as well as the DeNovo_non-spliced, we could see evidences of the expected binding motifs (Supplementary Figure 6), even if the number of peptides is significantly smaller than for the predicted spliced peptides of Liepe [3]. The differences between the binding specificities of DeNovo_spliced, DeNovo_non-spliced and UniProt peptides, as seen in case of the HLA-B27:05 in Mel15, are most likely related to a bias against identification of HLA-B27 peptides in PEAKS (Supplementary Figure 7), which has difficulty identifying such non-tryptic peptides.

Compared to the UniProt peptides, the DeNovo_spliced peptides found by our pipeline have a slightly higher absolute mass error and lower delta score, but they have very similar score and charge distribution. However, compared to the spliced peptides found by Liepe et al, the de-novo spliced peptides have better match characteristics: lower absolute mass error, higher Andromeda scores and delta scores, higher number of matching ions and less singly charged PSMs (Supplementary Figure 2 A-F).

**Sequence variants and spliced peptide conflicts**

Next, we searched for single amino acids variations (SAAV) obtained by exome sequencing of the Mel15 and Mel16 samples [10]. The spliced peptides found by MaxQuant for the Mel15 and Mel 16 samples were added to the fasta file used by Bassani-Sternberg et al., which contained all Ensemble human protein sequences and the sequence variants identified by exome sequencing. Out of the 600 DeNovo_spliced peptides, 17 (2.8%) unique peptides had the same sequence as an endogenous HLA-Ip with a single amino acid variation. Ten of the DeNovo_non-spliced sequences, which were identified by our pipeline, could also be explained by a peptide encompassing such variants. These results highlight the needs to carefully
evaluate spliced peptides identified by MS/MS and make sure that they do not have a different, potentially simpler explanation.

**Characterization of the splicing events**

Lastly, we found that in many *DeNovo_spliced* peptides, identified by both MaxQuant and Comet, the splicing position is at the N- and C-termini (Figure 4 A) in contrast to the *LM_spliced* peptides (Figure 5 C), which have more uniformly distributed splicing positions. The distribution of MaxQuant delta scores as a function of splice position indicates that certain splice positions may on average be more ambiguous than others (Figure 4 B). On the other hand, it could also be possible that single AA abundant in the proteasome or during sample processing could be attached to the peptide termini. This effect is known as transpeptidation and was observed in tryptic samples [26].

**Discussion**

Examples of proteasomal-spliced peptides have been reported and some of them were shown to be immunogenic [5-9, 15, 16, 27, 28]. A true estimation of the contribution of spliced peptides to the global immunopeptidome is critical in order to fundamentally understand the biological pathways involved. Consequently, advanced computational and experimental tools must be developed and benchmarked to facilitate their confident identification.

Liepe et al. [3] were the first to attempt to find PSPs by means of MS on a large scale. Their report concluded that about 30% of the HLA-Ip are produced by proteasomal splicing. Our reanalysis of their results revealed that their approach led to the identification of PSPs candidates that did not fit the consensus binding motifs, while the non-spliced UniProt HLA-Ip identified in the same experiment did. Additional parameters related to their MS/MS spectrum matches suggest that many of the spliced peptide matches reported in Liepe et al. are ambiguous and were ruled out when we used different search engines and included common PTMs or sequence variants in the search. We postulate that because of the huge
search space of potential spliced peptide, the bioinformatics approach applied by Liepe et al. led to uncontrollable propagation of false positives. Therefore, the true contribution of spliced peptides to the immunopeptidome has yet to be defined.

In a typical peptidomics setting, we match MS/MS spectra against a large set of theoretical peptide spectra, most of which are not present in the sample. This endeavor produces two types of PSMs: true matches and false positives. False positives are very common especially for spliced peptides since these peptides can produce similar MS/MS spectra to UniProt peptides with similar match scores. For example, the potential spliced peptide KRI-PLPTKK only differs from its UniProt counterpart RIKPLPTKK by a permutation of the first three AA. When using a very large proteasomal spliced peptide database there is an elevated chance that a potential spliced peptide will have a very similar spectrum to the UniProt peptide and produce a higher match score. Furthermore, if a spectrum has no match in the UniProt database (e.g. when it originates from a modified peptide, sequence variant or contaminant not considered in the search) it may still match a spliced peptide with a score that is significant.

The error in the multiple testing of MS/MS searches is controlled by using decoy database in order to calculate the FDR [29, 30]. One assumption behind this target-decoy approach is that the scores of the decoy peptides reflect the scores of wrongly assigned PSMs. When using decoys for spliced peptides, their similarity with the UniProt sequences may be lost and one would have to carefully evaluate whether the assumption mentioned above still holds. If it does not hold, the target-decoy approach might underestimate the FDR and lead to many false positives especially for large spliced peptide databases.

To overcome biases related to searching all possible splice peptides, we developed an alternative workflow based on de-novo sequencing and subsequent verification with multiple search tools including the most prevalent amino acid modifications and sequence variants detected by exome sequencing. We found that 1-2% of the high quality PSMs originate from potential proteasome spliced peptides. These peptides fitted
the HLA consensus binding motifs and had good spectrum match properties. Given that our de-novo sequencing approach finds about half of the peptides compared to a UniProt sequence search, we can say that the maximal amount of spliced peptide candidates is 2-4%. However, MS/MS based approaches cannot ultimately determine the creation mechanism of these peptides and different sequence interpretations may also be possible. For example, a significant number of detected HLA-Ip originates from transcripts, which do not fall into a UniProt protein coding region [31], and these non-canonical peptides could be misinterpreted as PSPs. Other ambiguities may be due to post-translational or chemical peptide modifications not considered in the search. Therefore, our results present only an upper bound for the proportion of spliced peptides, and the true contribution of PSPs to the HLA-I ligandome may be much smaller.

**Conflict of interest statement**

I.B is an employee of Adicet Bio Israel Ltd. All authors have no financial conflicts of interests.

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Figure legends

Figure 1. Motif deconvolution analysis with MixMHCp and GibbsCluster of the 9-mer LM-UniProt and LM-spliced peptides, and comparison to known logos from IEDB for HLA-A03:01, HLA-A23:01 and HLA-B08:01 (HLA-B15:18 has no experimental ligands in IEDB). Motifs found in LM-UniProt peptides are highly reproducible and comparable to the known motifs from IEDB, while this is not the case for motifs found in LM-spliced peptides. B) Length distribution of the LM-UniProt and LM-spliced peptide. C) Rate of differing PSMs for the peptides in LM-UniProt and LM-spliced. Only MS/MS scans where both search strategies reported a match at FDR of 1% were considered.

Figure 2. A) Example of MS/MS annotation of endogenous HLA-Ip identified as a LM_spliced peptide (DHAQQPYSM), (upper panel) MS/MS of the synthetic counterpart of the LM_spliced (lower panel). B) MS/MS annotation of the same endogenous HLA-Ip as an alternative UniProt peptide (DHRSEQSSM, upper panel), and MS/MS of the synthetic counterpart of the alternative UniProt peptide (lower panel). C) The cosine similarity score calculated for the 21 pairs of MS/MS spectra of LM_spliced peptides and their synthetic counterparts, and the pairs of the alternative sequences from UniProt and their synthetic peptides.

Figure 3. A) Scheme of the de-novo based pipeline to identify possible spliced peptides. B) The splicing gap in relation to the number of TagPep matches. The number of TagPep matches is the number of returned hits with a splicing gap shorter than a given value. Hits with the same splicing position and splicing gap are merged and counted as one hit. C) Distribution of 8-14 mer peptides predicted by NetMHCpan as binders and non-binders among the DeNovo_spliced, DeNovo_non-spliced and UniProt peptides identified in Mel16, Mel15, RA957 and Fib samples, and in addition also LM_spliced peptides.

Figure 4. A) Histogram of splicing positions within 9-mers for the DeNovo_spliced peptides identified by both Comet and MaxQuant. B) MaxQuant delta scores as a function of the splicing position within the – 9-
mers for the DeNovo_spliced peptides identified by both Comet and MaxQuant. C) Histogram of LM_spliced peptides splicing positions.
A.

| HLA-A03:01 | HLA-A23:01 | HLA-B08:01 | HLA-B15:18 |
|------------|------------|------------|------------|
| MixMHCp    | 856        | 357        | 161        | 471        |
| LM-UniProt | 836        | 352        | 175        | 450        |
| Gibbs-Cluster | 4,011    | 612        | 2,281      |            |
| IEDB       |            |            |            |            |
| MixMHCp    | 116        | 116        | 123        | 94         |
| LM-spliced |            |            |            |            |
| Gibbs-Cluster | 117      | 170        | 88         | 56         |

B.

Length distribution

Peptide length

LM-UniProt (n=2,282) vs LM-spliced (n=1,154)

C.

Different Peptide Rate

MaxQuant & Mascot: 0.763
Comet & Mascot: 0.832
Comet & MaxQuant: 0.444
Figure 2.

A

B

C

Cosine Similarity UniProt

Cosine Similarity LM_spliced

Cosine Similarity UniProt

0.0 0.2 0.4 0.6 0.8 1.0

0.0 0.2 0.4 0.6 0.8 1.0
Figure 3.

A

1. **PEAKS De novo**
   - **Input:** Raw MS files HLA-p
   - **Parameters:** Reference = UniProt
     - Score > 80
     - No modification
   - **Output:** DeNovo peptides not in UniProt

2. **TagPep**
   - **Input:** DeNovo peptides not in UniProt
   - **Parameters:** Reference = UniProt
     - Splicing gap < 25 AA
     - No trans-splicing
   - **Output:** Candidate sequences of DeNovo_spliced and DeNovo_non-spliced peptides

3. **MaxQuant & Comet**
   - **Input:** Raw MS files HLA-p
   - **Parameters:** Reference = UniProt + DeNovo_spliced + DeNovo_non-spliced sequences
     - FDR 1%
   - **Output:** DeNovo_spliced and DeNovo_non-spliced peptides

B

- Number of TagPep Matches vs Splice Gap

C

- Bar charts for Mel16, Mel15, RA957, and Fib
  - binders
  - non-binders
Figure 4.

A. DeNovo_spliced Peptides after MQ and Comet searches, no modifications

B. Peptides of Length 9

C. LM_spliced

Counts

Position

Counts

MaxQuant DeltaScore

Counts

Position