Thunder-DDA-PASEF enables high-coverage immunopeptidomics and identifies HLA class-I presented SarsCov-2 spike protein epitopes

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Thunder-DDA-PASEF enables high-coverage immunopeptidomics and identifies HLA class-I presented SarsCov-2 spike protein epitopes

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Abstract

Human leukocyte antigen (HLA) class I peptide ligands (HLA\textsuperscript{ps}) are key targets for developing vaccines and immunotherapies against infectious pathogens or cancer cells. Identifying HLA\textsuperscript{ps} is challenging due to their high diversity, low abundance, and patient-specific profiles. Here, we developed a highly sensitive method for identifying HLA\textsuperscript{ps} using liquid chromatography-ion mobility-tandem mass spectrometry (LC-IMS-MS/MS). The optimized method, Thunder-DDA-PASEF, semi-selectively fragments HLA\textsuperscript{ps} based on their IMS and m/z, thus increasing the coverage of immunopeptidomics analyses. Thunder-DDA-PASEF includes singly-charged peptides, which contributes to more than 35\% of the HLA\textsuperscript{p} identifications. Combined with MS\textsuperscript{2}Rescore, Thunder-DDA-PASEF improved ligandome coverage by 150\% compared to the original-DDA-PASEF method, and enabled in-depth profiling of HLA\textsuperscript{ps} from two human cell lines, JY and Raji, transfected to express the SARS-CoV-2 spike protein. We identified seventeen spike protein HLA\textsuperscript{ps}, thirteen of which had been reported to elicit immune responses in human patients.
Identifying ligands of the major histocompatibility complex (MHC) or human leukocyte antigen (HLA), also called immunopeptides, is key for developing vaccines and immunotherapies (extensively reviewed in [1, 2, 3]). Human HLA class-I complexes bind peptides (HLAIps) of typically 9 to 12 amino acids generated by a multi-step process called antigen processing, which involves multiple proteolytic events by the proteasome and aminopeptidases [4, 5, 6, 7, 8]. Loaded HLA complexes are then displayed on the cell surface, where CD8+ T-cells scrutinize them. Detection of a "non-self" antigen, e.g., HLAIps derived from viral proteins or mutated cancer-related proteins, leads to the efficient elimination of the presenting cell by cytotoxic T lymphocytes. Thus, non-self HLAIps constitute key targets for developing peptide or mRNA vaccines in the context of personalized immunotherapies, or diagnostic tools. Various in silico tools have been developed to predict HLA-binding peptides from genomic, transcriptomic, or riboSeq data. Still, most predictors are primarily based on HLA binding affinity, thus not fully considering the antigen processing and presentation mechanisms, resulting in discrepancies between predicted and presented HLAIps [9, 10]. Therefore, liquid chromatography mass spectrometry (LC-MS)-based immunopeptidomics is essential for directly identifying HLA class I presented peptides from cells, tissues, and biofluids [9, 11].

LC-MS immunopeptidomics faces different challenges than bottom-up proteomics, where proteins are usually digested using trypsin (reviewed in [3, 12]). HLAIps are generated by a complex multi-step process, including various proteolytic events [13, 14]. This results in peptides with restricted size and sequence patterns imprinted by the specificities of TAP transport and HLA binding. While these motifs differ between individual HLA alleles, they restrict the sequence space presented by a single allele. Thus, immunopeptidomics samples are more likely to contain isobaric peptides, potentially co-eluting from the LC, than enzyme-digested samples [2]. Since tryptic peptides are usually multi-charged, typical bottom-up proteomics workflows often omit the fragmentation and identification of singly-charged ions, which are more challenging to identify. In addition, singly-charged peptides are often masked by chemical noise, and their fragmentation generates many uncharged segments not detected by the MS [2]. Moreover, individual HLAIps are low abundant, and the sample preparation recovery yields are low (around 0.5-3% [15]). These factors demand tailored and high-sensitivity LC-MS methods and have major implications in database searches. The unspecific cleavage of HLAIps increases the search space by up to 2 orders of magnitude compared to tryptic digests. This impairs the discrimination of false positive from true positive peptide-spectrum matches (PSMs), negatively impacting peptide identification yield and confidence [16].

Coupling ion mobility separation (IMS) to LC-MS provides an extra dimension of separation, resolving ions in the gas phase by their size and shape. This enhances the signal-to-noise ratio and resolves isobaric ions, thus increasing the number and confidence of peptide identifications. In the timsTOF Pro instruments, a dual trapped ion mobility spectrometry (TIMS) analyzer is employed to perform a parallel accumulation–serial fragmentation (PASEF) of ions, resulting in a high sequencing speed without compromising sensitivity for data-dependent
acquisition (DDA-P ASEF) [17, 18], which has already been proven to perform well for immunopeptidomics [19].

During the ongoing Covid-19 pandemic, there have been significant efforts to identify SARS-CoV-2 HLAIps, mainly focusing on characterizing the immunogenicity in vitro or in vivo of large libraries of synthetic peptides of in silico predicted HLA-binders (25 studies reviewed in [20]). This has provided important insights into possible immunodominant regions in the viral proteome, HLA allele-dependent responses to SARS-CoV-2, and the protection capabilities of vaccines (reviewed in [20, 21, 22]). More than 2,000 possible HLA-binding peptides have been predicted from the SARS-CoV-2 genome [23]. However, only a few SARS-CoV-2 immunopeptides have been detected by LC-MS until now [24, 25, 26], including less than ten HLAIps for the spike glycoprotein [24, 26], the main target of vaccines and diagnostic tests. This emphasizes the challenges of LC-MS immunopeptidomics and the need for more sensitive and robust methods.

Here, we present Thunder-DDA-P ASEF, an optimized LC-IMS-MS method for immunopeptidomics and its application in the discovery of SARS-CoV-2 spike protein derived HLAIps. The optimized method uses an extended TIMS separation time (300 ms) to improve IMS resolution, and sensitivity [17, 27]. To include singly charged peptides while efficiently using instrument cycle time, precursors are selected by using a tailored isolation polygon for semi-selectively fragmenting potential HLAIps. Compared to the standard method (100 ms TIMS, bottom-up proteomics-optimized isolation polygon), Thunder-DDA-P ASEF increased the HLAIps identifications from JY cells by 2.3-fold, including more than 35% of identifications derived form singly-charged. Moreover, MS²Rescore-based rescoring [16] further boosted the identification to 3.5-fold relative to the non-rescored standard DDA-P ASEF. Subsequently, we employed Thunder-DDA-P ASEF to study the HLAIp ligandome repertoire of two cell lines recombinantly expressing the canonical spike protein of SARS-CoV-2. This resulted in deep coverage of 14,313 and 17,806 peptides from JY and Raji cells, respectively, including seventeen HLAIps derived from the SARS-COV-2 spike protein. Notably, thirteen of these peptides have been previously reported to elicit immune responses in human patients, confirming the potential of our improved method for efficient epitope discovery. In conclusion, optimized Thunder-DDA-P ASEF achieved deep and reproducible profiling of the HLA class I ligandome.

## 2 Results

### 2.1 General workflow for LC-IMS-MS immunopeptidomics

For our immunopeptidomics experiments, we followed the general procedure shown in Fig. 1 and described in Material and Methods. The settings used for the LC-MS methods and data processing are fully detailed in Supplementary Material S2a and the ready-to-use MS method for timsTOF Pro instruments is included in Supplementary Material S2b. Briefly, we enriched HLAIps from JY cells by immunoprecipitation (W6/32 antibody), and analyzed them by nanoLC-IMS-MS on a nanoElute coupled to timsTOF-Pro-2 in DDA-PASEF mode, using PEAKS XPro for subsequent peptide identification. We performed several iterations to optimize
our LC-IMS-MS method for identifying HLA class I ligands, as described in the following sections.

2.2 An HLA\textsuperscript{p}-tailored DDA-PASEF fragmentation scheme including singly-charged ions efficiently identified possible HLAIps

Contrarily to tryptic peptides, HLAIps originate from a large diversity of antigen processing events \cite{13, 14} and do not necessarily contain basic amino acid residues \cite{2}. Thus, many HLAIps can only be detected as singly-charged ions in LC-MS since only their N-ter residue can carry a positive charge (H\textsuperscript{+}). Although this varies depending on the HLA alleles, up to 40\% of singly-charged ions have been reported for peptides bearing hydrophobic anchor residues such as HLA-B07:02 \cite{28, 29}. In addition, HLAIps have a restricted size of typically 9 to 12 amino acids (AAs) \cite{2}, but between 8 to 13 in some instances \cite{30, 31}. For this reason, HLAIp-immunopeptidomics workflows have recently incorporated the fragmentation of singly-charged ions (with 2\textsuperscript{+} and 3\textsuperscript{+}) within the m/z range of possible HLAIps \cite{2, 19, 28, 32, 33, 34, 35, 36}. We hypothesized that the IMS separation and sensitivity of the timsTOF Pro-2 could provide high-quality MS\textsuperscript{2} spectra to identify singly-charged peptides confidently.

First, we tested the original-DDA-PASEF method for proteomics \cite{17} to analyze JY HLAIps samples (Fig. 2a, d, g). DDA-PASEF takes advantage of the charge-state-dependent mobility separation to selectively fragment ions detected within an isolation polygon on the inverse reduced ion mobility (1/K\textsubscript{0}) vs. m/z space. Since it was designed for tryptic peptides, the standard isolation polygon covers the multiply-charged ion cloud, clearly separated from the singly-charged ones (Fig. 2a). This resulted in almost 5,000 unique peptides from three injection replicates of JY HLAIps (Fig. 2a), mainly comprising doubly-charged ions (89\%, Fig. 2b) and almost 77\% of 8-13-mers (Fig. 2g, j). As expected, most singly-charged ions were excluded from fragmentation, and only a few were identified due to IMS peak tailing into the isolation polygon.

Our next step was to remove the isolation polygon (Fig. 2b). Omitting the isolation polygon enabled the fragmentation of singly-charged peptides, representing more than half (54.5\%) of all the peptides identified and 59.6\% of the 8-13-mers (Fig. 2i, h). Furthermore, the proportion of peptides with 8 to 13 AAs was 12.4\% higher than in the standard-polygon (Fig. 2j), corresponding to 72\% more 8-13-mers identified on average (p ≤ 0.0001, Fig. 2n). However, without an isolation polygon, many low m/z singly-charged ions and high mass multiply-charged ions were fragmented (Fig. 2b).

Therefore, we designed fragmentation isolation polygons covering the singly-charged and multiply-charged 8-13-mer peptides \cite{2}, h) (Table 1). This HLAIp-tailored scheme efficiently identified peptides within the isolation polygon (Fig. 2c, f, i), roughly maintaining the charge distribution of peptides identified, with 56.4\% of all the ions and 59.7\% of the 8-13-mers being singly-charged. The proportion of 8-13 mers was almost 92\%, which is 15\% and 2.6\% higher than the standard- and no-polygon, respectively (Fig. 2j, k, respectively). As a result, the HLAIp-tailored polygon increased the identification of 8-13-mers by 75\% relative to the standard (p ≤ 0.0001, 2n). Compared to no polygon, the HLAIp-tailored polygon resulted in 24\% fewer MS\textsuperscript{2} scans.
(p ≤ 0.001), but a similar yield of 8-13-mers identified (Fig. 2a). This 18% increase in the identification rate shows that the HLAIp-tailored polygon used the cycle time more efficiently to fragment 8-13-mers. In contrast, without an isolation polygon, a large proportion of the cycle time was used inefficiently to fragment ions that are not of interest for HLAIp profiling. These may include non-peptidic small ions or larger peptides (Fig. 2A, Supplementary Fig. S1a, b) originating from the degradation of HLA proteins, the antibody, or other co-enriched proteins (Supplementary Fig. S1c). Once having established the capabilities of DDA-PASEF with the HLAIp-tailored isolation scheme for immunopeptidomics, we optimized several other parameters of the MS method (detailed in Supplementary Material S2).

2.3 Optimized Thunder-DDA-PASEF enhanced the identification of 8-13-mers by 2.2-fold

In PASEF methods, each analysis cycle comprises several frames where the trapping TIMS tunnel accumulates a package of ions. Simultaneously, the second TIMS resolves the previous package of ions by ramping down the elution voltage. Increasing TIMS times enhances IMS resolution and accommodates more fragmentation events per MS2 frame while preserving the sensitivity [17]. Raising the TIMS time from 100 to 300 ms resulted in an 80% increase in peptide identification, while no substantial increase was observed between 400 ms and 300 (< 5% increase) (Supplementary Fig. S2a, b, c, d). However, the longer cycle times resulted in five-fold fewer MS1 frames and doubled the median coefficient of variation (CV) at 400 ms compared to 100 ms. Since the peak area reproducibility is essential for quantitative comparisons between samples (e.g., diseased vs. control), we compensated for this effect by decreasing the number of MS2 frames/cycle from 10 to 3, and the MS2 cycle overlap from 4 to 1 (Fig. S2e, f, g, h). This resulted in a cycle time of 1.2 s and reduced the median peak area CV from 19.3% to 10.3% (Fig. S2d,h). In addition, activating the high-sensitivity mode of the timsTOF Pro-2, which uses detector voltages optimized for low sample amounts, further increased the number of HLAIps identified by 30% (Supplementary Fig. S3).

In summary, the optimized method resolves ions using a 300 ms TIMS ramp, fragmenting mainly ions with $1^+$, $2^+$, and $3^+$ charges in 3 MS2 frames per MS1 frame within a 1.2 s cycle time and takes advantage of the high-sensitivity mode. Since the HLAIp-tailored isolation polygon resembles a lighting or thunder icon, we termed the fully optimized method Thunder-DDA-PASEF. In contrast, the original-DDA-PASEF designed for proteomics samples uses 100 ms ramps and selectively fragments multiply-charged ions in 10 MS2 frames per MS1 frame within a 1.2 s cycle time.

We compared Thunder-DDA-PASEF to the original-DDA-PASEF method by analyzing triplicate injections of JY HLAIps (equivalent to approximately 50 million cells/injection, Supplementary Material S3). Thunder-DDA-PASEF identified 2.2-fold the number of 8-13-mers than the original method ($p < 0.0001$, Fig. 3a). This was partly due to the inclusion of singly-charged peptides in Thunder-DDA-PASEF, constituting 48% of the 8-13-mers in this data set (Fig. 3a). Thunder-DDA-PASEF improved the dynamic range for identification...
by almost half an order of magnitude towards the low abundant species (Fig. 3). The number of peptides identified across all three replicates was 8.4% higher in Thunder-DDA-PASEF than in the original-DDA-PASEF, indicating a slight improvement in the data completeness (Fig. 3). Although 8.7% of the peptides were only identified in the original method (Fig. 3), this could be due to the sampling stochasticity of DDA. Then, we used NetMHCpan-4.1 via MhcVizPipe to predict peptide HLA-binding, which provides a ranking classifying the peptides into strong-binders (SB, rank ≤ 0.5%), weak-binders (WB, 0.5% < rank ≤ 2%) or non-binders (NB, rank > 2%). When focusing on the peptides predicted to bind JY HLA alleles, the 8-13-mers identified comprised 88.2% SB and 7.8% WB in the original method and 85.4% SB and 9.1% WB in Thunder-DDA-PASEF (Fig. 3, Supplementary Material S4). Altogether, these results proved a 2.2-fold increase in the coverage of the immunopeptidome using Thunder-DDA-PASEF compared to the original-DDA-PASEF (9,524 and 4,334 HLAIps, respectively).

2.4 Machine learning-based rescoring via MS²Rescore enhanced the identification of HLAIps and data completeness by more than 15%

Several post-processing tools have shown improvements in immunopeptide identification by rescoring peptide spectrum matches (PSMs) based on characteristics disregarded in the initial search. For instance, MS²Rescore (MS2R) integrates the machine learning prediction of retention and fragmentation peak intensity using DeepLC and MS²PIP, respectively, with the semi-supervised machine learning-based FDR calculation of Percolator. Since this strategy has shown the potential to boost immunopeptide identification, we decided to implement it in our workflow.

Rescoring the results of Thunder-DDA-PASEF from JY IP-enriched HLAIps (Supplementary Material S3) significantly increased the average number of 8-13-mer peptides identified per injection by 29.1% (p < 0.0001, Fig. 3a). The proportion of singly-charged peptides decreased (Fig. 3b) not due to a drop in their numbers but because most newly identified peptides were doubly charged (74.5%). Probably, the performance of MS²Rescore for singly-charged ions was lower due to the fewer singly-charged ions in the MS²PIP immunopeptidomics model training set. Thus, training a predictor model with orthogonal Thunder-DDA-PASEF data could improve its performance.

Novel identifications were obtained across the whole dynamic range indicating that rescoring performed well even for low-intensity ions (Fig. 3). Despite applying a stringent confidence filter independently for each file (PSM FDR ≤ 0.01), 77.1% of the peptides were consistently identified across all three replicates in the rescoring results, meaning a 16.7% increase in data completeness (Fig. 3). In addition, only a few peptide identifications were dropped by MS²Rescore (< 1.5%, Fig. 3), and it also recovered 263 peptides identified in the non-rescored original-DDA-PASEF but not in Thunder. The proportion of SB and WB was not affected by rescoring, indicating that no bias was introduced. The benefits of rescoring Thunder-DDA-PASEF identifications are summarized in a 14.7% increase in the number of predicted HLAIps identified, yielding a total
of 10,931 (Fig. 3f). Collectively, the Thunder-DDA-PASEF + MS2R strategy resulted in a 2.5-fold coverage of HLAIps compared to the non-rescored original-DDA-PASEF data for JY HLAIp IP-enriched peptides (Fig. 3, Supplementary Material S4), with an average of 9,821 HLAIps per injection.

In summary, combining the optimized Thunder-DDA-PASEF with MS²Rescore resulted in a highly sensitive and reproducible workflow. This level of coverage could enable deep profiling of immunopeptides in patient samples and the comparability between healthy and pathological tissue for the discovery of disease-specific antigens.

2.5 Thunder-DDA-PASEF enabled in-depth characterization of the HLA class I ligandome of JY and Raji cells

We tested our optimized workflow to characterize the HLA class-I immunopeptidome of JY and Raji cells transfected to express a segment of the SARS-CoV-2 spike protein (Supplementary Material S5). Thunder-DDA-PASEF + MS2R identified in total 23,147 peptides from JY and 29,397 peptides from Raji, comprising 78% of 8-13-mers, with a median length of 9 AAs (Fig. 4a), as expected for HLAIps. The reproducibility between biological replicates ranged between 35.8% and 62.7% 8-13-mers identified in all the samples of the same genotype, and 67.7% to 81.3% regarding the proteins covered (Supplementary Fig. S4). Based on the HLA-binding prediction (NetMHCpan-4.1 via MhcVizPipe [38], Supplementary Material S6), the 8-13-mers included 78.9% binders for JY (70% SB, 8.9% WB) and 77.6% for Raji (67.2% SB, 10.4% WB) (Fig. 4b), showing the respective peptide sequence motifs, as indicated by supervised clustering (GibbsCluster-2.0 [46], Fig. 4g, h). A lower proportion of HLAIps was detected as singly-charged ions in Raji, compared to JY (30.1% vs. 42.9%). This was due to the presence of basic amino acids at the anchor positions for Raji HLA alleles (Fig. 4k), including lysine or arginine at the C-ter (HLA-A03:01) or histidine at the second position (HLA-B15:10, HLA-C04:01). In contrast, the anchor residues binding JY HLA alleles were dominated by apolar amino acids (Fig. 4l).

Thunder-DDA-PASEF achieved an extensive coverage of protein-HLAIp representation. A total of 14,074 and 17,469 HLAIps were detected in JY and Raji, respectively, summing up to 30,948 peptides (Fig. 4l, top). These peptides corresponded to 5,660 protein groups in JY, 6,170 in Raji, and 8,214 in total (Fig. 4l, bottom). Each protein group was represented by a median of 2 HLAIps per protein group and 75% of them with one to three peptides for both cell lines (Fig. 4l). As a comparison, the DIA analysis of JY HLAIps provided a median of one HLAIp per protein [32] despite a deep coverage of 7,627 peptides. This further shows the potential of our workflow to provide an in-depth characterization of the immunopeptidome, which may unravel novel antigen processing and presentation mechanisms.

Although only 1.8% of all HLAIps were detected in both JY and Raji, 44% of all the protein groups were covered by the ligandomes of the two cell lines (Fig. 4, top and bottom, respectively). A gene ontology (GO) enrichment analysis using GOrilla [47] indicated a significant over-representation ($FDR \leq 0.001$) of proteins
involved in essential processes, such as the metabolism of nucleic acids (GO:0090304), macromolecule biosynthesis (GO:0034645), macromolecule localization (GO:0033036), and regulation of the cell cycle (GO:0022402) (Supplementary Material S7 and S8). Thus, the cell lines presented complementary peptides for these same crucial proteins due to their different HLA alleles and probably also due to differences in the antigen processing pathway. Because of the large number of HLAIps covered (30,984 binders) (Fig. 4b, d), including more than 11,000 singly-charged peptides (Supplementary Material S3), this combined immunopeptidome of JY and Raji cells constitutes an essential resource for future exploitation.

2.6 Thunder-DDA-PASEF identified seventeen spike HLAIps in JY and Raji spike-transfected cells

To explore the potential of Thunder-DDA-PASEF on a clinically relevant subject, we focused on the transfected SARS-CoV-2 spike protein, and the GFP reporter included in the construct. Importantly, peptides from these proteins were only detected in the transfected cells and not in the wild-type cells. Three GFP-derived HLAIps were identified in JY and six in Raji cells (Fig. 5b), serving as a control for successful antigen processing of the transfected constructs. Five spike HLAIps were identified in JY and thirteen in Raji (Fig. 5a) across a large dynamic range corresponding to four orders of magnitude (Fig. 5c). While the Raji spike HLAIps were distributed across the whole dynamic range, they were mainly in JY’s middle to low range. The sequence and characteristics of the spike HLAIps are shown in Fig. 5d and detailed in the Supplementary material S9. Nomenclature in Fig. 5: and d denotes identified spike HLAIps (e.g., SIIAYTMLs0691-0699) both by peptide sequence and position (N- to C-ter) in the full-length spike protein. Notably, six of the thirteen spike HLAIps were singly charged, showing the advantage of the Thunder HLAIp-tailored isolation polygon for identifying potential clinically relevant immunopeptides.

In addition to the 1% FDR threshold applied, the spike HLAIps were assessed based on the number of identifications across biological and technical replicates (n BR, n TR; Fig. 5l, yellow to green scales) and by the similarity of their fragmentation spectra against synthetic peptides or in silico predictions, based on the Pearson correlation coefficient (PCC) [48] (Fig. 5l, blue scale with letters, S = synthetic, P = predicted). The mirrored spectra comparisons are shown in Supplementary Material S10. At the same time, SIIAYTMLs0691-0699 and TLKSFTVEKs0302-0310 are shown in Fig. 6 as examples of the confident identification of peptides with high and low abundance, respectively. Around 82% of the reported spike HLAIps were identified in two biological replicates with a PCC >= 0.85, indicating both robust sample preparation and high-confidence identifications. The synthetic peptides analyzed independently with the same method were eluted at similar indexed retention times (iRT) as the corresponding endogenous peptides (ratio iRT endogenous/synthetic >= 0.99). Even though peptides GVLTESNKS0550-0558 from Raji and RLQSLQTYV1000-1008 from JY were identified in only one injection replicate in one of the biological replicates, their PCC were 0.94 and 0.96, respectively (FDR < 0.005).

Peptide AIHVSGTNGTKS0067-0077 showed a low PCC (0.47) against the predicted spectra but was detected in
five injection replicates across both biological replicates with an FDR < 0.0005, thus validating its detection. While a large proportion of the HLAIps were predicted to be strong binders (Fig. 5), there was a deficient number of HLAIps for both the HLA-C alleles in Raji (HLA-C04:01) and JY (HLA-C07:02). This could be due to the low expression of this gene in JY cells, whose effect on its immunopeptidome has been previously reported. Interestingly, some spike HLAIps were predicted to bind to the HLA alleles of both cell lines, but only SIIAYTMSLs0691-0699 was identified in both cell lines. Once more, this highlights the need for direct validation of in silico-predicted HLA class I binders. However, the challenge of LC-MS immunopeptidomics is exemplified here since only one of the seventeen spike HLAIps had been previously reported by MS (SIIAYTMSLs0691-0699) [40]. Moreover, four represent completely novel identifications (AIHVSGTNGTKs0067-0077, YGVSPTKLS0380-0387, RVYSTGSNVFQTRs0634-0646, NRALTGIAVs0764-0772). The remaining thirteen spike HLAIps have been reported to exhibit positive results in T-cell or MHC ligand assays according to the IEDB [50] (December 18, 2022) (Fig. 5, dot range plot). This shows the capabilities of Thunder-DDA-PASEF for identifying potential HLA class I-restricted immunogenic targets which could be employed for vaccine development.

In summary, we report seventeen spike peptides identified with high stringency and confidence, which are predicted to bind HLA class I in two cell lines expressing different HLA alleles. Accordingly, this set of peptides constitutes a key resource, comprising novel spike HLAIps, and confirms many previously reported peptides capable of eliciting a T-cell response.

3 Discussion

Here, we present Thunder-DDA-PASEF, an LC-IMS-MS method tailored and optimized for identifying HLA class I peptide ligands (HLAIps). We showed that the HLAIp-tailored isolation polygon enabled the identification of singly-charged peptides, expanding the universe of identifiable MHC peptide ligands. Thunder-DDA-PASEF uses a thunder-shaped isolation polygon (Fig. 2), optimized detector voltages (high sensitivity mode), enhanced IMS resolution (300 ms TIMS), and fewer MS2 frames (3 MS2 frames/cycle, 1 cycle overlap), resulting a cycle time of 1.2 s, compatible with nanoLC peak width (Supplementary Fig. S2h, Supplementary Material S2a and S2b). Altogether, this resulted in more than a 2.2-fold higher number of 8-13-mers identified from JY cells, compared to the standard DDA-PASEF optimized for proteomics samples (excluding singly-charged ions, 100 ms TIMS ramp, 10 MS2 frames/cycle, 4 overlap) (Fig. 3). MS2Rescore further boosted the identifications up to 2.5-fold compared to the standard, unrescored DDA-PASEF. In addition, Thunder + MS2Rescore improved the identification data completeness, reliably and constantly identifying 77.1% of the peptides across three technical replicates (Fig. 3).

Field asymmetric waveform ion mobility spectrometry (FAIMS) has been combined with LC-MS to identify singly-charged HLAIps [29]. However, FAIMS acts as a gas-phase fractionation device, filtering ions in function of their mobility in the electric field. Since only a population of ions can be analyzed simultaneously, identifying
multiply and singly-charged HLAIps requires dividing the cycle time within an LC-MS run between or perform-
ing multiple injections per sample [29]. In contrast, TIMS-MS profiles ions across a $1/K_0$ range. In addition,
PASEF maximizes the duty cycle by trapping a package of ions while the previous is being separated and syn-
chronizing ion fragmentation with TIMS elution. We adapted this concept for HLAIps by taking advantage of
their size- and charge-dependent separation forming two distinct ion clouds for the singly and multiply-charged
8-13-mer peptides. Thus, PASEF-MS2 frames are efficiently used to fragment singly-charged ions during the
first half of the TIMS ramp and multiply-charged during the second half.

Additional adaptations could further improve the identification of immunopeptides. For instance, the Thun-
der isolation polygons could be more restrictive towards 9 to 12-mers to improve fragmentation selectivity for
more challenging samples. For example, soluble HLAIps enriched from plasma samples tend to include larger
peptides resulting from the degradation of proteins adhering non-specifically to the beads, such as blood clotting
and other plasma proteins [51]. Here, we decided to employ broad limits to account for variability between HLA
alleles and to accommodate slight variations in the instrument (e.g., IMS variations between days). In addition,
disease-associated HLAIps can be composed of larger sequences [30, 52, 53] or include modifications that are
key for their immunogenicity [11, 54, 55].

Sensitivity and reproducibility could be further improved by using a data-independent acquisition (DIA)
method including singly-charged ions. Although DIA requires spectral libraries for peptide identification, recent
publications have shown its value for immunopeptidomics [32, 40]. For instance, using Orbitrap instruments,
more than 97% of the combined identifications from 3 DDA runs used to create the library were identified in each
single DIA injection of HLAIp-enriched peptides from cell lines. Using this strategy, Pak et al. [32] identified
7,627 HLAIps per injection of JY cell W6/32 IP-enriched peptides. However, sample fractionation by SPE or
in the gas phase, or at least multiple DDA injections, is required to obtain the spectral libraries. In contrast,
Thunder-DDA-PASEF can achieve higher HLAIps identification coverage in a single run (10,000 on average).
Considering this, we propose a future strategy where a spectral library is acquired using Thunder-DDA-PASEF
and then used to identify the peptides for quantitative DIA analysis.

Thunder-DDA-PASEF enabled the deep profiling of the HLA class I ligandomes from two cell lines with
distinct HLA alleles. We detected 14,074 predicted HLAIps from JY and 17,469 from Raji, with a median
coverage of two HLAIps per protein, surpassing the number of HLAIps identified for a single cell line in previous
publications [32, 40, 19]. In total, 30,984 HLAIps were identified (Fig. 4b, d), including more than 11,000 singly-
charged peptides (Supplementary Material S3). Thus, this combined data set constitutes an important resource
for future exploitation (data available via ProteomeXchange, identifier: PXD040385). For instance, using the
identifications for training DeepLC and MS2PIP prediction models could further improve the performance of
MS2Rescore on timsTOF immunopeptidomics data [16], and other prediction algorithms could be explored
(31, 40, 55). In addition, different strategies for data analysis remain to be evaluated (Fragpipe, MSmill).
Besides, a deeper PTM search could be performed using the PTM algorithm from PEAKS [57], or PROMISE
The onset of the ongoing SARS-CoV-2 pandemic has fueled the discovery of antigen candidates for vaccination, employing in silico prediction algorithms, genetic screens, or peptide library T-cell response assays. Even though immunogenicity testing of hypothesized vaccine candidates yielded some positive outcomes (reviewed in [20, 21, 22]), direct evidence of MHC peptide ligand antigens relies mainly on direct identification by LC-MS. The 17 SARS-CoV-2 spike HLA Ips (Fig. 4) identified included thirteen peptides with proven immunogenicity (IEDB) and four possibly novel antigens that could be explored as targets for therapy development. Notably, six of the seventeen spike peptides were only identified as singly charged ions, and only the peptide identified in both cell lines (SIIAYTMSL<sup>0691-0699</sup>) was reported by MS before ([40]). Altogether these results show that Thunder-DDA-PASEF substantially expands the MS-detectable immunopeptidome providing the means for reproducible antigen discovery and direct validation of immunopeptides hypothesized by non-MS methods.

In summary, Thunder-DDA-PASEF enables an in-depth coverage of HLA Ips in a highly reproducible manner. This opens new opportunities to dig deeper into the immunopeptidome in our search to discover novel and specific antigens to target infectious diseases and cancer.

4 Methods

4.1 Cell culture
The human B lymphoblastoid cell line JY expressing HLA-A02:01, B07:02, C07:02 was purchased from ATCC and the human Burkitt lymphoma cell line Raji expressing HLA-A03:01, B15:10, C03:04, 04:01 was obtained by the DSMZ-German Collection of Microorganisms and Cell Cultures. Both cell lines were maintained in RPMI1640 medium supplemented with 10 % FCS (Gibco), 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin and 100 µg/ml streptomycin. Cells were harvested at 220 x g for 10 min and washed three times with 1x PBS prior counting and freezing at -80°C until further use.

4.2 Cell transfection
The pcDNA3.1-SARS2-spike vector containing the full-length cDNA encoding for the SARS-CoV2 spike protein was obtained from Fang Li (Addgene plasmid #145032 ; https://www.addgene.org/145032/) [58]. The spike S cDNA was split into S1 (2016 bp) and S2 (1761 bp) subunits for cloning by PCR into the NheI and XhoI restriction sites from the multiple cloning site of the pcDNA3.1+P2AeGFP vector (Genscript). The following oligonucleotides (all purchased by Sigma) were used : GCAT GCT AGC ATG TCT CAG TGC GTG AAC CTG ACT ACT AGA ACC and GCAT CTC GAG ACG GGC ACC CCT CCT TGG GGA GTT GGT CTG GGT CTG for the S1 cDNA and GCAT GCT AGC ATG AGC GTG GCC AGC GAC CTG GGA TGT CTC GGT GGA G for the S2 cDNA cloning. To generate stable JY and Raji transfectants expressing either the S1 or the S2 protein fragments
(Supplementary Material S1, Material and Methods), 2 million cells were exposed to 230 V and 500 µF in the presence of 10 µg plasmid DNA using the Bio-Rad Gene Pulser II. After electroporation, cells were cultured 24 h before starting G418 (Gibco) selection at a concentration of 400 µg/ml for JY cells and 800 µg/ml for Raji cells. G418-resistant and eGFP-expressing cells were selected by three rounds of screening using a FACS Aria (BD Biosciences) at the Core Facility of the Research Center for Immunotherapy (University Medical Center, Johannes Gutenberg University Mainz).

4.3 Immuno-affinity purification of HLA peptide ligands

HLA class I ligands were enriched by immunoprecipitation as described by [59] with modifications [60]. Briefly, 500 million cells were washed three times with PBS, harvested, flash-frozen, and stored at -80°C until further preparation. The cell pellets were thawed and lysed in a non-denaturant buffer (1% CHAPS in PBS (m/v)) aided by sonication. Immunoprecipitation was performed using an anti-panHLA Class I antibody (W6/32, anti-HLA-A, -B, -C), immobilized on CNBr-activated beads. After overnight incubation, the beads were washed once with PBS and once with water before peptide ligands were eluted under acidic conditions (0.2% TFA (v/v)). Next, peptides were ultrafiltered (10 kDa cutoff) and then desalted by SPE on a Hydrophilic-Lipophilic-Balanced sorbent (HLB, Waters Corp.), applying 35% ACN (v/v) + 0.1% TFA (v/v) for elution. Finally, dried peptides were dissolved in 15 µL of water with 0.1% FA (v/v) for subsequent LC-MS/MS analyses.

4.4 LC-MS/MS

NanoLC-MS analysis was performed using a nanoElute coupled to a timsTOF-Pro-2 mass spectrometer. The desalted peptides were directly injected in a C18 Reversed-phase (RP) analytical column (Aurora 25 cm x 75 µm ID, 120Å pore size, 1.7 µm particle size, IonOpticks, Australia) and separated using either a 47 min or 110 min gradient (Supplementary Material S2a) increasing the proportion of phase B (ACN + 0.1% FA (v/v)) to phase A (water + 0.1% FA (v/v)), as detailed in Supplementary Material S2. A Captive Spray source was used for ionization, with a capillary voltage of 1600 V, dry gas at 3.0 L/min, dry temperature at 180 °C, and TIMS-in pressure of 2.7 mBar. MS data were acquired in DDA-PASEF mode. Different MS parameters were evaluated during method development, as detailed in Supplementary Material S2a. The JY and Raji spike-transfected data set was acquired using the optimized conditions described in the following lines. HLAIP IP-enriched, ultrafiltered, and desalted peptides were analyzed in three injection replicates each, using a volume of 1.5 µL/injection, equivalent to 50 million cells from the original sample. Peptides were separated in a 110 min. gradient from 2 % to 37 % of ACN +0.1% FA (v/v). The MS was configured with the optimized Thunder-DDA-PASEF method, employing an HLAIP-tailored isolation polygon (Fig. 2), a 300 ms TIMS ramp, three MS2 frames/cycle, one cycle overlap, using the high-sensitivity mode (optimized detector voltages). The settings used for LC-MS are detailed in Supplementary Material S2a and the timsTOF Pro method is included as Supplementary Material S2b.
4.5 Peptidomics database search

Data analysis was performed in PEAKS XPro (v10.6, build 20201221). Raw LC-MS files were loaded with the configuration for timsTOF DDA-PASEF data with CID fragmentation. The option timstof_feature_min_charge (in file PEAKSStudioXPro\algorithm\para\feature_detection_para.properties) was set to 1 to allow the identification of singly-charged features. The protein database was composed of the UniProtKB (Swiss-Prot) reference proteomes of Homo sapiens (Taxon ID 9606, downloaded 02/Feb/2020), Epstein-Barr virus (strain GD1, Taxon ID 10376, downloaded 06/Feb./2022), GFP from Aequorea victoria (P42212), and SARS-CoV-2 (Taxon ID 2697049, downloaded 10/March/2021), as well as the SiORF1 reported by [61, 62], supplemented with a list of 172 possible contaminants. For database searches, protein in silico digestion was configured to unspecific cleavage and no enzyme. Methionine oxidation, cysteine cysteinylation, and Protein N-terminal acetylation were set as variable modifications. Peptides were identified with mass accuracy thresholds of 15 ppm for MS1 and 0.03 Da for MS2. Results were filtered at FDR ≤ 0.01 for peptides and −10lgP ≥ 20 for proteins. For rescoring, spectra were exported in MGF format and identifications in mzIdentML format, including decoys and without any score filter (−10lgP ≥ 0 for peptides and proteins). Identifications were then rescored using MS²Rescore [16] using the Immuno-HCD MS2PIP model and an MS2 mass accuracy tolerance of 0.03 Da. The settings used for data processing are also detailed in Supplementary Material S2a.

4.6 Experiment design

For method development, pooled samples of IP-enriched HLAİps from JY WT cells were used. For the final JY and Raji data set, the IP protocol was used to enrich the HLAİps from three cultures of each WT cell line (JY_WT, and Raji_WT) and two different cultures of each transfected cell line (JY_S1, JY_S2, Raji_S1, and Raji_S2). In every experiment, each sample was analyzed in three LC-MS injection replicates.

4.7 Data analysis and statistics

MHC-binding was predicted using NetMHCpan 4.1 [57] and GibbsCluster 2.0 [10] through MhcVizPipe (v0.7.9) [38]. R scripts [33] were used for data analysis, including merging the MS²Rescore [16] output with PEAKS peptide results, as well as with MhcVizPipe output. The main R packages used were as follows; the statistical difference was assessed by two-sided t-test using ggpurbr (v. 0.4.0) [64]; plots were generated using ggplot2 (v. 3.4.0) [65]; Venn plots with ggvenn (v. 0.1.9) [66]; and upset plots with ggupset (v. 0.3.0) [67].

We employed the Universal Spectrum Viewer (USE) [48] to compare the spectra acquired from the cells against spectra obtained from synthetic peptides (n= 7) or predicted in silico (n= 10), based on the similarity Pearson correlation coefficient (PCC). Prosit [34, 56] was used for in silico prediction since it’s an orthogonal model to MS²Rescore [16] used for rescoring.
4.8 Data availability

The mass spectrometry immunopeptidomics data have been deposited to the ProteomeXchange Consortium [68] via the jPOSTrepo partner repository [69] with the dataset identifiers PXD040385 for ProteomeXchange and JPST002044 for jPOSTrepo.

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6 Author contributions


7 Competing interests

The authors have no conflicts of interest to declare.

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References


9 Figures

Graphical abstract

[Diagram with various components and labels, including "Raji ± spike", "JY ± spike", "HLA class ligands", "DDA-PASEF", "1/K", "z = 1 59.7%", "m/z", "MHCzippe", "NetMHCpan", "GibbsCluster", "PEAKS XPLO", "MS Rescore"]
Figure 1: **Immunopeptidomics workflow using Thunder-DDA-PASEF.** (a) Sample preparation: 500 million cells of the human JY or Raji cell lines were harvested, then lysed by sonication in 1% CHAPS in PBS buffer (m/v). (b) MHC-ligand peptide enrichment: was performed by immunoaffinity using the W6/32 anti-human-MHC-A, B, C antibody coupled to CNBr-activated agarose beads; after overnight incubation and several washes, peptides were eluted with 0.2% trifluoro-acetic acid, ultrafiltered on molecular weight cutoff filters (MWCO, 10 kDa cutoff) and desalted in HLB plates (Waters Corp.). (c) NanoLC-MS: analysis was performed using a nanoElute coupled to timsTOF-Pro-2 in DDA-PASEF [17] with different parameters to optimize the MS acquisition. (d) Data analysis: Database search was performed in PEAKS XPro using unspecific cleavage. Data analysis was performed in R and predicted MHC-binding affinity was evaluated using NetMHCpan 4.1 [37] and GibbsCluster 2.0 [46] through MhcVizPipe (v0.7.9) [38].
Figure 2: Evaluation of the different fragmentation isolation filters: "standard", "None" and "HLAIp-tailored". (a, b, c): Exemplary heatmaps of ion intensities (gray-scale) across the inversed ion mobility (1/K_0) vs m/z dimensions showing fragmentation events (red rhombus). (d - i): Correspondent peptides identified across the 1/K_0 vs m/z dimensions colored by charge state, including all peptides (d, e, f) or only those with 8 to 13 amino acids (AAs) (g, h, i). (j, l, m): Length distribution and percentage of peptides (pie-charts) with 8 to 13 AAs or other lengths; cut-off at 20 AAs dropping 5.4%, 1.6% and 0.26% of peptides identified for standard, None and HLAIp-tailored, respectively. (m) Average number of unique peptides identified per injection in each method (3 injection replicates, mean ± sd). (n) Average number of MS2 scans triggered per injection in each method (3 injection replicates, mean ± sd). Two-sided t-test, ns: p > 0.05, *: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001, ****: p ≤ 0.0001.
Figure 3: Evaluation of the original-DDA-PASEF method (original) compared to the optimized Thunder-DDA-PASEF (Thunder) profiling of JY immunopeptides, and the effect of identification rescoring using MS\textsuperscript{2}Rescore (Thunder + MS2R), considering only peptides of 8 to 13 amino acids long. (a) Average number of unique peptides identified per injection in each method (3 injection replicates, mean ± sd; two-sided t-test, ****: p ≤ 0.0001). (b) Proportion of peptides (considering modifications) identified in function of their charge state. (c) Dynamic range plot showing the peptides identified (considering modifications), ranked in descending order (x-axis) in function of the average peak area across three replicates (y-axis); the dashed gray line indicates the lowest limit of identification for the original method. (d) Identification data completeness, measured as the proportion of peptides identified across three, two, or only one replicate. (e) Upset plot showing the number (barplot) and percentage (text) of 8-13-mers identified uniquely in each method or their combinations; the intersection matrix at the bottom indicates that the same peptides shown above (columns) were detected in the methods (rows) highlighted with a blue dot. (f) Total number of peptides identified in each workflow and the proportion predicted as strong-binders (SB, rank ≤ 0.5%), weak-binders (WB, 0.5% < rank ≤ 2%) or non-binders (NB, rank > 2%) by NetMHCpan 4.0 [37].
Figure 4: HLA class I ligandome of JY and Raji cells employing Thunder-DDA-PASEF, combining wild type and spike-transfected cells. (a) Size distribution of total peptides identified from JY and Raji cells. (b) Number of 8-13-mer peptides identified in each workflow and the proportion predicted as strong-binders (SB, rank ≤ 0.5%), weak-binders (WB, 0.5% < rank ≤ 2%) or non-binders (NB, rank > 2%) by NetMHCpan 4.0 against the matched HLA alleles expressed by each cell line (JY = HLA-A02:01, B07:02, C07:02; Raji: HLA-A03:01, B15:10, C03:04, C04:01). (c) Charge distribution for the predicted HLA class I binders (HLAIps, SB & WB). (d) Total number of predicted HLAIps (SB & WB) identified (top) and protein groups covered (bottom) for JY, Raji, and in total. (e) Distribution of the number of HLAIps per protein group represented as boxplots (center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range) (top) and histogram (bottom); y-axis cut-off at 12 for simplicity, excluding 0.7% of JY HLAIps (13 to 34 Binders/Protein) and 1.4% of Raji HLAIps (13 to 53 Binders/Protein). (f) Overlap of HLAI ligand peptides (top) and protein groups (bottom) between JY and Raji. (g, h) Supervised clustering (GibbsCluster-2.0 via MhcVizPipe) showing the peptide sequence motifs corresponding to the specific allele motifs for JY and Raji HLAIps, respectively.
Figure 5: Spike HLA class I binder peptides (HLAIps) identified in JY and Raji transfected cells. 
(a, b) Count of protein-specific HLAIps predicted strong-binders (SB, rank ≤ 0.5%) and weak-binders (WB, 0.5% < rank ≤ 2%) using NetMHCpan 4.0 [37] for spike (a) and the reporter GFP (b). (c) Peptide peak area distribution of the spike peptides (black dots) and all the HLAIps identified in JY (orange) and Raji (purple). (d) Characteristics of spike HLAIps identified in JY (top) and Raji (bottom) transfected cells. From left to right: sequence code name indicating their position within the protein sequence [s[N-ter]-[C-ter], e.g., s0691-0699 for SIIA YTMSL]; sequence, with common peptides highlighted in gray; charge state (number of H+); the number of biological replicates (BR) and technical replicates (TR) where the peptide was identified; Log2 of the peptide peak area; Pearson’s correlation coefficient (PCC) comparing the fragmentation spectrum of the endogenous peptide against synthetic peptides (S) or Prosit-predicted (P) [56, 34] calculated employing the Universal Spectrum Explorer (USE) [48]; indexed retention times (iRT) ratio (endogenous/synthetic); Immune Epitope Database and Analysis Resource (IEDB) [50] immune response frequency (RF) = proportion of subjects with positive immune response in B-cell or T-cell assays (dots = RF, lines = 95% confidence interval (CI) range, color scale = lower 95% CI, empty = not reported), relative to the total number of subjects tested for the corresponding peptide; binding affinity to JY and Raji HLA alleles predicted by NetMHCpan 4.0 [37], with labels indicating SBs and WBs.
Figure 6: Mirrored fragmentation spectra showing the spectrum from endogenous peptides at the top and synthetic or predicted spectra for two spike peptides. (a) SHAYTMSL<sup>s0691-0699</sup> (bottom = synthetic), and (b) TLKSFTVEK<sup>s0302-0310</sup>, (bottom = Prosit predicted); obtained by USE [48]; PCC = Pearson’s correlation coefficient, SA = spectral (contrast) angle.
Supplementary Files

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- S2Extendedmethodssettings.xlsx
- S10spectraspikeUSEmirror.pdf
- S7gorillajyrajipepbinder.xlsx
- S1ThunderDDAPASEFSpike20230224.pdf
- S2bThunderDDAPASEF1600V300ms3rampsLSA.m.zip
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