MAETi: Mild acid elution in a tip enables immunopeptidome profiling from 25,000 cells

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2. Main messages / Highlights

MAETi is a simplified and optimized MAE workflow enabling MHC1-ligandomics profiling from sub-million cell samples down to 25,000 cells

- We present a minimal, simplified sample preparation protocol termed Mild Acid Elution in a Tip (MAETi) enabling the MHC-1 ligandome analysis using 25,000 to 100,000,000 cells per sample as initial input
- Replacing citric acid in the elution buffer by β-alanine reduced unspecific contaminants, resulted in cleaner LC-MS chromatograms and boosted 8-13mer identifications by over 50% in MAEti samples.
- Bulk β-alanine-based MAE provides similar coverage of MHC class 1 ligands compared to bulk immunoprecipitation

3. Abstract

The identification of MHC class I presented ligands by mass spectrometry (MS)-based immunopeptidomics is an essential tool to characterize antigen processing pathways and to define targets for tumor immunotherapies. However, existing sample preparation workflows typically require large sample inputs, limiting the applicability in high throughput drug screenings, kinetic immunopeptidome studies and for scare samples in clinical contexts. To address this challenge, we developed Mild Acid Elution in a Tip (MAETi), an antibody-free approach for low input MHC-1 immunopeptidome profiling. Using an optimized β-alanine MAE-buffer for MAETi reduces background interferences, enhances peptide coverage, and boosts reproducibility. Comparing bulk β-alanine-based MAE with bulk immunoprecipitation (IP), achieves similar or complementary immunopeptidome depth. Using two protocol layouts we further profiled initial inputs from 25,000 to 1 Million cells with HLA-tailored DDA- and DIA-PASEF schemes yielding on average over 1,000 and 3,000 predicted binders, respectively (DIA). This renders MAETi a facile, fast and scalable method enabling robust MS-based immunopeptidomics for minimal sample inputs.

4. Introduction

Mass spectrometry (MS)-based immunopeptidomics has become key for understanding immune surveillance and developing immunotherapies.[1–3] The field advances quickly and rapid technological, instrumental and bioinformatic developments along with higher isolation efficiency in the past 10 years enabled astonishing progress in the field[4]. However, despite these advancements, the need for high sample amounts of most workflows substantially limits translation into the clinic, where patient input material for characterization and analysis is typically scarce[5,6].

There are two major immunopeptide enrichment strategies (Figure 1a). The most commonly used immunoprecipitation (IP), employs a bead-immobilized antibody to enrich MHC-peptide complexes from a cell lysate[4,7]. In contrast, mild acid elution (MAE) takes advantage of the dissociation of MHC class I complexes in mild-acidic conditions thereby eluting MHC1ps directly from ideally intact cells[8,9]. In both methods, peptides are further purified and analyzed by LC-MS[9].

Historically, MAE was the first method used to isolate immunopeptides, but later widely replaced by IP to achieve higher specificity. More recent work by Sturm *et al.*[9] challenged this view and demonstrated that an optimized MAE workflow can achieve immunopeptide coverage and enrichment specificity comparable to IP. These findings indicate that MAE is a viable alternative to IP. Nevertheless, both methods involve labor-intensive sample cleanup steps such as centrifugation, ultrafiltration, and offline solid-phase extraction (SPE, Figure 1b, top) [4,7,9]. This results in a lengthy process associated with a low recovery yield, resulting in high sample input requirements[10–14]. More recently, microfluidics-based approaches have gained traction in immunopeptidomics for their ability to handle small sample volumes, making them well-suited for low-input samples[10,15,16] (Figure 1a, top). However, requirement for specialized equipment and expertise to manufacture and operate the microfluidic chips have so far hindered broader adoption and throughput[10,15,16].

To provide a simple, viable alternative to complex microfluidics layouts, we developed an optimized Mild Acid Elution in a Tip workflow (MAETi) (Figure 1b, bottom). Briefly, pre-washed cells are placed onto glass fiber membrane-tapered pipette tips (GF-tips) and directly MAE-stripped onto disposable trap columns (Evotips). This eliminates most steps required in bulk immunopeptidomics enrichment workflows, improves overall sensitivity, and enables the direct analysis of input cell numbers down to 25,000 cells. After desalting on the Evotip, the MAE-eluate is finally injected into the Evosep One coupled to a timsTOF mass spectrometer.

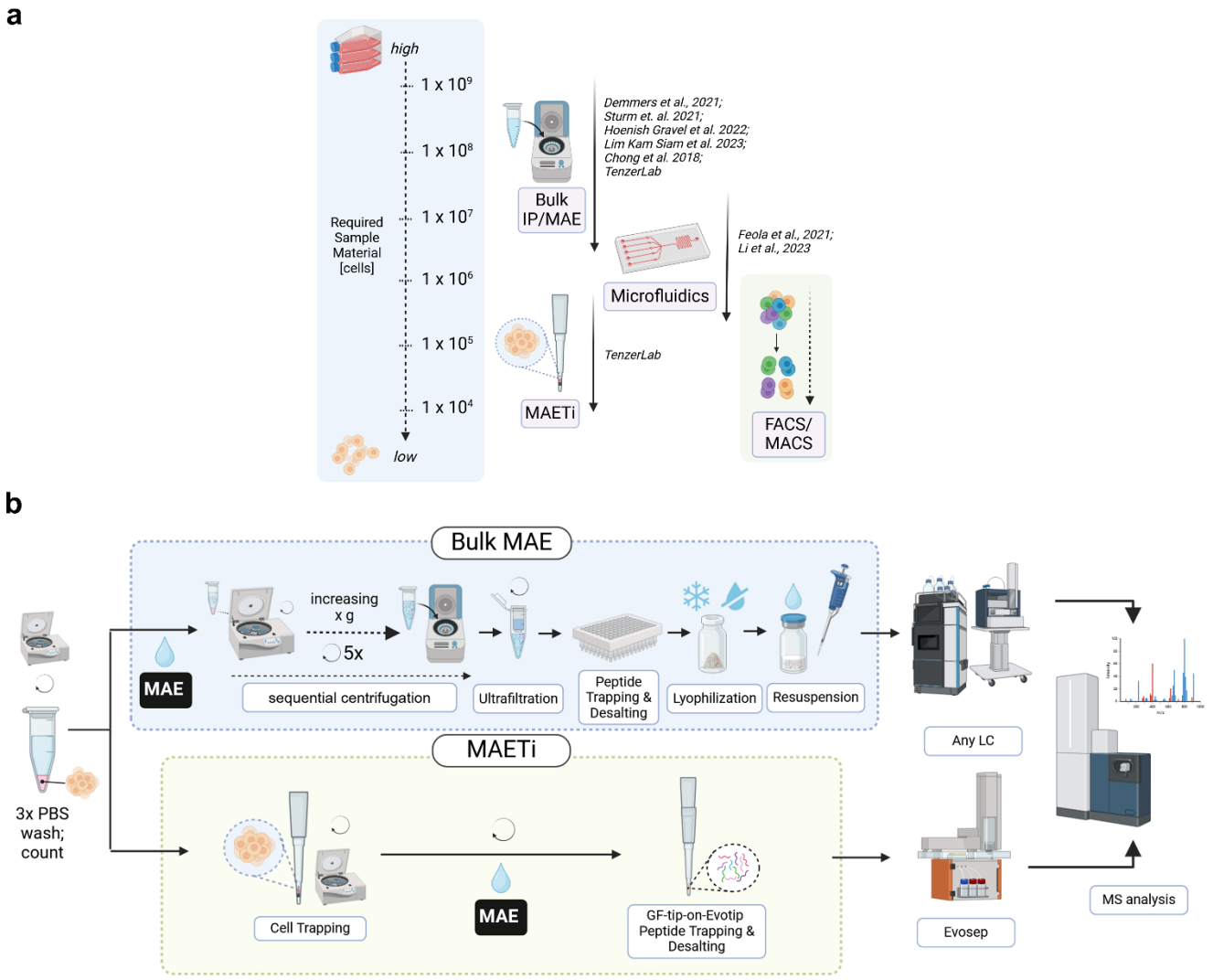


Figure 1: Schematic Overview showing current immunopeptidomics methodologies and experimental steps in Mild Acid in a Tip (MAETi)

Schematic Overview showing current immunopeptidomics methodologies in the context of required sample input material including bulk, microfluidics and our Mild Acid Elution in a Tip (MAETi) workflow (**a**). MAETi profits from the substantial reduction of sample handling steps compared to a bulk MAE workflow (**b**) and enables immunopeptidome profiling of 50,000 cells and below. MAETi utilizes a glass fiber membrane tapered into a pipette tip retaining MAE-stripped cells while directly recovering eluted peptides on disposable trap columns (Evotips) prior desalting and MS analysis. IP, immunoprecipitation; MAE, mild acid elution; FACS, fluorescence-assisted cell sorting; MACS, magnetically-assisted cell sorting.

5. Results

5.1. A minimal MAE-based MHC1p enrichment workflow enables immunopeptidomics profiling from 50,000 cells

To demonstrate the feasibility of our minimal protocol (Figure 1b, bottom) after initial protocol optimizations, we employed the MAETi method to process individual aliquots of 50,000 JY cells. Peptides were resolved using the 40 samples per day (SPD) Whisper gradient and mass spectrometry data were acquired on a timsTOF-SCP (Figure 2a) using DDA-PASEF with two distinct isolation polygons (n = 3 each) (Figure 2b). The stepped polygon was designed to fragment all possible peptides by using a stepped isolation polygon covering the 1/K0 vs. m/z ion clouds corresponding to all multiply charged ions, and singly charged ions above 600 m/z. In contrast, Thunder-DDA-PASEF efficiently uses the instrument time by focusing the MS/MS events on the ion cloud corresponding to 8-13mers based on their IMS vs. m/z distribution using a Thunder-bolt icon-shaped isolation polygon[17].

First, we assessed the peptide length distribution using an unbiased approach (Stepped-DDA-PASEF, Figure 2b-left). This resulted in 1574 total peptides (Figure 2c) identified across three replicates, and 1030 ± 133 per sample (mean ± sd). The enriched peptides presented the expected length distribution for MHC1ps with 9mers forming the apex (Figure 2e) and were mostly composed of 8-13mers (82%, Figure 2f), corresponding to 849 ± 118 8-13mers per sample (mean ± sd; Figure 2g). These results indicate that MAETi enables the direct identification of peptides with a MHC-1 -typic peptide length distribution and does not lead to co-enrichment of longer peptides.

Next, we implemented the MHC1p-tailored Thunder polygon into the DDA-PASEF method (Figure 2b-middle,[17]), which resulted in a 10% increase of peptides in total and on average (Figure 2c, 2d) compared to the stepped polygon. In addition, the Thunder polygon slightly increased the number of 9mers (Figure 2e) and the proportion of 8-13mers (from 83 to 86%; Figure 2f). Data completeness was comparable between the stepped (36%) and Thunder polygons (38%; Figure 2h). However, acquisition using the Thunder polygon increased the quantitative precision as indicated by decreasing the median coefficient of variation (CV) of the MS1 area of 8-13mers from 30% to 22% (Figure 2i).

Finally, to verify the capability of MAETi to enrich MHC class I ligands, we used MHCvizPipe[18] to predict peptide binding to JY MHC alleles (via NetMHCPan-4.1[19]) and generate unsupervised and supervised sequence clustering analyses (via GibbsClustering[20]). Although both MS methods yielded nearly identical proportions of predicted binders (strong and weak binders, Figure 2j), Thunder generally detected more binders than the stepped polygon (*p* = NS; Figure 2k). Importantly, the enriched peptides displayed the expected sequence motifs

previously described in other studies including IP-based enrichments[9,17] for the JY MHC-Alleles (HLA-A*0201, B07*02, C*0702), as revealed by unsupervised Gibbs Clustering (Figure 2l for thunder_DDA and Suppl. Figure S2d for both MS-methods) and allele-supervised Gibbs Clustering (Suppl. Figure S2e for both MS-methods).

These results demonstrate that MAE-stripping whole cells on a glass-filter tip can be used to enrich MHC class 1 immunopeptidomes. This initial version of MAETi enabled the MHC1p immunopeptidomics profiling of as low as 50,000 cells, yielding on average 785 ± 40 predicted binder in timsTOF SCP with Thunder-DDA-PASEF.

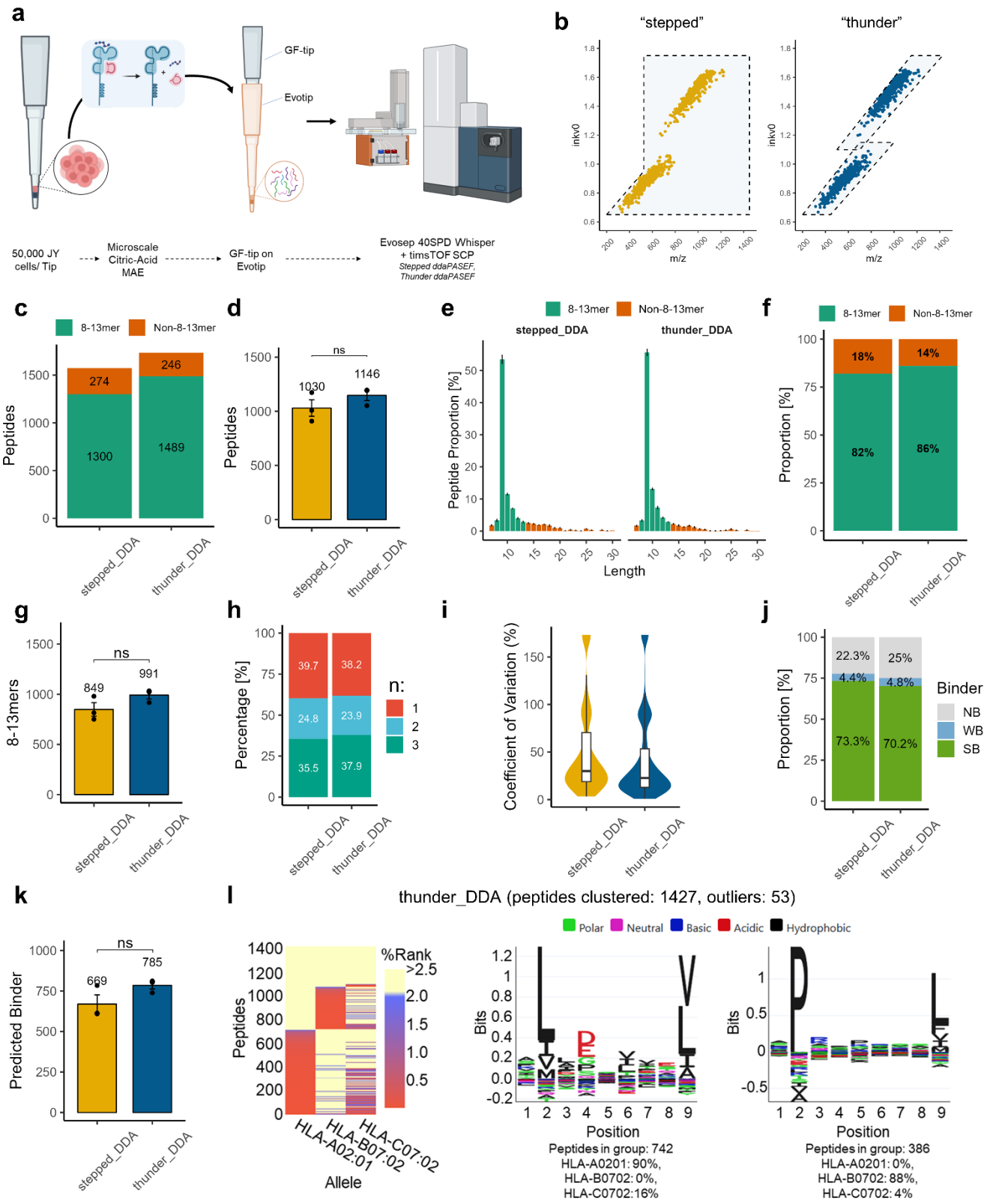


Figure 2: Proof of Principle - Mild Acid Elution in a Tip (MAETi)

a-b Layout of Mild Acid Elution in a Tip (MAETi) proof of principle experiment (**a**) and acquisition schemes used (**b**). **a** Samples of 50,000 JY cells each were prepared by MAETi, directly loaded into Evotips, and analyzed in an Evosep One coupled to a timsTOF SCP, using a Whisper 40 SPD gradient and two distinct acquisition schemes (n = 3, each). **b** 1/K0 (inverse ion mobility, invkv0) vs. m/z peptide distribution showing the polygons used for unbiased Stepped-DDA-PASEF and MHC-tailored Thunder-DDA-PASEF. **c-i** Experimental

characteristics of MHC-1 immunopeptide enrichment using MAETi. (**d, g, k**): Unique peptides identified on average per injection. **c** Total peptides identified per acquisition scheme across three replicates, divided in 8-13mers and other peptide lengths. **d** Peptides identified on average per sample (n = 3). **e** Histograms showing the peptide length distribution for both acquisition schemes. **f** Proportion of peptides with the expected peptide length for MHC class I ligands (8-13mer). **g** Peptides with the expected size (8-13mers) identified on average per sample. **h** Data completeness shown as the proportion of 8-13mers identified in one, two, or all the three replicates. **i** Violin and boxplots of the peak area coefficient of variation (CV) of 8-13mers identified in all 3 replicates (center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range). **j** Proportion of 8-13mers predicted to bind JY MHC alleles (NB, non-binders; WB, weak-binders; SB, strong binders). **k** MHC predicted binders (unique SB and WB) identified on average per sample. **l** Unsupervised sequence clustering of the peptides identified, matching the expected motifs for JY MHC alleles.
Bars with error bars correspond to *average* ± *sd* with points indicating the individual values, and asterisks designate statistical differences (two-sided t-test; * $p = 0.05$, ** $p = 0.01$, *** $p = 0.001$).

5.2. Optimized β-alanine-based MAE Buffer provides cleaner samples and enhances immunopeptidome coverage and reproducibility

High-sensitivity immunopeptidome profiling of minute sample amounts demands for high purity substances to minimize introducing contaminants during sample preparation. After initial validation of the citric acid-based MAETi protocol, closer inspection of the raw data revealed a high level of background in the acquired LC-MS/MS datasets. To reveal the source of this potential contamination, we analyzed pure 131 mM citric acid (pKa ~ 3.13) loaded into Evotips (Figure 3a-top). The resulting data showed an up to 7-fold higher maximal base peak chromatogram (BPC) baseline than a blank injection of 0.1% formic acid (FA) (Figure 3b), with several highly intense ions tailing across the whole gradient (Figure 3c). Thus, citric acid introduced a high level of background ions potentially interfering with analyte ionization despite extensive washing of the Evotips (3x 0.1% FA in water).

Therefore, we explored β-alanine as an alternative substance buffering at the pH used for MAE, with a pKa of ~ 3.55. Notably, the analysis of pure β-alanine resulted in cleaner chromatographic traces with an average baseline over 8-fold lower than citric acid, showing only a few distinct peaks (Figure 3b). Additionally, the ion mobility spectrometry (IMS) versus retention time (RT) heatmaps of pure β-alanine were substantially cleaner than the heatmaps of pure citric acid (Figure 3c).

Subsequently, we assessed the suitability of β-alanine as an alternative buffer substance for MHC1p enrichment in the MAETi protocol. Towards this purpose we prepared MAETi samples with 50,000 JY cells each using MAE buffers with either citric acid or β-alanine at the same concentration (131 nM, n=3 per buffer) and analyzed them using the 40SPD Whisper gradient and the stepped DDA-PASEF method in a timsTOF SCP (Figure 3a-bottom).

In comparison to citric acid, β-alanine resulted in 36% more peptides in total and 57% more peptides on average (Figure 3d, e, p = 0.01). Both buffers resulted in the expected MHC1p length distribution with > 40% 9mers (Figure 3f) and > 75% 8-13mers (Figure 3g). Notably, β-alanine yielded 54% more 8-13mers than citric acid (1587 vs. 1029; Figure 3h; p = 0.001). In addition, β-alanine improved data completeness across replicates (Figure 3i), and decreased the 8-13mer area CV median from 35% to 26% (Figure 3j). This indicates that peptide enrichment is more robust and reproducible with the β-alanine-based MAE, compared to citric acid.

Despite showing a lower proportion of 8-13mers predicted to bind JY MHC alleles (Figure 3k), the overall higher yield of β-alanine resulted in 30% more predicted binders than citric acid (p = 0.05, Figure 3l), corresponding to 733 ±29 and 956 ±68 predicted binders, respectively. Interestingly, only 32% of the predicted binders were detected in both buffers, but the peptide sequence motifs (Figure 3n) were similar, showing that their amino acid composition was alike

(Suppl. Figure S3). Thus, the differences could be due to biological, preparation, and analytical variations.

Taken together, these experiments prove that replacing citric acid by β-alanine in the MAE buffer results in lower background ions and improve the MHC1p coverage and reproducibility of our workflow. Hence, β-alanine-based MAETi robustly identifies almost 1000 predicted binders from only 50,000 JY cells. To further assess quality of MHC1p enrichment using a β-alanine MAE buffer, we compare bulk β-alanine MAE buffer enrichments to bulk immunoprecipitation in the next section.

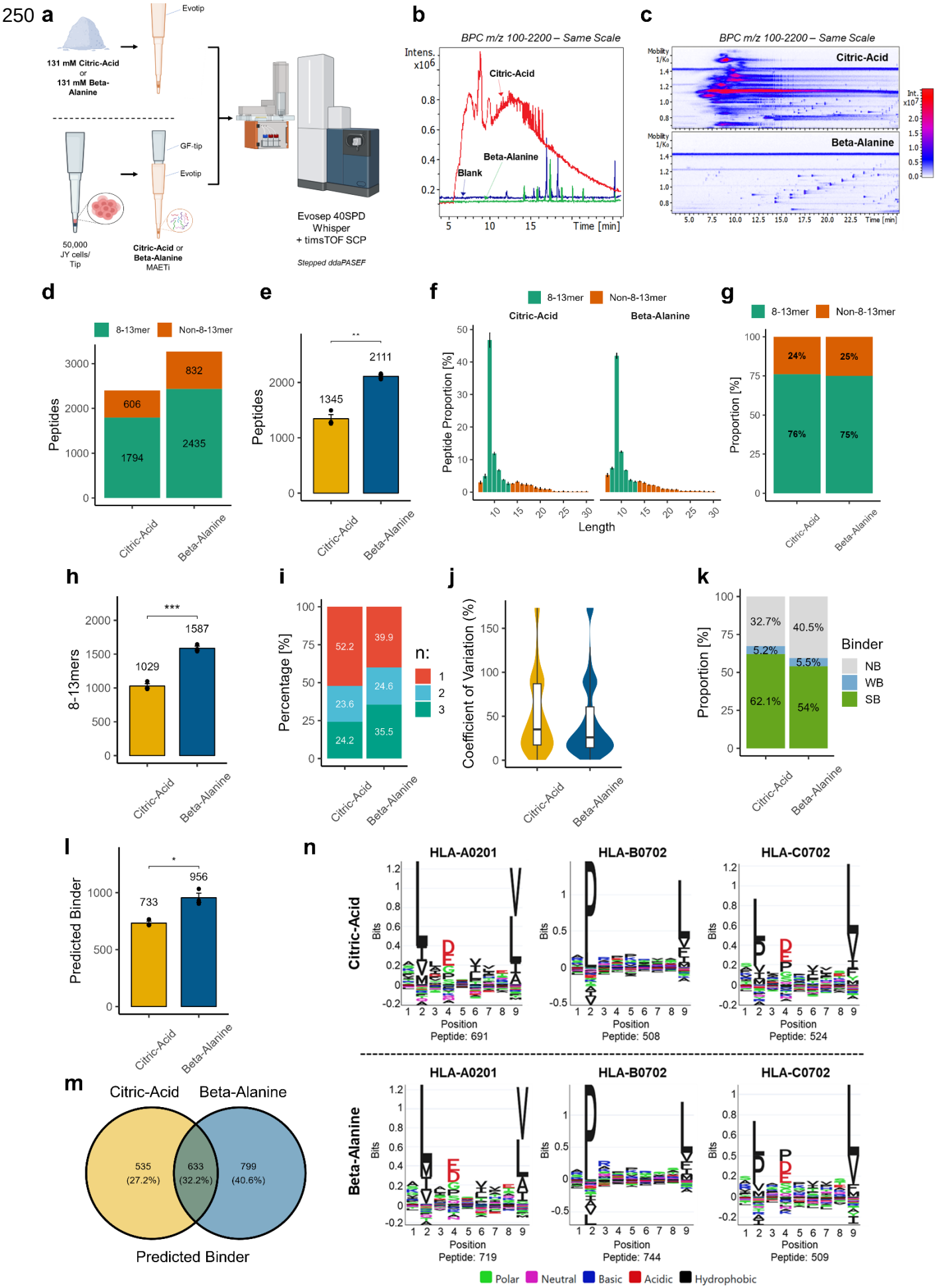

a
Evotip
131 mM Citric-Acid
or
131 mM Beta-Alanine
GF-tip
Evotip
50,000
JY cells/
Tip
Citric-Acid or
Beta-Alanine
MAETi
Evosep 40SPD
Whisper
+ timsTOF SCP
Stepped ddaPASEF
b
BPC m/z 100-2200 – Same Scale
Intens.
x10^6
Citric-Acid
Beta-Alanine
Blank
Time [min]
c
BPC m/z 100-2200 – Same Scale
Mobility
1/Ko
Citric-Acid
Beta-Alanine
Int.
x10^7
Time [min]
d
8-13mer
Non-8-13mer
Peptides
606
832
1794
2435
Citric-Acid
Beta-Alanine
e
Peptides
1345
2111
Citric-Acid
Beta-Alanine
f
8-13mer
Non-8-13mer
Citric-Acid
Beta-Alanine
Peptide Proportion [%]
Length
g
8-13mer
Non-8-13mer
Proportion [%]
24%
25%
76%
75%
Citric-Acid
Beta-Alanine
h
8-13mers
1029
1587
Citric-Acid
Beta-Alanine
i
Percentage [%]
52.2
39.9
23.6
24.6
24.2
35.5
n:
1
2
3
Citric-Acid
Beta-Alanine
j
Coefficient of Variation (%)
Citric-Acid
Beta-Alanine
k
Proportion [%]
32.7%
40.5%
5.2%
5.5%
62.1%
54%
Binder
NB
WB
SB
Citric-Acid
Beta-Alanine
l
Predicted Binder
733
956
Citric-Acid
Beta-Alanine
m
Citric-Acid
Beta-Alanine
535
(27.2%)
633
(32.2%)
799
(40.6%)
Predicted Binder
n
Citric-Acid
HLA-A0201
HLA-B0702
HLA-C0702
Bits
Position
Peptide: 691
Peptide: 508
Peptide: 524
Beta-Alanine
HLA-A0201
HLA-B0702
HLA-C0702
Peptide: 719
Peptide: 744
Peptide: 509
Polar
Neutral
Basic
Acidic
Hydrophobic

Figure 3: Optimization of alternative MAETi buffer composition

a-n Experimental layout for MAE buffer optimizations (**a**), testing pure buffer substances (**b-c**) and benchmarking alternative buffer performance for MAETi analysis of JY cells (**d-n**). **a** Either 131 mM citric acid or β-alanine were directly loaded into Evotips to assess background contaminant levels; Then whole MAETis (n = 3) using either a citric acid- or β-alanine-based MAE buffer and with 50,000 JY cells per tip were analyzed in an Evosep coupled with timsTOF SCP using Stepped-DDA-PASEF. **b** Base peak chromatograms (BPC) of 131 mM citric acid, β-alanine, or a blank (1% FA in water) directly loaded into Evotips. **c** Corresponding heatmaps of the MS1 ions projected in the inversed IMS vs. m/z space. **d** Total peptides identified per buffer substance across three replicates of JY cells, divided in 8-13mers and other peptide lengths. **e** Peptides identified on average per sample. **f** Histograms showing peptide length distributions for both buffer substances. **g** Proportion of peptides with the expected peptide length for MHC class I ligands (8-13mer). **h** Peptides with the expected size (8-13mers) identified on average per sample. **i** Data completeness shown as the proportion of 8-13mers identified in one, two, or all the three replicates. **j** Quantitative accuracy shown as Violin and boxplots of the peak area coefficient of variation (CV) of 8-13mers identified in all 3 replicates (center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range). **k-n** Binding predictions and sequence motifs. **k** Proportion of 8-13mers predicted to bind JY MHC alleles (NB, non-binders; WB, weak-binders; SB, strong binders). **l** MHC predicted binders (unique SB and WB) identified on average per sample. **m** Venn diagram showing the overlap of predicted binders identified in DIA (n=3) for citric acid and β-alanine. **n** Supervised Gibbs sequence clustering of the peptides identified, matching the expected motifs for JY MHC alleles.

Bars with error bars correspond to *average* ± *sd* with points indicating the individual values, and asterisks designate statistical differences (two-sided t-test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

5.3. β-alanine-based MAE provides a complementary immunopeptidome coverage compared to immunoprecipitation

Next, we assessed the performance of the β-alanine MAE buffer and its influence on MHC1p enrichment by comparing bulk β-alanine-based MAE with bulk immunoprecipitation (IP) using the anti-pan-HLA class 1 antibody W6/32 (Figure 4a). We profiled the two lymphoblastoid human B-cell lines JY and Raji covering 6 distinct MHC alleles and low immunopeptidome overlap (<2%, Gomez-Zepeda *et al.*[17]). Briefly, three identical aliquots of JY and Raji cells (at $4x10^7$ cells each) were prepared using MAE and IP in parallel, and each sample was injected in triplicate ($2x10^6$ cell equivalents/injection, n = 9 total injections). For this experiment, all samples were ultrafiltered, desalted, and analyzed in a nanoElute-2 coupled with a timsTOF Pro 2 using stepped DDA-PASEF with settings previously optimized for this instrument (Gomez-Zepeda *et al.*[17]).

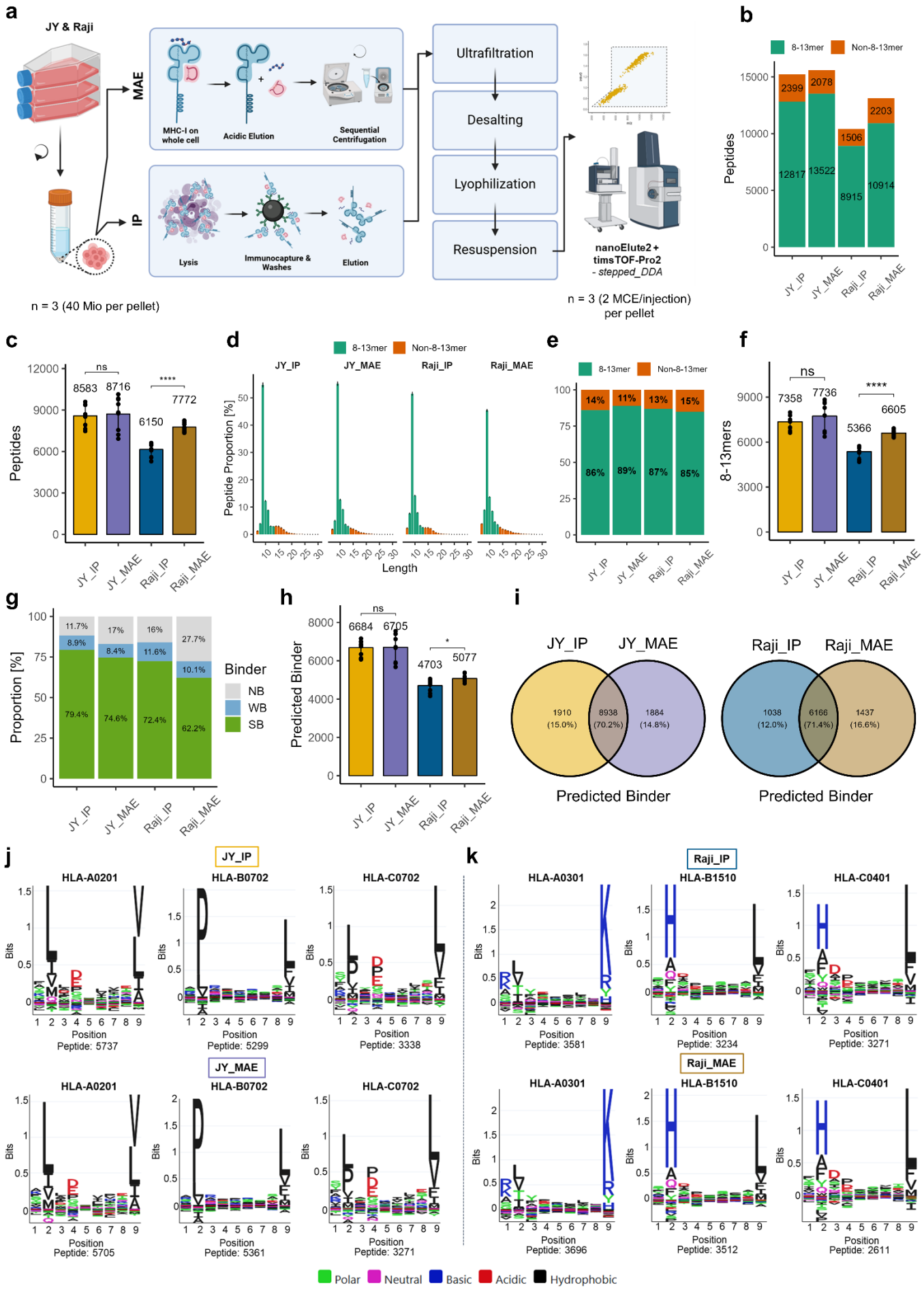
For JY cells, MAE and IP resulted in similar numbers of peptides both in total (15,600 and 15,216, respectively) and on average per sample (8,716 ± 1,210 and 8,583 ± 783, respectively). In contrast, for Raji cells, MAE outperformed IP by yielding 26% more peptides in total (13,117 and 10,421, respectively) and on average (7,772 ± 324 and 6,150 ± 456, respectively, $p \leq 0.0001$). Notably, the peptide length distribution remained comparable across both methods for JY and Raji (85% to 89% 8-13mers) (Figure 4d, e). Thus, MAE produced a significantly higher 8-13mer yield for Raji (6,605 ± 213 versus 5366 ± 383, $p \leq 0.0001$), but not for JY (Figure 4f). Notably, data completeness for 8-13mer detection across four replicates was comparable between IP and MAE within each cell line (Suppl. Figure S3, d).

Although the proportion of predicted binders was slightly lower in MAE than in IP for both JY and Raji (Figure 4g), the average number of predicted binders identified was similar for JY and slightly higher for Raji using MAE (Figure 4h, $p \leq 0.05$). Furthermore, the overlap of predicted binders identified by MAE and IP was over 70% for both JY and Raji (Figure 4i), with similar peptide sequence motifs obtained by supervised Gibbs Clustering (Figure 4j-k). The similarities and differences in the peptide sequences - such as motifs, isoelectric points, or hydrophobicity - are provided in the supplementary Figure S4e-k.

In fact, despite the distinct alleles and accordingly peptide motifs for Raji and JY, we observed a slight overlap in 8-13mers (~2.5%, Suppl. Figure S3h) but not predicted binder (Suppl. Figure S3i) suggesting minimal shared non-binder elutions by MAE. However, this did not negatively impact binder identification (Figure 4h) and comparison of predicted binder sequence motifs (9mers, without clustering) of shared or unique identifications for MAE or IP showed no apparent motif differences for both cell lines (Suppl. Figure S3j-k).

Collectively, these findings indicate that our β-alanine mild acid elution (MAE) results in a similar and complementary enrichment of MHC class I ligands compared to

immunoprecipitation (IP) rendering it a cost-effective alternative to antibody-based enrichment methods.


a
JY & Raji
MAE
IP
MHC-I on whole cell
Acidic Elution
Sequential Centrifugation
Lysis
Immunocapture & Washes
Elution
Ultrafiltration
Desalting
Lyophilization
Resuspension
nanoElute2 + timsTOF-Pro2
- stepped_DDA
n = 3 (40 Mio per pellet)
n = 3 (2 MCE/injection) per pellet
b
8-13mer
Non-8-13mer
Peptides
JY_IP
JY_MAE
Raji_IP
Raji_MAE
c
Peptides
ns
****
8583
8716
6150
7772
d
Peptide Proportion [%]
Length
e
14%
11%
13%
15%
86%
89%
87%
85%
f
8-13mers
7358
7736
5366
6605
g
Proportion [%]
Binder
NB
WB
SB
11.7%
8.9%
79.4%
17%
8.4%
74.6%
16%
11.6%
72.4%
27.7%
10.1%
62.2%
h
Predicted Binder
6684
6705
4703
5077
i
JY_IP
JY_MAE
1910 (15.0%)
8938 (70.2%)
1884 (14.8%)
Raji_IP
Raji_MAE
1038 (12.0%)
6166 (71.4%)
1437 (16.6%)
Predicted Binder
j
JY_IP
HLA-A0201
HLA-B0702
HLA-C0702
Position
Peptide: 5737
Peptide: 5299
Peptide: 3338
JY_MAE
Peptide: 5705
Peptide: 5361
Peptide: 3271
k
Raji_IP
HLA-A0301
HLA-B1510
HLA-C0401
Peptide: 3581
Peptide: 3234
Peptide: 3271
Raji_MAE
Peptide: 3696
Peptide: 3512
Peptide: 2611
Bits
Polar
Neutral
Basic
Acidic
Hydrophobic

318

Figure 4: β-alanine-based mild acid elution (MAE) provides a complementary immunopeptidome coverage compared to immunoprecipitation (IP) in bulk experiments.

a Experimental layout for bulk MHC-1 ligand enrichment from JY and Raji cells using MAE or IP analyzed in a nanoElute-2 coupled with a timsTOF Pro 2 using Stepped-DDA-PASEF. **b** Total peptides identified per enrichment method and cell line across three preparation and injection replicates (n = 9), divided in 8-13mers and other peptide lengths. **c** Peptides identified on average per sample. **d** Histograms showing the peptide length distribution. **e** Proportion of peptides with the expected peptide length for MHC-1 ligands (8-13mer). **f** Peptides with the expected size (8-13mers) identified on average per sample. **g** Proportion of 8-13mers predicted to bind JY MHC alleles (NB, non-binders; WB, weak-binders; SB, strong binders). **h** Predicted binders identified on average per sample. **i** Venn diagram showing the overlap of binders identified in MAE and IP for JY (left) and Raji (right). **j** Supervised Gibbs sequence clustering of the peptides identified in both protocols, matching the expected motifs for the corresponding MHC alleles JY (left), and Raji (right).

Bars with error bars correspond to *average* ± *sd* with points indicating the individual values, and asterisks designate statistical differences (two-sided t-test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

5.4. MAETi enables the MHC1p-ligandome quantitative profiling from 25,000 to 1 Million cells

To evaluate the quantitative performance of the optimized MAETi protocol across different input cell numbers and layouts (on-GF-tip and 96-well plate) we processed samples ranging from 25,000 (25k) to 1 Million (1M) JY cells (Figure 5a). Captured peptides were separated using the 30SPD gradient on an Evosep One coupled with a timsTOF-Ultra2. To assess both coverage and quantitative performance, we acquired the data using Thunder-DDA-PASEF (n=8 each) and MHC-tailored diaPASEF (n=4 each). The combined PEAKS database search results from the DDA-PASEF acquisitions were then used to build spectral libraries used to process the DIA data.

The number of detected peptides and 8-13mers increased in function of the sample input in both DDA and DIA, until reaching a plateau between 500,000 and 1 million cells (Figure 5b, c). Importantly, ≥77% of the identified peptides were 8-13mers across all cell inputs, regardless of the protocol layout used (Suppl. Figure S5b). DIA provided deeper coverage, yielding on average 1.6-fold more peptides and 1.8-fold more 8-13mers per sample compared to DDA. The results ranged from 2,009 ± 83, to 4,750 ± 302 (*mean* ± *sd*) peptides (Figure 5b) and 1,568 ± 54, to 3,884 ± 273 (*mean* ± *sd*) 8-13mer (Figure 5c) as sample inputs increased from 25,000 to 1 Million JY cells, respectively. Unique identifications ranged from 2,573 to 6,519 total peptides from 8 DDA runs and from 3,339 to 6,556 total peptides from 4 DIA runs across the sample input range of 25,000 to 1 million cells (Figure 5d). In addition, DIA expanded the dynamic range of peptides identified for all the sample inputs (Suppl. Figure S5c) and increased the data completeness up to 50% of peptides reproducibly identified across four replicates, compared to >25% in DDA (Suppl. Figure S5d).

Although the proportion of predicted binders (strong binders and weak binders) was slightly lower compared to immunoaffinity purified samples, the range remained high between 63.1% to 72.8% in DDA and 66% to 76.2% in DIA (Figure 5e). These proportions corresponded to an average of 585 ± 73 to 1,872 ± 175 predicted binders in DDA and 1,117 ± 37 to 3,176 ± 248 in DIA from 25,000 to 1 million cells, respectively (Figure 5f).

Altogether, these results show that our MAETi workflow can be applied from as low as 25,000 on a GF-Tip to up to 1 Million cells in a 96-well plate enriching MHC1ps with high specificity. This renders MAETi a versatile tool for immunopeptidomics analyses of limited sample amounts. Furthermore, the advantages of DIA, including its increased sensitivity, dynamic range, and improved data completeness for MHC1p identifications, make the workflow particularly well-suited for robust quantitative profiling of MHC1p- ligandomes across a wide range of cell inputs.

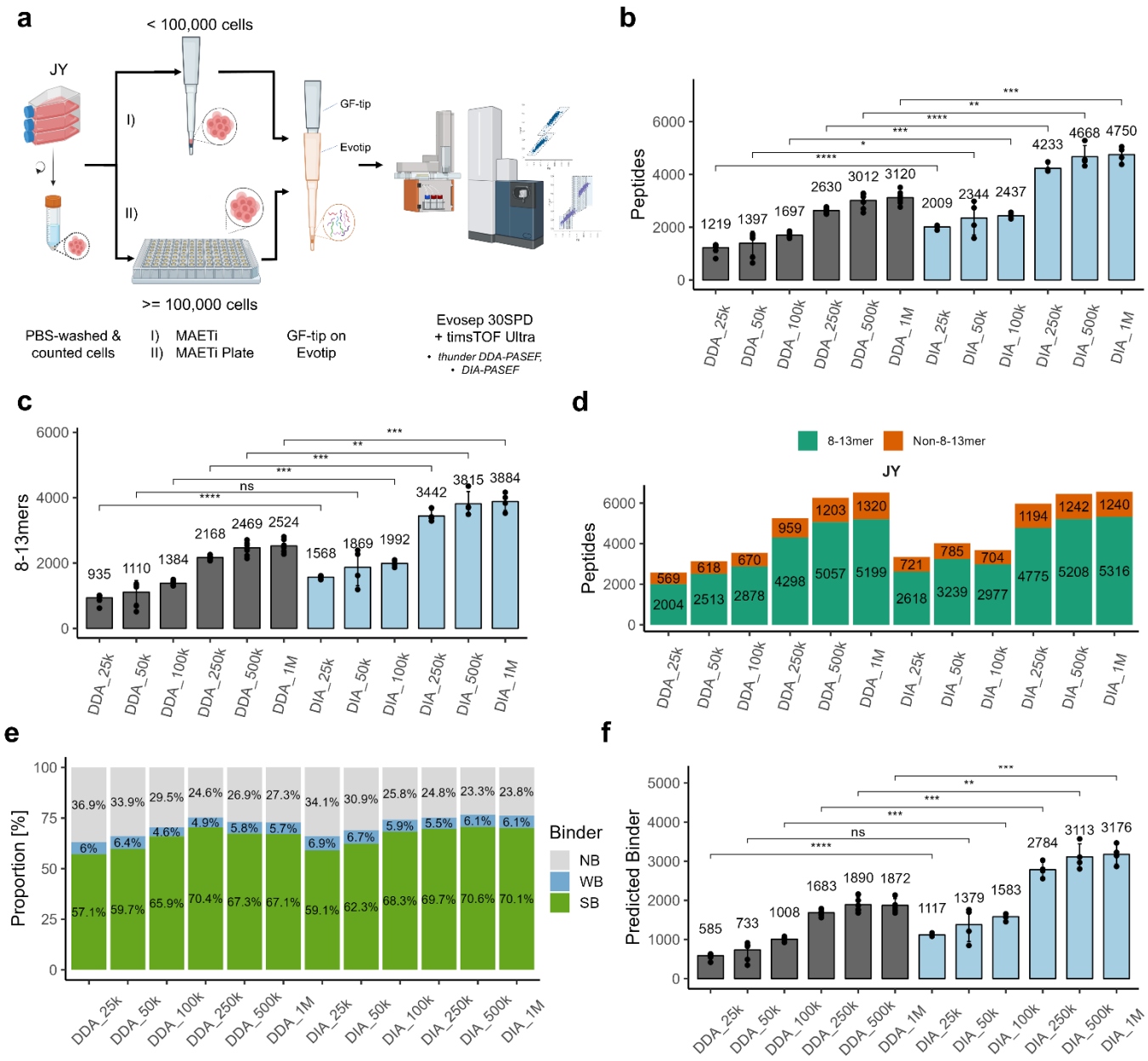


Figure 5: MAETi enables immunopeptidome profiling analyzing 25,000 (25k) to 1,000,000 (1M) JY cells per sample.

a MHC-1 ligands enriched from the human lymphoblastoid cell line JY were either MAE-stripped on the GF tip and directly eluted into the Evotip (cell inputs < 100,000 (100k); **I**) or MAE-stripped in a 96-well plate utilizing 100,000 (100k) to 1,000,000 (1M) cells and supernatants were transferred and passed through the GF-tip on Evotip (**II**). Loaded and washed Evotips were analyzed using an Evosep (30SPD) coupled to a timsTOF Ultra in Thunder-DDA-PASEF and MHC-tailored DIA-schemes. **b** Peptides identified on average per sample. **c** 8-13 mers identified on average per sample. **d** Total peptides identified in DDA (n=8) and DIA (n=4), divided in 8-13mers and other peptide lengths. **e** Proportion of 8-13mers predicted to bind JY MHC alleles (NB, non-binders; WB, weak-binders; SB, strong binders). **f** Predicted binders identified on average per sample.

Bars with error bars correspond to *average* ± *sd* with points indicating the individual values, and asterisks designate statistical differences (two-sided t-test; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

6. Discussion

The optimized MAETi protocol introduced in this study demonstrates sensitive, reproducible and low-cost alternative to conventional and microfluidics immunoprecipitation approaches particularly in low-sample settings. Replacing citric acid with β-alanine in the MAE buffer resulted in lower background ions and improved the MHC1p coverage and reproducibility of our workflow. Our results showed that also on a larger scale, the β-alanine-based MAE showed comparable identification characteristics to conventional bulk immunoprecipitation (IP). We demonstrated that our MAETi workflow can be applied from as low as 25,000 cells on a GF-Tip to up to 1 Million cells in a 96-well plate enriching MHC1ps with high specificity. Using these two protocol layouts (Figure 5a), MAETi is scalable for different types of applications. MAETi-prepared samples can readily be analyzed with both DDA and DIA approaches, enhancing its versatility and analytical depth. Spectral library-based DIA-PASEF improved coverage, dynamic range and data completeness for MHC1p identifications and yielded on average 1117 predicted binders per 25,000 cells and 3176 predicted binders per 1 Million JY cells.

In the past years, the landscape of immunopeptidomics enrichment techniques was dominated by IP[4,9,26]. This is partly due to concerns about yield and unspecific MHC1p elution using MAE[9,26]. However, more recently, Sturm *et al.* proved that an optimized MAE protocol can yield comparable and complementary results to bulk IP, with similar MHC1p enrichment specificity.[9] Thus, we based our initial MAETi buffer on the protocol proposed by Sturm *et al.*[9], and further improved it by replacing citric acid with β-alanine. This minimized the background noise and improved MHC1p coverage in MAETi, and yielded similar coverage and comparable specificity to IP in bulk experiments.

Peptide adsorption on plastic surfaces during proteomics or peptidomics sample handling is known to substantially affect sample yield.[27–30] In fact, several studies reported substantial peptide losses for IP enrichments as they typically involve multiple sample handling steps, including washing steps after MHC-peptide complex capture[10–14]. Due to minimal sample handling compared to bulk experiments, MAETi is not only compatible with lower sample input material but also substantially quicker, providing ready-to-analyze samples in less than 3 hours starting from washed cells.

More recently, IP-based microfluidic approaches have been explored for low-input immunopeptidomics.[10,15,16] In comparison to a microfluidic IP layout ("peptiCHIP") using JY cells[15], MAETi provides enhanced MHC class I-specific peptide length profiles and higher 8-13mer proportions at equal (1 Mio) or lower input cell numbers. In contrast to microfluidics, MAETi does not require specialized equipment and expertise[10], enabling easy and quick adoption by other labs.

One limitation of our study is the use of self-fabricated glass fiber (GF) tips. Although the procedure of fabricating filter-tapered pipette tips has been described before (Rappsilber et.al.[31]), throughput and technical reproducibility of MAETi may be increased by replacing self-fabricated GF-Filter tips with commercially manufactured versions. Exploring automated workflows or integration with high-throughput systems could further expand the utility of MAETi, making it even more accessible for large-scale immunopeptidome studies. In addition, novel diaPASEF methods such as slicePASEF[21] and midiaPASEF[22] continue to push sensitivity and analytical depth. Coupled with novel instrument platforms and advanced processing solutions enabling directDIA searches (e.g. FragPipe v22.0[23] or Spectronaut[24]) or DIA analysis using de novo predicted immunopeptide spectral libraries (e.g. PeptDeep/PeptDeep-HLA[25]), these technologies can further enhance the sensitivity of MAETi.

The MAETi workflow offers a cost-effective and scalable solution for MHC-1 peptide enrichment, providing comparable results to IP while enabling immunopeptidome analysis from as few as 25,000 cells. We anticipate that MAETi will enable immunopeptidomics experiments requiring high sensitivity across various biological applications, such as kinetic drug screenings and profiling immunopeptidomes from FACS- or MACS-sorted cells. MAETi thus holds significant potential for advancing tumor immunology, personalized immunotherapy, and drug discovery.

7. Methods

7.1. Materials and substances

Analytical grade substances and reagents were used for sample preparation, and were acquired from Sigma Aldrich (Merck), unless otherwise stated. Water, acetonitrile, and formic acid were LC-MS grade products acquired from Carl Roth. LoBind tubes (Eppendorf) were employed to minimize sample loss.

7.2. Cell culture

The human B lymphoblastoid cell line JY (CVCL_0108) was purchased from ATCC and the human Burkitt lymphoma cell line Raji (CVCL_0511) was obtained from the DSMZ-German Collection of Microorganisms and Cell Cultures. Both cell lines were maintained in RPMI1640 medium supplemented with 10 % FCS (Gibco (v/v)), 2mM glutamine, 1mM sodium pyruvate, 100 units/ml penicillin, and 100µg/ml streptomycin. Suspension cell cultures were harvested at 220xg for 10min and washed three times with 1x PBS at 4°C prior counting and freezing at −80 °C until further use.

7.3. MAE Filter Tip (MAETi) Preparation

Glass fiber filter tips were prepared as described by Rappsilber *et al.*[31] using a blunt-cut injection needle (BD microlance 3, 1.2 x 40 mm, 18G) and a 1.7 µm glass fiber (GF) membrane (GE Healthcare, Whatman™ CAT No. 1820-030) tapered into a Protein LoBind tip (Greiner BioOne Sapphire Tips). Prior utilization in the MAETi procedure (Section 7.4), fabricated tips were washed twice with PBS and once with MAE buffer at 220 x g, for 1 min.

Evotips were prepared as described in the manufacturer's manual. Briefly, after washing, conditioning and equilibration, Evotips were placed on a rack filled with 0.1% FA (v/v) in MS-grade water prior sample loading (Section 7.4) to avoid drying of the resin and stored at 4°C until further use.

7.4. Immunopeptide enrichment by mild acid elution in a tip (MAETi)

All next steps were either performed at 4°C or on ice to ensure sample integrity. Depending on the input cell number, we used two protocol layouts (also see Figure 5a) to profile input amounts across a range from 25,000 to 1 million cells.

In brief, cells were washed and prepared according to their input number, transferred to the respective protocol layout (onGFTip or inPlate, Figure 5a), followed by MAE buffer treatment and peptide capture on the Evotip. For both recommended protocol versions, eluted peptides captured by the Evotip were subsequently washed three times with 20 µL 0.1% FA (v/v) in Water and finally topped up with 200 µL 0.1% FA (v/v) in Water.

The original citric acid-based MAE buffer formulation was adapted from Sturm *et al.*[9] adjusted to pH 3.3. Note, that for section 5.2 the citric acid- and a β-alanine-based MAE buffer were compared but used at equimolar concentrations. Pure substances were compared using equimolar amounts of pure buffer substances prepared in glass bottles, directly transferred to the Evotip.

7.5. Immunopeptide enrichment by bulk mild acid elution (MAE)

The initial mild acid elution (MAE) protocol and buffer recipe was based on a protocol published by Sturm *et al.*[9], with slight adaptations. Throughout the MAE procedure, all steps were performed at 4°C or on ice. Briefly, pre-washed and counted whole cells (see section 8.3) were pelleted at 220 x g, 10 min and 40 Million cells per replicate (n = 3) were resuspended in ice-cold MAE buffer (pH 3.3) adapted from Sturm *et al.*[9] by pipetting up and down three times and gently tapping the tubes during a 1 min incubation on ice. After incubation, the MAE supernatant was immediately removed by centrifugation at 300 x g for 5 min and further cleared by centrifugation at 350 x g for 10 min and 3,500 x g for 15 min. To get rid of potential cellular debris, retrieved MAE supernatant was further cleared twice at 16,000 x g for 20 min, transferring the supernatant to a new tube in between.

7.6. Immunopeptide enrichment by bulk immunoprecipitation (IP)

Class I MHC immunoprecipitation was performed as previously described by Gomez-Zepeda *et al*.[17] (detailed protocol in Gomez-Zepeda *et al.* 2024[32]). Briefly, frozen aliquoted and PBS-washed cell pellets at 40 Million cells per replicate (n = 3) were thawed in lysis buffer (1% CHAPS (v/v) in PBS), subjected to sonication (Diagenode™ Bioruptor Plus: 10 cycles, 30s on/off for a total duration of 10 min) and incubated overnight at 4°C with W6/32 antibody (Leinco™ Anti-Human HLA-A, B, C – purified in vivo GOLD functional grade, P/N H263) coupled to CNBr-activated Sepharose (Cytiva™; P/N 17098101). Captured MHC class I peptide complexes were washed with PBS, MS-grade H_2O and eluted with 0.2% trifluoroacetic acid (TFA, (v/v)).

7.7. Ultrafiltration and peptide desalting of bulk IP and MAE samples

Samples prepared by bulk IP and bulk MAE were ultrafiltered (Sartorius® Vivacon 500, 10.000 MWCO Hydrosart) prior desalting by SPE on a Hydrophilic-Lipophilic-Balanced (HLB) sorbent (Oasis HLB 96-well µElution Plate, 2 mg Sorbent per Well, 30 µm, Waters Corp.). Peptides were eluted from the HLB resin with 35% ACN (v/v) in MS-grade H_2O + 0.1% TFA (v/v), lyophilized and reconstituted in 15 µL 0.1% FA (v/v) in MS-grade H_2O prior LC-MS analysis (Section). Samples were injected utilizing approximately 1 µL (~ 50 ng; ~ 2 million cell equivalents (MCEs)) of each replicate in technical triplicates.

7.8. Bulk IP and MAE LC-IMS-MS acquisitions on timsTOF Pro 2

Samples for the bulk comparison (Section 5.3; Figure 5) were separated using a NanoElute2 coupled to a timsTOF Pro 2. Desalted peptides obtained from bulk-MAE and bulk IP preparations (Section 7.5-7.7) were directly injected in a C18 Reversed-phase (RP) Aurora 25 cm analytical column (25 cm x 75 µm ID, 120 Å pore size, 1.7 µm particle size, IonOpticks, Australia) and separated using gradients increasing the proportion of phase B (ACN + 0.1% FA (v/v)) to phase A (water + 0.1% FA (v/v)) over 47 min. 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. Bulk IP and MAE samples (as obtained under Section 7.5-7.7) were analysed in three injection replicates for each biological/preparation replicate, using a target of 50 ng (~2 MCEs) per injection (detailed outline in Supplemental) acquiring data utilizing a non-MHC-tailored “stepped” DDA-PASEF precursor selection polygon including singly-charged ions for unbiased comparison.

7.9. MAETi LC-IMS-MS acquisition on timsTOF SCP

MAETi-eluted peptides captured on Evotips in section 7.4 were separated at 50 °C on a C18 RP Aurora Elite 25 cm analytical column (15 cm x 75 µm ID, 120 Å pore size, 1.7 µm particle size, IonOpticks, Australia) using the 40SPD whisper method on the Evosep One nanoLC system coupled to a timsTOF SCP. A Captive Spray source was used for ionization, with a capillary voltage of 1400 V, dry gas at 3.0 L/min, dry temperature at 200 °C, and TIMS-in pressure of 2.7 mBar. For proof of principle experiments (Section 5.1) 50,000 cell samples were injected utilizing a non-MHC-tailored “stepped” polygon and the MHC-tailored “thunder” polygon[17] (Figure 3, b). Buffer comparisons (Section 5.2) were acquired applying the same settings using the “stepped” polygon for unbiased comparison. All acquisitions on the timsTOF SCP utilized a 100 ms TIMS ramp, a scan range of from 100-1400 m/z, 10 PASEF ramps and a cycle time of 1.17 sec.

7.10. MAETi LC-IMS-MS acquisition on timsTOF Ultra

MAETi-eluted peptides captured on Evotips in section 7.4 were separated at 50 °C on a C18 Reversed-phase (RP) analytical EV1137 Performance column (ReproSil-Pur C18, 1.5 µm

beads by Dr Maisch. 15 cm x 150 µm) using the 30SPD method on the Evosep nanoLC system and 20 µm zero-dead volume CaptiveSpray 2 Emitters (Bruker, Part No:1811112). An Ultra source was used for ionization, with a capillary voltage of 1500 V, dry gas at 3.0 L/min, dry temperature at 200 °C at 1 mBar Funnel and 2.38 mBar TIMS-in pressure. Ions were accumulated and separated utilizing a 60 ms TIMS ramp. Acquisitions in Thunder-DDA-PASEF utilized the "thunder" MHC-tailored precursor isolation polygon[17] selecting precursors (charge +1 to +3) in the range of 100-1700 m/z and 0.65 to 1.76 V*s/cm^2 for fragmentation utilizing 16 PASEF ramps per cycle, resulting in total cycle time of 1.12 sec. Precursors were isolated with 2 Th windows below 700 m/z and 3 Th above and, when reaching a target intensity of 20,000 and an intensity threshold of 500 arbitrary units, actively excluded for 0.4 min. High-sensitivity mode was activated.

7.11. Peptidomics database search

Data analysis was performed in PEAKS XPro (v10.6, build 20201221). 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), supplemented with a list of 172 possible contaminants. For database searches, protein in silico digestion was configured to unspecific cleavage and no enzyme. Up to two variable modifications per peptide were allowed, including Methionine oxidation, cysteine cysteinylation, and Protein N-terminal acetylation. For timsTOF data, the option *timstof_feature_min_charge* (in file *PEAKSStudioXpro\algorithmpara\feature_detection_para.properties*) was set to 1 to allow the identification of singly-charged features. Raw LC-MS files were loaded with the configuration for timsTOF DDA-PASEF data with CID fragmentation. Mass tolerance was set to 15 ppm for MS1 and 0.03 Da for MS2.

7.12. Experiment design

Experimental descriptions including number of replicates per conditions are detailed in the respective result sections.

7.13. Data analysis and statistics

Data analysis, statistical analysis and visualization were done mostly using in house R scripts[33]. The main R packages used were as follows; the statistical difference was assessed by two-sided t-test using ggpubr (v. 0.4.0)[34]; plots were generated using ggplot2 (v. 3.4.0)[34]; Venn plots with ggvenn (v. 0.1.9)[35]; upset plots with ggupset (v. 0.3.0)[36]; and sequence logos were generated using ggseqlogo[37].

Binding prediction was performed using NetMHCpan-4.130 and sequence clustering with GibbsCluster-2.064, both via MhcVizPipe (v0.7.9)[18]. The MHC selected allotypes used for

prediction were as follows: JY (HLA-A*0201, -B*0702, -C*0702), Raji (HLA-A*03:01, -B*15:11, -C*03:04, -C*04:01).

Note that throughout the manuscript “Peptides” refers to peptides including their modifications, “Sequences” refers to the “stripped peptide” (no modifications) and binders refers to “Sequences” predicted to be weak or strong MHC binders for the sample-specific HLA alleles.

7.14. Data availability

The mass spectrometry immunopeptidomics data will be deposited to the ProteomeXchange Consortium[38].

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10. Author contributions

Conceptualization: DGZ, ST. Methodology: JB, DGZ, ST. Software: JB. Validation: JB. Formal analysis: JB. Investigation: JB, UD, DGZ. Resources: UD, DGZ, ST. Data Curation: JB, DGZ. Writing - Original Draft: JB, DGZ. Writing - Review & Editing: all the co-authors. Visualization: JB. Supervision: DGZ, ST. Project administration: DGZ, ST. Funding acquisition: ST.

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12. Competing interests

The authors declare no competing interests.