Innovative CRISPR/Cas9-Based Strategy for Allele-Specific HLA Peptidome Analysis Using a Pan-HLA Antibody

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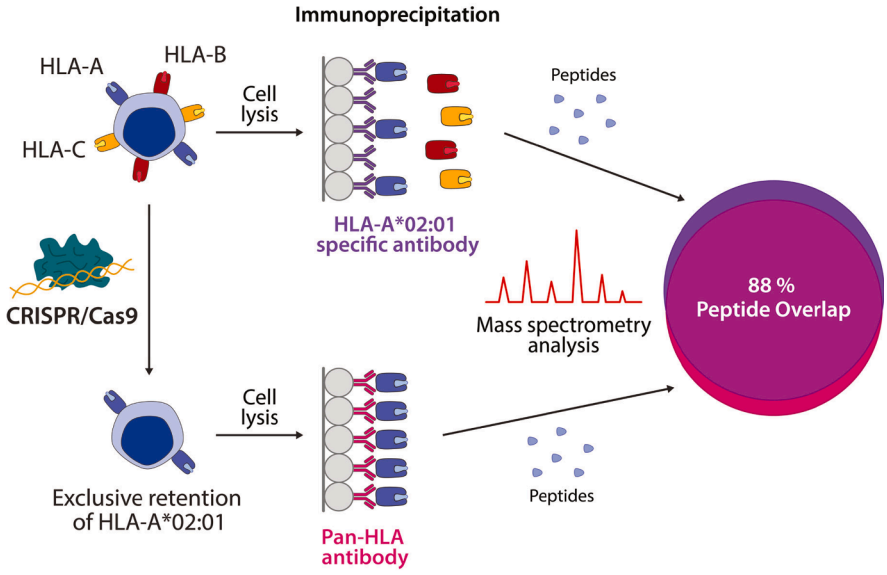
Graphical Abstract



In Brief

This study introduces a CRISPR/Cas9-based strategy that enables allele-specific HLA immunopeptidome analysis without relying on allele-specific antibodies. By selectively deleting undesired HLA class I alleles while preserving endogenous expression of a single targeted allele, this approach allows accurate peptide profiling using a pan-HLA antibody. The method expands immunopeptidomics to underrepresented HLA alleles and provides a scalable, potentially more physiological framework for antigen discovery and immune monitoring.

Highlights

- CRISPR/Cas9-mediated selective HLA deletion enables allele-specific immunopeptidomics.
- Endogenous expression of a single HLA allele allows pan-HLA–based peptide profiling.
- Pan-HLA capture in HLA-A2ΔBC clones mirrors WT peptide yield with specific antibody.
- High peptide overlap validates the specificity and fidelity of the CRISPR approach.

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Innovative CRISPR/Cas9-Based Strategy for Allele-Specific HLA Peptidome Analysis Using a Pan-HLA Antibody

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Human leukocyte antigen (HLA) immunopeptidomics is restricted by the limited availability of allele-specific antibodies and by potential artifacts introduced by HLA overexpression systems. To address these challenges, we developed a CRISPR/Cas9-based strategy that selectively deletes undesired classical class I alleles while preserving a single endogenous allele, thereby enabling allele-resolved peptidome profiling with a pan-HLA class I antibody. As a proof of concept, we edited JY cells to eliminate HLA-B*07:02 and HLA-C*07:02 while retaining HLA-A*02:01 (ΔBC clones). Peptide–HLA complexes were immunoprecipitated from WT and ΔBC clones using either the pan-HLA class I antibody W6/32 or the A*02:01-specific antibody PA2.1, followed by nanoLC-MS/MS and computational HLA assignment. Deletion of HLA-B and HLA-C alleles caused an expected ~55% reduction in total class I surface expression. Despite this, W6/32 immunoprecipitation from ΔBC clones recovered a comparable peptide yield to PA2.1 in WT cells. Binding predictions showed that most peptides identified in ΔBC clones using W6/32 were assigned to HLA-A*02:01, with near-complete loss of HLA-B*07:02– and HLA-C*07:02–derived peptides. Sequence logo analysis confirmed the canonical A*02:01 motif across conditions. The ΔBC W6/32 immunopeptidome exhibited a high degree of overlap (~88%) with the WT PA2.1 repertoire, supporting the specificity and fidelity of the approach. These findings establish CRISPR-based editing of HLA alleles as a viable strategy for allele-specific immunopeptidome analysis using pan-HLA antibodies, supporting its potential application beyond this proof-of-concept system, reducing reliance on allele-specific reagents and facilitating the study of underrepresented HLA alleles.

Human leukocyte antigen (HLA) classical class I molecules (HLA-A, HLA-B, and HLA-C) are encoded within the HLA locus on chromosome 6. They are expressed in almost all nucleated cells and present intracellularly processed peptides to T cells, playing a fundamental role in distinguishing self from non-self and shaping immune responses against infections, cancer, and autoimmune diseases.

The study of HLA-bound peptide repertoires is crucial for understanding antigen presentation, immune surveillance, and the development of immune-based therapies (1). Identifying and characterizing these peptide repertoires, collectively referred to as the immunopeptidome, provides insights into disease mechanisms and informs the design of vaccines and immunotherapies. However, current methodologies still face significant challenges.

Mass spectrometry–based immunopeptidomics has become the gold standard for characterizing HLA-presented peptides (2). One of the main challenges in this field is the need for immunoprecipitation (IP) to selectively isolate peptides bound to specific HLA alleles (3). While pan-HLA antibodies, such as W6/32 (4), allow the capture of peptides from all HLA class I molecules, they do not enable precise characterization of allele-specific peptide repertoires. To address this limitation, allele-specific antibodies have been developed for some well-characterized HLA alleles, such as HLA-A*02:01 (5, 6). However, given the extreme polymorphism of the HLA system, with 28409 HLA class I alleles recorded as of March 2025 (7), most alleles still lack specific antibodies, significantly complicating their study.

Since the 1990s, researchers have circumvented this limitation using transfection-based approaches, in which full-length, naturally occurring HLA molecules are overexpressed in cell lines with low or no endogenous HLA class I expression, such as C1R, K562, or B721.221 (8–10). Additionally, recombinant soluble HLA molecules, which lack transmembrane and cytosolic domains, have also been overexpressed (11). These approaches have been well established and widely used for decades. Indeed, they continue to be employed in recent studies (12, 13).

However, the HLA-peptidome is influenced by a variety of factors, including gene expression (14). In the approaches

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mentioned, HLA molecules are overexpressed in an artificial context, bypassing natural regulatory mechanisms. This can influence the repertoire of peptides presented, potentially skewing it from physiological conditions. Moreover, the cell surface expression of peptide-loaded HLA molecules is determined more by the presence of empty HLA molecules than by peptide availability (15).

Nowadays, affinity prediction algorithms are widely used for classifying HLA-peptide binding when cells expressing multiple HLA molecules are analyzed (16, 17). However, these algorithms remain inaccurate for underrepresented HLA alleles, requiring further experimental efforts to improve their predictive capabilities. Some studies aim to enhance model accuracy by analyzing the immunopeptidome in mono-allelic cell models, but these are typically generated using the previously mentioned cell lines that lack natural gene regulation and may introduce artifacts due to HLA overexpression (12, 18).

To provide a more physiologically relevant alternative, we explored a CRISPR/Cas9-based approach to selectively eliminate specific HLA alleles while retaining a single classical HLA class I molecule in its natural cellular context. This strategy enables the study of individual HLA peptide repertoires using a general pan-HLA class I antibody, offering a potential solution for investigating alleles that lack specific antibodies.

Here, we report the implementation of a CRISPR strategy to selectively retain the HLA-A*02:01 allele as a proof of concept. This configuration enables the pan-HLA class I antibody W6/32 to function effectively as an allele-specific probe. The availability of the HLA-A*02:01-specific antibody, PA2.1, allowed us to validate this approach through direct comparison. We used the JY cell line, which homozygously expresses HLA-A*02:01, HLA-B*07:02, and HLA-C*07:02, and generated CRISPR-edited clones deficient in HLA-B*07:02 and HLA-C*07:02 (ΔBC clones). By comparing immunoprecipitations using W6/32 and PA2.1, we aimed to assess whether the peptide repertoire captured from ΔBC clones with W6/32 would closely match that of WT clones with PA2.1.

Our results demonstrate that this CRISPR/Cas9 strategy enables the isolation of HLA-specific peptide repertoires even in the absence of allele-specific antibodies. This approach broadens the scope of immunopeptidomics, providing a valuable tool to study underrepresented HLA alleles and furthering our understanding of antigen presentation in diverse populations.

EXPERIMENTAL PROCEDURES

Cell Lines

Human JY cell line, an Epstein-Barr virus–immortalized B-lymphoblastoid cell line, part of the HLA typed collection maintained by the European Collection of Cell Cultures, was used. This cell line is homozygous for the HLA class I molecules HLA-A*02:01, HLA-B*07:02, and HLA-C*07:02. Unedited (WT) clones and clones deficient in HLA-B and HLA-C genes generated in a previous study (ΔBC) were used (19). The deficient clones were generated using a one-step CRISPR/Cas9 strategy targeting the simultaneous disruption of both HLA-B*07:02 and HLA-C*07:02 class I molecules. Guide RNA design was restricted to exons 2 and 3, which encode the most polymorphic regions of the antigen-binding groove. The selected guide (g615; 5′-CGACGCCGCGAGTCCGAGAG-3′) targets a sequence conserved between HLA-B*07:02 and HLA-C*07:02, but not in HLA-A*02:01, thereby enabling selective editing of these alleles. JY cell clones were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 1% penicillin (10,000 UI)-streptomycin (10 mg/ml), and 1% L-glutamine (200 mM), under standard conditions (37 °C, 5% CO2).

Antibodies

The monoclonal antibodies used in this study were generated in-house from hybridoma cell lines, W6/32 (specific for a monomorphic HLA class I) (20) and PA2.1 (specific for polymorphic sites of the HLA-A*02:01) (6).

Major Histocompatibility Complex Class I–bound Peptide Isolation

HLA-bound peptides were isolated from two independent preparations of 3×10^9 cells from WT 1, WT 2, ΔBC 1, ΔBC 2 cell clones as previously described (21). Cells were lysed for 1h at 4 °C in 20 ml of lysis buffer containing 1% Igepal CA-630 (Sigma-Aldrich), 20 mM Tris–HCl buffer, and 150 mM NaCl, pH 7.5, in the presence of a protease inhibitor cocktail (11697498001; F. Hoffmann-La Roche Ltd). After centrifugation, the supernatants were passed first through a control precolumn containing CNBr-activated Sepharose 4B (GE Healthcare) to remove nonspecific peptides. HLA–peptide complexes were isolated by affinity chromatography from the soluble cell extract using either the PA2.1 or W6/32 monoclonal antibody immobilized on CNBr-activated Sepharose beads. Antibodies were coupled to the beads at a ratio of 2 mg antibody per 1 ml Sepharose by overnight incubation at 4 °C. A total of 1.5 ml of antibody-conjugated Sepharose was used to prepare the affinity columns. Cell lysates were then passed through the columns at 4 °C to capture HLA–peptide complexes. Next, the columns were successively washed with 20 mM Tris–HCl, pH 8.0, containing the following: 1) 150 mM NaCl; 2) 400 mM NaCl; 3) 150 mM NaCl; and 4) buffer without NaCl. The HLA-bound peptides were eluted with 1% aqueous trifluoroacetic acid (Sigma-Aldrich), separated and concentrated from the HLA molecules by ultrafiltration using a Vivaspin 2 filter (5000 MWCO HY; Sartorius Stedim Biotech), and further concentrated in a SpeedVac (Savant; Thermo Fisher Scientific). Finally, peptide mixtures were desalted using OMIX Tips (C18, Agilent Technologies).

Mass Spectrometry Analysis

Desalted samples were analyzed by LC-MS/MS using a Q-Exactive-Plus mass spectrometer fitted with an Ultimate 3000 RSLC nanocapillary U-HPLC (Thermo Fisher Scientific). The peptides were resolved on homemade Reprosil C18-Aqua capillary columns (Dr Maisch GmbH) (22) with a 7 to 40% acetonitrile linear gradient for 180 min in the presence of 0.1% formic acid at a flow rate of 0.15 μl/min (22). The dynamic exclusion was set to 20 s. The selected masses were fragmented from the survey scan of m/z 300 to 1800 AMU at a resolution of 70,000. The ten most intense masses from each full mass spectrum were fragmented by higher-energy collisional dissociation. Tandem mass spectrometry (MS/MS) spectra were acquired with a resolution of 17,500 at m/z 200. The target value of the MS/MS was set to 1×10^5 and the isolation window to 1.8 m/z.

The maximum injection time was set to 100 ms, and normalized collision energy was set to 25 eV.

HLA-Peptidome Identification

Peptides were identified from MS/MS spectra obtained independently for each clone and immunoprecipitation against the human UniProt/Swiss-Prot database of May 2023 (73,101 entries). Database searches were performed using MaxQuant (version 2.4.9.0) (23) with a precursor ion mass and fragment mass tolerance of 20 ppm. False discovery rates were set to 0.01 for the protein and site level and 0.05 for the peptide-spectrum match level. Additionally, searches were performed with no protease specificity selected, oxidation (M) set as a variable modification, and excluding contaminants and reverse sequences.

Immunopeptidome Data Analysis

All downstream data analyses following peptide identification by PEAKS and MaxQuant were performed using RStudio (version 2025.05.1 + 513; Posit, PBC). Peptide identifications from biological replicate clones were pooled for each immunoprecipitation condition. Only peptides ranging from 8 to 15 amino acids in length were included, as this range encompasses the majority of HLA class I ligands. For sequence logo generation and Venn diagrams, ggseqlogo and eulerr or VennDiagram R packages were employed. HLA-peptide binding predictions were performed using NetMHCpan 4.1 (16).

Experimental Design and Statistical Rationale

Peptides bound to HLA-A*02:01, HLA-B*07:02, and HLA-C*07:02 were isolated from two independent biological replicates for each condition (WT1 and WT2 for WT cells; ΔBC1 and ΔBC2 for edited clones). Each replicate consisted of 3×10^9 cells processed following established protocols. Two technical replicates were analyzed per biological replicate (1.5×10^9 cells per technical replicate), resulting in four independent LC-MS/MS runs per condition, which supports robust statistical evaluation and minimizes experimental variability.

Statistical analyses were performed using GraphPad Prism (version 10; GraphPad Software). Statistical tests included one-way Kruskal-Wallis tests with Dunn's post hoc correction and two-way ANOVA followed by Šidák's multiple comparisons test. Results are presented as mean ± SEM (or mean ± SD where indicated), and the number of biological replicates is reported in each figure legend. A p-value <0.05 was considered statistically significant.

RESULTS

WT and ΔBC Clones Using PA2.1 and W6/32 Antibodies Yield a Similar Number of Identified Peptides

In this study, we evaluated whether CRISPR/Cas9-mediated deletion of all classical HLA alleles except the one of interest provides a viable strategy for studying HLA peptide repertoires in the absence of allele-specific antibodies. To this end, we used both JY WT clones (HLA-A*02:01$^{+/+}$; HLA-B*07:02$^{+/+}$; HLA-C*07:02$^{+/+}$) and JY ΔBC clones (HLA-A*02:01$^{+/+}$; HLA-B*07:02$^{-/-}$; HLA-C*07:02$^{-/-}$), in which HLA-B and HLA-C alleles were disrupted by CRISPR/Cas9, as previously described (19) (Fig. 1*A*). We analyzed total HLA-Class I and HLA-A*02:01 expression levels by flow cytometry in these clones, labeling cells with W6/32 and PA2.1 monoclonal antibodies, respectively (Fig. 1*B*). No significant differences in HLA-A*02:01 expression were observed between WT and ΔBC clones. However, a significant 55% decrease in total HLA-class I expression was detected in ΔBC clones compared to WT, consistent with the loss of HLA-B and HLA-C molecules. We then sought to determine whether the peptide repertoire of JY ΔBC clones immunoprecipitated with the pan-specific HLA antibody W6/32 matches that of JY WT clones immunoprecipitated with the HLA-A*02:01-specific antibody PA2.1. Thus, protein extracts from two WT and two ΔBC clones were individually subjected to affinity chromatography–based immunoprecipitation using W6/32 (α-pan HLA class I) or PA2.1 (α-HLA-A*02:01) antibodies (Fig. 1*C*).

In WT clones, immunoprecipitation with PA2.1 resulted in the identification of 2519 peptides similarly to ΔBC clones immunoprecipitated with W6/32, which yielded 2821 peptides (Supplemental Table S1). These results confirm that despite the overall reduction in HLA-class I expression due to the deletion of HLA-B and HLA-C, the ΔBC clones retain robust HLA-A*02:01 expression and yield a comparable number of peptides when immunoprecipitated with the pan-HLA class I antibody W6/32.

*Immunoprecipitation of ΔBC Clones with a Pan-Specific Antibody Retrieves HLA Class I Peptides from HLA-A*02:01*

Utilizing the NetMHCpan 4.1 tool from the Technical University of Denmark (DTU) (16), we assigned each identified peptide to the HLA molecule for which it exhibited sufficient binding affinity (threshold rank <2). In cases where a peptide showed strong affinity for multiple HLA molecules, it was assigned to the one with the highest binding score.

In WT clones immunoprecipitated with the pan-HLA class I specific antibody W6/32, approximately 45% of the peptides were assigned to HLA-A*02:01, ~41% to HLA-B*07:02, ~1% to HLA-C*07:02, and ~12% remained unassigned. When the same immunoprecipitation was performed in ΔBC clones lacking HLA-B*07:02 and -C*07:02 alleles, we clearly observed how peptides associated with these alleles were lost, dropping to ~0 to 0.5%, while peptides associated with HLA-A*02:01 increased to ~84%. These clones retained ~16% unassigned peptides (Fig. 2*A*, left). Notably, only a very small fraction of peptides was associated to HLA-C*07:02, in accordance with the idea that in basal conditions, HLA-C is considerably less expressed on the cell surface than HLA-A and -B (24, 25).

On the other hand, when immunoprecipitation was performed with the HLA-A*02:01–specific antibody PA2.1, as expected, both WT and ΔBC clones exhibited ~75 to 80% HLA-A*02:01–assigned peptides and ~18 to 21% unassigned peptides (Fig. 2*A*, right). Interestingly, a small but detectable fraction (~3%) of HLA-B*07:02-associated peptides were identified in WT clones immunoprecipitated with PA2.1. This percentage was nearly absent (~0.6%) in ΔBC clones under the same conditions or in ΔBC clones immunoprecipitated

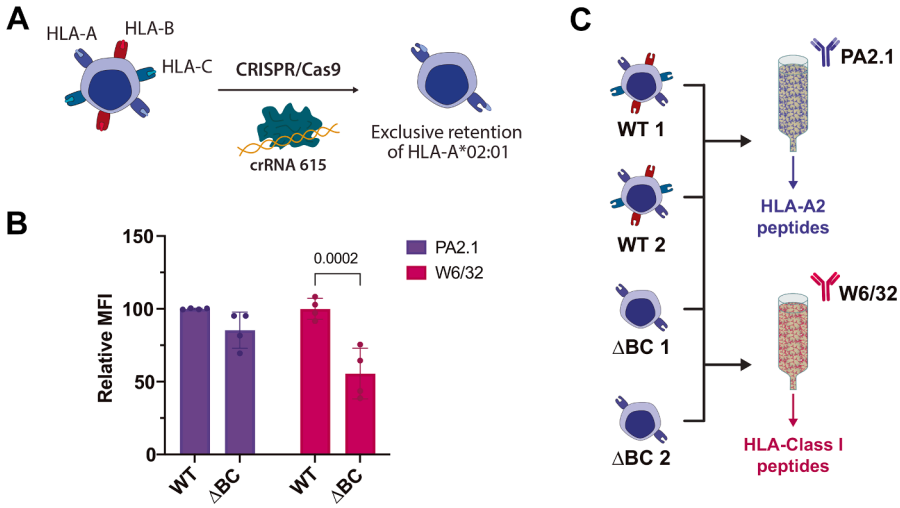

FIG. 1. **Characterization of edited clones and immunoprecipitation strategy. *A*, schematic representation of the CRISPR/Cas9-based strategy used to selectively eliminate HLA-B and HLA-C expression in JY cells.** *B*, flow cytometry analysis of HLA-A*02:01 and total HLA class I surface expression in unedited (WT) and edited (ΔBC) JY clones, stained with PA2.1 and W6/32 antibodies, respectively. Data represent mean ± SD from two independent experiments using two independent clones. Statistical analysis was performed using two-way ANOVA. *C*, schematic representation of the workflow used for the immunoprecipitation of HLA complexes. WT and ΔBC clones were individually subjected to immunoprecipitation using α-HLA-A*02:01, PA2.1 (*purple*) and α-pan-HLA class I, W6/32 (*dark pink*).

with W6/32. The presence of HLA-B*07:02–associated peptides in the HLA-A*02:01–specific immunoprecipitation could be attributed to a limitation in the NetMHCpan algorithm or to the assignment of certain peptides to both HLA molecules. However, we have checked the affinity ranks calculated by the algorithm, and none of them could be attributed to the binding motif of HLA-A*02:01, but rather to the binding motifs of HLA-B*07:02. Moreover, the fact that this percentage was higher in WT clones than in ΔBC clones suggests a higher purity of results in the edited clones.

The 10 to 20% unassigned peptides across all clones and conditions could be attributed to peptides bound to nonclassical HLA class I molecules in the W6/32 immunoprecipitation. However, in the PA2.1 immunoprecipitation, this fraction would need to be explained by the absence of the expected binding motifs required by the NetMHCpan algorithm for classification.

To further explore the reasons behind the lack of HLA assignment, we analyzed the peptide length distribution across the different clones and immunoprecipitation conditions. As expected, peptides assigned to HLA-A*02:01 were predominantly 9-mers, although ten-mers and 11-mers were also detected (Fig. 2*B*), with no significant differences observed among groups. In contrast, when analyzing the "Not assigned" peptides, we observed a more variable length distribution in which, although 9-mers still represent the majority, there was a noticeable increase in peptides ranging from 10 to 15 amino acids. This suggests that longer peptides are less likely to be confidently assigned to a specific HLA molecule, potentially due to reduced binding prediction scores. It is also possible that a fraction of the unassigned peptides corresponds to common contaminants that copurify with the affinity column (26).

Our CRISPR/Cas9-edited ΔBC clones, in combination with the pan-HLA class I antibody W6/32, effectively isolate HLA-A*02:01-associated peptides.

*W6/32 ΔBC Clones Immunoprecipitation Reveals an HLA-A*02:01–Specific Peptide Signature*

Having established that peptides identified in ΔBC clones are predominantly associated with HLA-A*02:01, we next aimed to confirm the specificity of this repertoire by comparing peptide identities and binding motifs across conditions.

To assess the overlap of peptides, we generated a comprehensive heatmap that included all peptides identified in at least one sample. Peptides were organized according to amino acid properties (H, R, K, I, L, M, V, C, A, W, F, Y, N, Q, T, S, G, P, D, E), and binary values (1 for presence, 0 for absence) were assigned to each peptide per sample.

We observe that WT clones immunoprecipitated with W6/32 show a broad distribution across all peptides, whereas several peptide bands clearly disappear when PA2.1 was used (Fig. 3*A*). Given that PA2.1 is specific for the HLA-A*02:01 allele, we assume the absent peptide bands correspond to HLA-B*07:02 and HLA-C*07:02, while the present bands correspond to HLA-A*02:01. This pattern becomes even more pronounced in ΔBC clones in both IPs. Moreover, the HLA-A*02:01 bands in these edited clones with W6/32 appear stronger, supporting the higher ability of W6/32 to capture peptides compared to PA2.1.

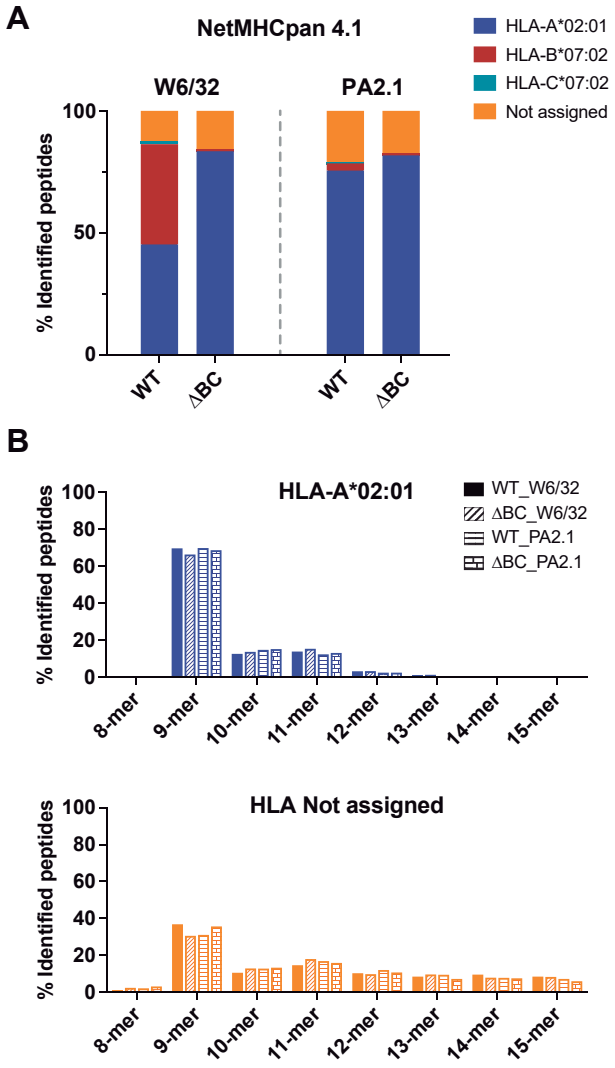

FIG. 2. **NetMHCpan 4.1 predictor associates ΔBC clone peptides identified with W6/32 to the HLA-A*02:01 allele.** *A*, stacked bar plots showing the proportion of unique peptides from unedited (WT) and edited (ΔBC) JY clones, immunoprecipitated with α-HLA-A*02:01 (PA2.1) or α-pan-HLA class I (W6/32), predicted by NetMHCpan 4.1 to bind HLA-A*02:01 (*blue*), HLA-B*07:02 (*red*), or HLA-C*07:02 (*green*), or left unassigned (*yellow*). *B*, length distribution of peptides assigned to HLA-A*02:01 (*blue*, *top*) and unassigned peptides (*yellow*, *bottom*).

To ensure peptide specificity, we plotted the binding motifs of the groups for the 9-mers, as this is the most prevalent peptide length among MHC class I ligands. WT clones immunoprecipitated with PA2.1 displayed the expected HLA-A*02:01 motif, characterized by leucine at position 2 (P2) and valine/leucine at P9, with occasional aspartate or glutamate at P4 (Fig. 3*B*). As expected, this pattern is present in the PA2.1 IP of edited clones, but what is crucial is that this pattern is also present in the edited clones immunoprecipitated with W6/32.

To evaluate whether the peptides exclusively identified in WT_W6/32 samples originated from HLA-B*07:02 or HLA-C*07:02, we subtracted peptides shared across WT_PA2.1, ΔBC_PA2.1, and ΔBC_W6/32 from the WT_W6/32 dataset. The remaining peptides displayed a motif consistent with HLA-B*07:02, with proline at P2 and a hydrophobic residue (leucine, methionine, phenylalanine, isoleucine, or valine) at P9. Peptides associated with HLA-C*07:02 were underrepresented, consistent with the low abundance observed in Figure 2*A*. Although HLA-C*07:02 shares the P9 anchor (L/M/F) with HLA-B*07:02, its additional motif features, a tyrosine at P9 and a tyrosine or arginine at P2, were detectable but barely visible, reflecting their low frequency.

Collectively, these findings validate that ΔBC clones lacking HLA-B and HLA-C molecules present a highly specific HLA-A*02:01 peptide repertoire. The consistent identification of the HLA-A*02:01–binding motif in both PA2.1 and W6/32 immunoprecipitations from edited clones, together with the absence of HLA-B*07:02 and HLA-C*07:02 motifs, further validate the specificity and utility of this genome editing strategy to isolate the peptide repertoire of a single HLA allele in the absence of allele-specific antibodies.

High Immunopeptidome Similarity Between PA2.1 Immunoprecipitated WT Clones and W6/32 Immunoprecipitated ΔBC Clones

Since we have demonstrated that peptides identified in edited clones using the pan-HLA class I antibody W6/32 are specific to the HLA-A*02:01 molecule, we now aim to explore the overlap in peptide repertoires across different conditions, focusing on a defined cohort of peptides shared by both WT clones immunoprecipitated with W6/32. This cohort was carefully selected to minimize variability stemming from biological replicates and the mass spectrometry (MS) workflow. It includes 1110 peptides, representative of the previously described dataset, of which 51.71% were assigned to HLA-A*02:01, 39.46% to HLA-B*07:02, 1.08% to HLA-C*07:02, and 7.75% remained unassigned (Fig. 4*A*).

Within this reference cohort, we detected 619 peptides in WT clones immunoprecipitated with PA2.1, 533 in ΔBC clones with PA2.1, and 596 in ΔBC clones with W6/32 (Fig. 4*B*). In all three conditions expected to isolate HLA-A*02:01 peptides, approximately half of the peptide cohort was recovered—consistent with the theoretical proportion of HLA-A*02:01–associated peptides predicted by the NetMHCpan algorithm.

Venn diagrams were constructed to visualize peptide overlap, revealing a substantial intersection: 477 peptides were common among WT_PA2.1, ΔBC_PA2.1, and ΔBC_W6/32 conditions, representing 77 to 90% of the total peptide identifications in each (Fig. 4*C*). In contrast, the 432 peptides uniquely identified in WT_W6/32 are likely derived from HLA-B*07:02 and HLA-C*07:02.

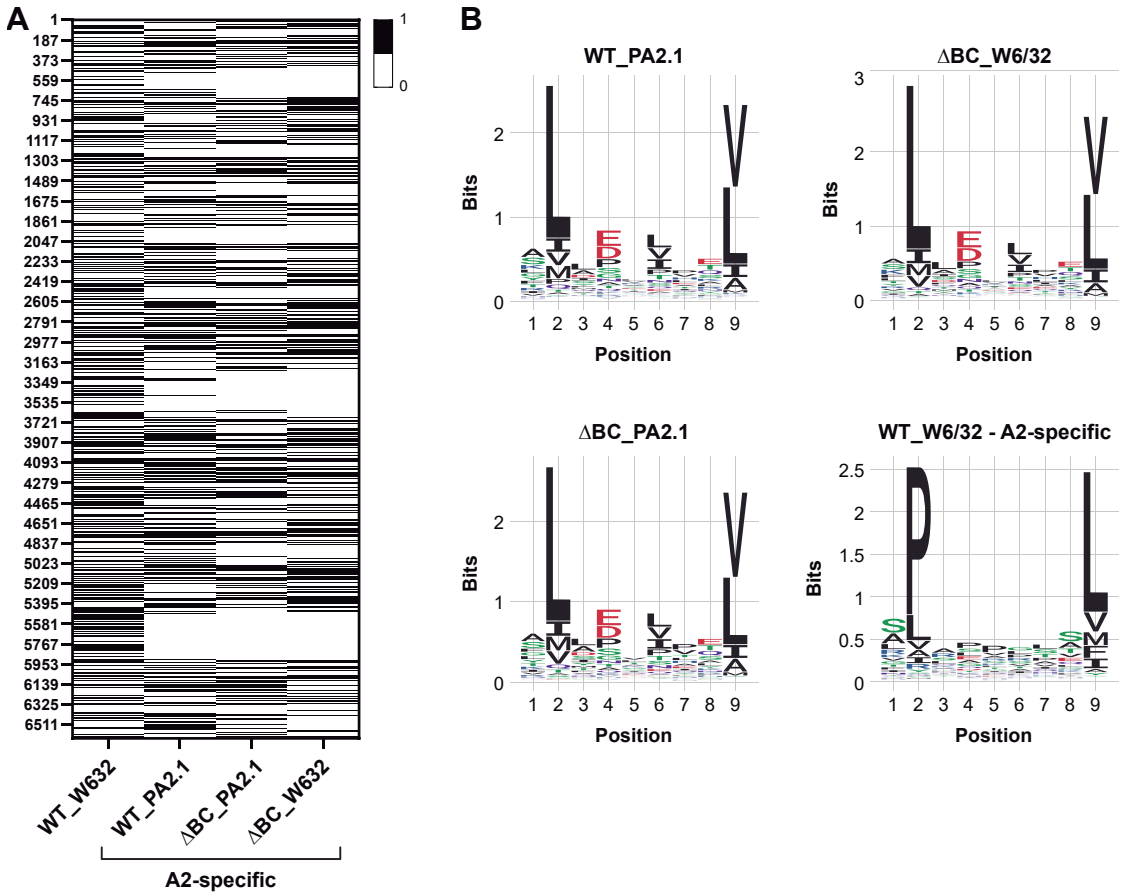

FIG. 3. **Edited clones immunoprecipitated with α-pan-HLA class I reveal the HLA-A*02:01-specific peptide-binding motif.** *A*, heatmap representation of all unique peptide identified in unedited (WT) and edited (ΔBC) JY clones, immunoprecipitated using α-HLA-A*02:01 (PA2.1) or α-pan-HLA class I (W6/32). Numbers 1-6632 correspond to each unique peptide identified across all conditions. Peptides are assigned a value of 1 (*black*) if present in a given condition or 0 (*white*) if absent. *B*, sequence logos representing binding motifs of the 9-mer peptides identified in WT clones immunoprecipitated with PA2.1 (WT_PA2.1), ΔBC clones immunoprecipitate with PA2.1 (ΔBC_PA2.1), ΔBC clones immunoprecipitated with W6/32 (ΔBC_W6/32), and those identified in WT clones immunoprecipitated with W6/32, excluding peptides already assigned to previous conditions (WT_W6/32 - A2-specific). Sequence logos were generated with the ggseqlogo package in RStudio. Amino acid colors reflect their chemical properties: acidic (*red*), basic (*blue*), hydrophobic (*black*), neutral (*purple*), and polar (*green*).

Additionally, we performed pairwise comparisons between conditions (Fig. 5*A* and Supplemental Fig. S1). When comparing WT and ΔBC clones, both immunoprecipitated with PA2.1, we identified 483 shared peptides, representing 81% of the WT_PA2.1 repertoire, indicating that our CRISPR strategy does not negatively impact the identified repertoire. Assessing our edited clones across both immunoprecipitations, we observed an overlap of 480 peptides, corresponding to 93.5% of the ΔBC_PA2.1 repertoire. Finally, our key comparison was the overlap between ΔBC clones immunoprecipitated with W6/32 (pan-HLA class I antibody) and WT clones immunoprecipitated with PA2.1 (HLA-A*02:01-specific antibody). Notably, this comparison yielded the highest number of overlapping peptides (528), covering 88% of the WT_PA2.1 repertoire, which represents the standard approach.

To investigate the nature of non-overlapping peptides, we analyzed their MS detection intensities (Fig. 5*B*). We found that overlapping peptides tend to show higher signal intensities, suggesting that the lower abundance of non-overlapping peptides may limit their detectability across conditions.

Overall, the high degree of overlap, particularly between WT clones IP with PA2.1 and ΔBC clones IP with W6/32, validates our approach as a sensitive and specific method for HLA-A*02:01 peptidome profiling using a general HLA class I antibody.

DISCUSSION

By selectively deleting HLA-B and HLA-C alleles while retaining HLA-A*02:01, we were able to immunoprecipitate peptides using a pan-HLA class I antibody (W6/32). The resulting peptide repertoire closely resembled that obtained using the HLA-A*02:01–specific antibody PA2.1 in WT clones, yielding comparable peptide counts and a high overlap (88%)

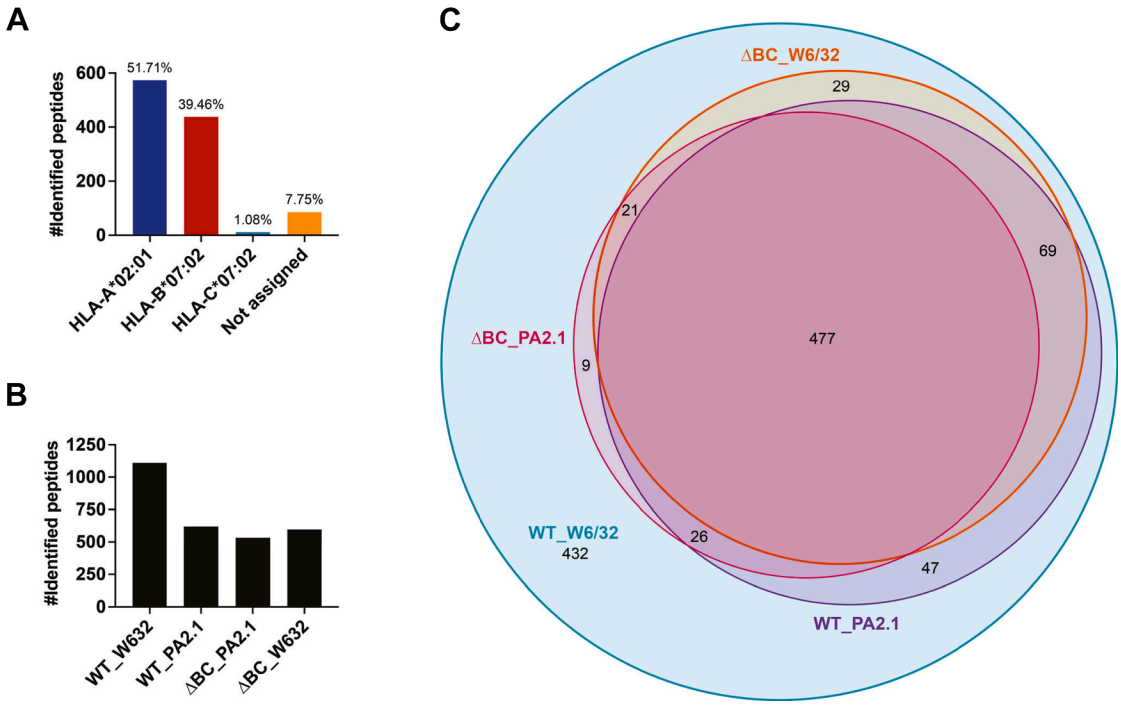

FIG. 4. **High peptide overlap among HLA-A*02:01-specific expected conditions.** *A*, proportion of peptides within this cohort predicted by NetMHCpan 4.1 to bind HLA-A*02:01 (*blue*), HLA-B*07:02 (red), or HLA-C*07:02 (*green*), or unassigned (*yellow*). *B*, number of unique peptides identified in unedited (WT) and edited (ΔBC) JY clones, immunoprecipitated using α-HLA-A*02:01 (PA2.1) or α-pan-HLA class I (W6/32), restricted to the peptide set shared by both WT_W6/32 samples. *C*, Venn diagram representation of WT clones immunoprecipitated with PA2.1 (WT_PA2.1, *purple*), WT clones immunoprecipitated with W6/32 (WT_W6/32, *blue*), ΔBC clones immunoprecipitated with PA2.1 (ΔBC_PA2.1, *pink*), and ΔBC clones immunoprecipitated with W6/32 (ΔBC_W6/32, *orange*).

between repertoires, which underscores the robustness and specificity of this strategy.

One of the most significant advantages of this method is its ability to exclude peptides derived from undesired alleles. In the ΔBC clones, the frequency of HLA-B*07:02 and HLA-C*07:02 peptides dropped to nearly 0%, confirming the specificity of the retained HLA-A*02:01 presentation. Furthermore, the binding motifs identified in ΔBC clones immunoprecipitated with W6/32 were consistent with those captured using the PA2.1 antibody, validating the approach at both the sequence and motif level. Compared to conventional systems, such as C1R transfectants, which still express HLA-B35 and HLA-Cw4 to some degree (27), models like K562 and B721.221 (9, 10), or soluble MHC class I transfectants (26), all of which require overexpression of the HLA allele of interest, our strategy presents a clear advantage by avoiding such overexpression. This is particularly important given that HLA expression levels influence antigen presentation. Overexpression systems can distort the natural immunopeptidome by artificially increasing peptide loading capacity and altering peptide selection (14, 15). Studies have shown that, at least in the context of HLA class II, elevated HLA expression levels can promote the binding of lower-affinity peptides, potentially broadening the immunopeptidome, while possibly reducing its biological relevance (28); whether comparable effects occur in HLA class I is still unclear.

By preserving physiological HLA expression levels, our approach provides a complementary strategy to overexpression-based systems, enabling the study of antigen presentation under endogenous regulatory contexts. This may be particularly relevant in settings where HLA expression is dynamically modulated, such as in response to cytokines or infection, allowing the investigation of how such perturbations impact naturally expressed HLA molecules. For instance, Goncalves *et al*. (29) supporting the findings of Komov *et al*. (15) demonstrated that IFN-γ stimulation increases the peptide repertoire without a corresponding increase in source proteins at either the proteomic or transcriptomic level. Additionally, they observed that IFN-γ treatment favors the presentation of longer peptides, suggesting that increased availability of HLA molecules could be driving both effects. These findings reinforce the value of our system to study the effects of molecules regulating the antigen processing and presenting machinery.

Although the use of the JY cell line, which is homozygous for HLA-A, -B, and -C, facilitated the implementation of our genome editing strategy and made it particularly suitable for proof-of-concept validation, this approach is conceptually

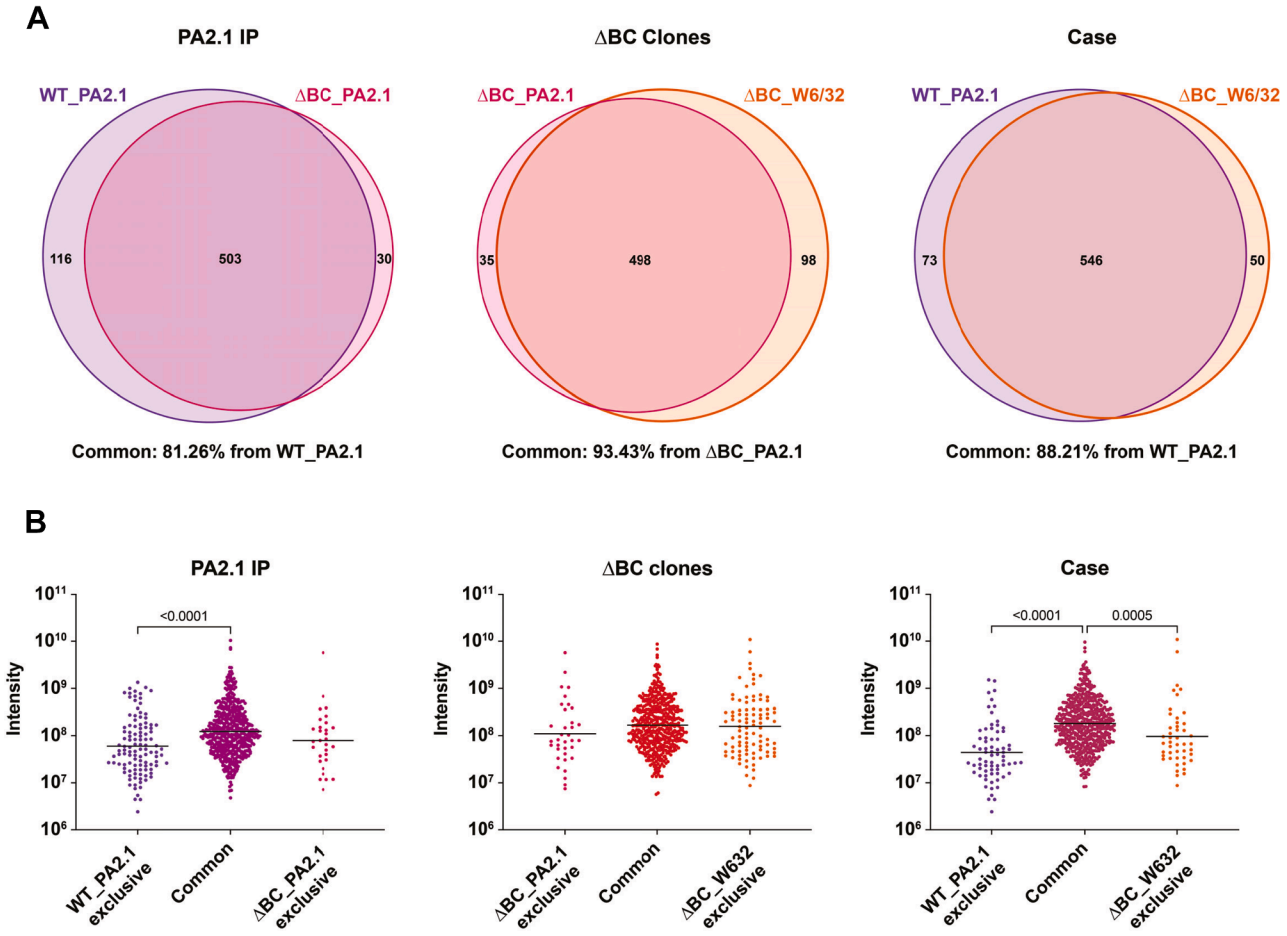

FIG. 5. **Edited clones immunoprecipitated with α-pan-HLA class I show a substantial peptide overlap with unedited clones immunoprecipitated with α-HLA-A*02:01.** *A*, Venn diagram representations of unique peptide overlap in three pairwise comparisons: unedited (WT) and edited (ΔBC) clones immunoprecipitated with PA2.1 (WT_PA2.1, *purple*; ΔBC_PA2.1, *pink*) (*left*), ΔBC clones immunoprecipitated with both, PA2.1 and W6/32 antibodies (ΔBC_PA2.1, *pink*; ΔBC_W6/32, *orange*) (*middle*), and the key comparison of interest, WT clones immunoprecipitated with PA2.1 (WT_PA2.1, *purple*) and ΔBC clones immunoprecipitated with W6/32 (ΔBC_W6/32, *orange*) (*right*). *B*, distribution of mass spectrometry intensities for overlapping and non-overlapping peptides across the three comparisons. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post hoc test. A *p*-value <0.05 was considered statistically significant.

transferable to more complex, heterozygous genetic contexts. In such cases, adaptation would primarily require either allele-specific guide RNA design or a sequential editing strategy to selectively eliminate undesired alleles. While this may require additional optimization, it does not represent a fundamental limitation of the method. Importantly, previous studies have demonstrated that CRISPR/Cas9 can effectively target HLA loci despite their high sequence homology, as polymorphic regions often provide sufficient specificity for selective allele disruption (30).

HLA-binding prediction models have emerged as powerful tools to predict epitope specificity, especially when analyzing cells that express multiple HLA molecules, avoiding the need for mono-allelic cell lines (16, 17). However, continuous efforts are being made to improve their accuracy, as detection limitations persist. Our results provide evidence of these challenges. While we show high specificity and overlap, a fraction of unassigned peptides (~10–20%) was observed across all conditions. These peptides may correspond to those bound to nonclassical HLA molecules in W6/32 immunoprecipitation but not in PA2.1, suggesting that certain HLA-A*02:01–binding motifs remain uncharacterized in the algorithms, especially when larger peptides are identified. Comparable findings (data not shown) were obtained using the MHCflurry2.0 algorithm. This highlights a substantial proportion of peptides that current models fail to classify, emphasizing the need for more empirical data to refine and enhance predictive algorithms.

To enhance model accuracy, studies such as those by Abelin *et al*. (2017) and Sarkizova *et al*. (2019) generated 95 mono-allelic B721.221 HLA transfectant cell lines, providing high-quality training datasets for refining prediction

algorithms (12, 18). While these cell lines enable the study of specific HLA-bound peptide repertoires, as mentioned, they could introduce potential artifacts in processing and presentation due to the overexpression of HLA molecules. In light of this, there is a continued need for additional studies employing complementary strategies that yield high-quality datasets in more physiological settings, which are essential not only for the refinement of current algorithms but also for ensuring broader and more accurate coverage of the extensive diversity of HLA alleles.

This work lays the foundation for extending the strategy to other HLA alleles lacking specific antibodies, including those less represented in population databases. It can also be applied to different cell types or primary cells to investigate tissue-specific immunopeptidomes, particularly relevant for immunotherapy development and vaccine safety.

Although we consider the observed 88% overlap between the peptide repertoire identified in edited clones with the general antibody and the one in unedited clones with the HLA-A*02:01–specific antibody substantial and sufficient for the approach to be extended in the field, we would like to remark that the non-overlapping peptides (12% of the WT_PA2.1 repertoire) exhibit a decrease in detection intensity. Highlighting a limitation of the mass spectrometry technique in consistently detecting peptides present in low abundance across samples under our experimental conditions. Increasing input material or technical replicates may further enhance detection and reduce variability.

Variability between technical replicates (identical HLA ligand samples analyzed in separate MS runs) is a well-recognized phenomenon in immunopeptidomics. However, when protocols are robust and optimized, this variability is generally moderate and acceptable. Typically, a peptide overlap of approximately 60 to 80% is observed across technical replicates (31–33). Several factors contribute to this variability, including instrument sensitivity and resolution, depth of sampling (*e.g.,* gradient length, fractionation), sample complexity (*i.e.,* the number of co-eluting peptides competing for fragmentation), and peptide loading. Importantly, the most common source of discrepancy is not quantitative variation but stochastic detection failure: some peptides are identified in one run but not another simply due to their low intensity or failure to be selected for MS/MS fragmentation.

One potential limitation of our approach is that CRISPR editing may introduce clonal heterogeneity. However, we did not observe significant differences between the peptide repertoires of edited and unedited cells in our analyses. Although our editing strategy specifically targets HLA gene expression, compensatory or downstream effects on the antigen processing machinery—such as TAP or ERAP1/2—could theoretically occur. Nevertheless, the strong similarity between the HLA-A*02:01–presented peptides in WT and edited clones suggests that such effects are minimal or absent in this context.

Overall, our results demonstrate that our CRISPR-based strategy enables highly specific, physiologically relevant immunopeptidome analysis without relying on allele-specific antibodies. By deleting alleles of no interest rather than overexpressing a single allele, this approach overcomes key limitations of traditional overexpression systems. Additionally, the ability to capture a comparable peptide repertoire with a general antibody broadens the scope of HLA immunopeptidomics, making it particularly valuable for studying rare or less-characterized HLA alleles and expanding research on antigen presentation and immune recognition. Extending this strategy to patient-derived primary cells could provide highly relevant insights for personalized immunotherapy and diseases.

While our approach focused on HLA class I alleles, future work could explore its application to class II loci, which pose additional challenges due to their heterodimeric structure and more restricted expression patterns.

DATA AVAILABILITY

The mass spectrometry proteomics data have been deposited to the MassIVE repository (http://massive.ucsd.edu) with the dataset identifier MSV000099922 (ftp://MSV000099922@massive-ftp.ucsd.edu, password: PRI_3796).

Supplemental data—This article contains supplemental data

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Abbreviations—The abbreviations used are: HLA, Human leukocyte antigen; IP, Immunoprecipitation; MS, Mass spectrometry; MS/MS, Tandem mass spectrometry.

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