Identification of Native and Posttranslationally Modified HLA-B*57:01-Restricted HIV Envelope Derived Epitopes Using Immunoproteomics

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The recognition of pathogen-derived peptides by T lymphocytes is the cornerstone of adaptive immunity, whereby intracellular antigens are degraded in the cytosol and short peptides assemble with class I human leukocyte antigen (HLA) molecules in the ER. These peptide-HLA complexes egress to the cell surface and are scrutinized by cytotoxic CD8+ T-cells leading to the eradication of the infected cell. Here, naturally presented HLA-B*57:01 bound peptides derived from the envelope protein of the human immunodeficiency virus (HIVenv) are identified. HIVenv peptides are present at a very small percentage of the overall HLA-B*57:01 peptidome (<0.1%) and both native and posttranslationally modified forms of two distinct HIV peptides are identified. Notably, a peptide bearing a natively encoded C-terminal tryptophan residue is also present in a modified form containing a kynurenine residue. Kynurenine is a major product of tryptophan catabolism and is abundant during inflammation and infection. Binding of these peptides at a molecular level and their immunogenicity in preliminary functional studies are examined. Modest immune responses are observed to the modified HIVenv peptide, highlighting a potential role for kynurenine-modified peptides in the immune response to HIV and other viral infections.

1. Introduction

Posttranslational modification (PTM) of proteins is an essential process that controls protein function. Accordingly, it is unsurprising that the immune system is not only regulated through PTM, but it also has the capacity to differentiate PTM antigens. The constitutive presentation of PTM peptides including phosphorylation, deamidation, and dimethylation has been reported in several studies that describe the repertoire of peptides bound to various HLA molecules (also coined immunopeptidomes).

The presence of PTMs in these immunopeptidomes highlights the high-fidelity representation of the cellular proteome in the context of HLA-peptide complexes, which in turn facilitates immune surveillance by T lymphocytes (T cells). Moreover, pathological changes in the PTM of
Significance of the study

This study shows that the viral specific peptidome of envelope protein of HIV is a minor component of the overall peptidome constituting <0.1% of all bound peptides. We report several novel HIV epitopes including a peptide found in both native and modified states that bears a tryptophan to kynurenine modification. This is the first time that peptides bearing this modification have been validated as naturally presented HLA class I ligands. We go on to show that this modification of the native peptide can occur through the peroxidase activity of IDO1 and spontaneous formation of this peptide modification is negligible under the conditions used to isolate the HLA bound peptides. Immune responses to both native and modified forms of this peptide can be detected in HIV-naïve and infected patients suggesting a role for this modification in the immune response. Such recognition was further substantiated by examining the binding of these peptides to HLA and the X-ray crystallographic structures of the native and modified peptides in complex with HLA-B*57:01, where the molecular structure showed that the modification was not altering the stability nor the conformation of the bound peptide.

the cellular proteome have also been correlated with disease states in humans and animal disease models (reviewed in Ref. 11). For example, changes in protein phosphorylation have been linked to cancer and the presentation of phosphorylated peptides can act to alert the immune system and promote eradication of cancerous cells.[1,12–14] Other PTMs play a central role in the pathogenesis of autoimmune diseases[15] such as arginine citrullination in arthritis,[16–18] deamidation of glutamine residues in wheat proteins in coeliac disease,[19–23] citrullination and/or phosphorylated epitopes in systemic lupus erythematosus (SLE).[27,28] Redox modifications of antigens and their derived HLA ligands during infection have also been previously documented with cysteine di- and tri-oxidation, cysteinylolation, and S-glutathionylation of class I-restricted T-cell epitopes frequently observed.[29] The molecular dissection’s role of PTMs in engendering T cell responses is complex and almost certainly dependent upon the HLA-restriction and the type of PTM. In some circumstances, the PTM can impact on HLA binding while in other cases the PTM is directly required for interactions with the T-cell receptor (TCR) (reviewed by Ref. 30)

Kynurenine is one of the PTMs of tryptophan formed by catalytic conversion by either the tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO) enzymes, the latter existing as two isoforms IDO1 and IDO2. This is the rate-limiting step of tryptophan catabolism,[31,32] Tryptophan catabolism and the accumulation of kynurenine has wide-ranging effects on the cells of both the humoral and adaptive immune systems. These effects include the reduction of antibody-secreting plasma cells,[33] inhibition of NK (natural killer) cell proliferation, decline of CTL (cytotoxic T lymphocyte) activity[34,35] and reduced proliferative abilities in CD4+ and CD8+ T-cells.[34,36] Moreover, IDO1 activity can lead to localized immune suppression by metabolic reprogramming of cells due to tryptophan deficiency and an accumulation of kynurenine pathway by-products.[37,38]

It is unclear whether the appearance of kynurenine within polypeptides is due to kynurenine incorporation during synthesis or if the IDO1 enzyme can act upon mature proteins and/or peptides.

A sub-population of HIV-infected individuals control the infection and do not progress to AIDS even decades post infection.[39,40] These long-term non-progressor individuals (LTNPs) maintain normal CD4+ T-cell levels (typically >500 cells/μL) and low levels of viremia.[41–43] How LTNPs control infection remains unclear,[40,44,45] however, genome-wide association studies (GWAS) have shown that the HLA-viral peptide interaction is one of the most significant factors controlling HIV infection.[46–48] Certain HLA class I allotypes, particularly HLA-B*57:01, are significantly over-represented in LTNPs.[40,46,49] CD8+ T-cells from such LTNPs demonstrate sustained poly-functional activity, are not suppressed by regulatory T-cells,[50,51] and are more resistant to apoptosis than those from patients who fail to control the virus.[52] Though the genetic and environmental factors have been mapped for these subpopulations, there is no systematic study of epitopes naturally presented by antigen presenting cells (APCs) in HLA-B*57:01+ individuals. Therefore, we undertook an immunopeptidomics approach to identify HLA-B*57:01-restricted peptides from envelope protein of HIV (HIVenv), an antigen that is under-represented among the known HLA-B*57:01-restricted epitopes. Out of a large constitutive immunopeptidome (>8700 nonredundant peptides), only seven were identified from HIVenv binding to HLA-B*57:01. Of note, both native and PTM forms of two peptides were observed—native and kynurenine modified RVKEKYQHLW/Kyn (RVK or RVK(Kyn)) and the native and a deamidated form of KSLEQWNNMTW (KSLE and KSLE(N9D)). Here, we discuss the identification and detailed characterisation of these modified peptides, specifically focusing on the kynurenine-modified RVK peptide due to its novelty. T cell-based assays were also performed to evaluate the immunogenicity of the native and RVK(Kyn) peptides using T cells derived from HLA-B*57:01+ HIV-infected and HIV-naïve donors.

2. Experimental Section

2.1. Experimental Design, Cell Lines, and Culture

The human B-lymphoblastoid cell line C1R[53,54] expressing HIV envelope were generated by electroporating a plasmid encoding either HIV NL(AD8)-gp160 or -gp140 and expression evaluated by ELISA as described previously.[55,56] Stably transfected clones expressing high amounts of HIVenv protein were selected and maintained in RF10 containing 0.5 mg mL−1 G418 (Roche). These cells were then super transfected with a HLA-B*57:01 containing plasmid and maintained under hygromycin (0.3 mg mL−1) selection in RF10. Cells expressing significant levels of HLA were sorted using flow cytometry (using W6/32 anti-HLA pan-class I antibody[57]) and subjected to limiting dilution to obtain clones. Clonal cells were expanded in roller bottles at 37 °C and 5% CO2, and the cell pellets (three
replicates per plasmid) snap frozen in liquid nitrogen prior to analysis.

2.2. Affinity Purification of HLA-Peptide Complexes

HLA-peptide complexes were affinity purified from six C1R cell pellets (5 × 10⁶ each) expressing HIVenv and HLA-B*57:01 as described previously. Briefly, immunoaffinity captured (W6/32 anti-HLA pan class I antibody) HLA-B*57:01-peptide complexes were eluted by acidification with 10% acetic acid. The eluted mixture of peptides was fractionated on a 4.6 mm internal diameter × 50 or 100 mm long RP C18 endcapped HPLC column (Chromolith SpeedRod, Merck) using an AKTA Ettan HPLC system (GE Healthcare) running a mobile phase consisting of buffer A of 0.1% TFA and buffer B of 80% acetonitrile (ACN)/0.1% TFA. The HLA-peptide mixtures were loaded onto the column at a flow rate of 1 mL min⁻¹ with separation based on a gradient of 2–15% in 1 min, 15–40% B for 8 min, 40 to 45% for another 4 min, and a rapid 2 min increase to 100% B. Fractions (500 μL) were collected, vacuum concentrated to 10 μL, and diluted in 0.1% formic acid to reduce the concentration of ACN.

2.3. Analysis of HLA-B*57:01-Bound Peptides

For LC–MS/MS, peptide-containing fractions were loaded onto a microfluidic trap column packed with ChromXP C18-CL 3 μm particles (300 Å nominal pore size; equilibrated in 0.1% formic acid/2% ACN) at 5 μL min⁻¹ using a NanoUltra chipHPLC system (Eksigent). An analytical (75 μm x 15 cm ChromXP C18-CL 3 μm, 120 Å; Eksigent) microfluidic column was switched in line, and peptides separated using linear gradient elution starting with buffer concentration of 5% buffer B (80% ACN, 0.1% formic acid) and 95% buffer A (0.1% formic acid), 5–10% buffer B over 1 min, 10–30% buffer B over 50 min, and 30–80% over 5 min flowing at 300 nL min⁻¹. Separated peptides were analyzed using a SCIEX TripleTOF 5600+ mass spectrometer equipped with a Nanospray III ion source and accumulating up to 20 MS/MS spectra per second. The following instrument parameters were used: ion spray voltage (ISVF) was set at 5000 V, curtain gas (CUR) at 25 L min⁻¹, ion source gas (GS1) at 10 L min⁻¹, and an interface heater temperature (IHT) setting of 150 °C. MS/MS switch criteria included ions of m/z >200 amu, charge state +2 to +5, intensity >40 cps, and the top 20 ions meeting this criterion were selected for MS/MS per second. The instrument was calibrated every four LC runs using [Glu1]-Fibrinopeptide B peptide in TOF-MS and -MS/MS modes.

2.4. Data Analysis

LC–MS/MS data was searched against the human proteome (UniProt/SwissProt v2014_10) using ProteinPilot software (version 4.5, SCIEX) and resulting peptide identities were subject to strict bioinformatic criteria including the use of a decoy database to calculate the false discovery rate (FDR). A 5% FDR cut-off was applied, and the filtered dataset was further analyzed manually to exclude redundant peptides as we have reported for non-tryptic and HLA-bound peptides. The data from C1R and C1R-B*57:01 cells that were negative for HIV protein expression were searched against same database as a control to identify false positive assignment to HIVEnv.

Table 1. HLA-B*57:01 restricted HIVenv peptides identified in this study.

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>HXB2 AA position in Protein</th>
<th>Sequence in Literature and HLA-restriction if known (HIV LANL DATABASE EPITOPE ID in parenthesis)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAYDTEVHNVW</td>
<td>59–69</td>
<td>KAYDTEVHNVW (56054)</td>
<td>[Hh]</td>
</tr>
<tr>
<td>KSLEQIWNMTW</td>
<td>617–628</td>
<td>Not known to be immunogenic</td>
<td>[Kt]</td>
</tr>
<tr>
<td>KSLEQIWNMDMTW (Deamidated N9)</td>
<td>617–628</td>
<td>RIKQINMW (793) B57, A*32:01</td>
<td>[Hh]</td>
</tr>
<tr>
<td>RVKEYQHLW</td>
<td>2–11</td>
<td>MRVKEYQHLWRGW (57674)</td>
<td>[P]</td>
</tr>
<tr>
<td>RVKEYQHL(Kyn)</td>
<td>2–11</td>
<td>MRVKEYQHLWRGW (57674)</td>
<td>[P]</td>
</tr>
</tbody>
</table>

Newly identified peptides are in bold and a representative HIV LANL epitope ID is given in parentheses (mapped using the PepMap tool at HIV Molecular Immunology database[63]).

3. Results

3.1. Identification of HLA-B*57:01 Bound Peptides from HIV AD8 Envelope Transfected APC

A cell-based model to identify antigen processing and presentation of HIVenv-derived HLA-B*57:01-restricted peptides was developed. A total of 8773 endogenous peptides were identified from HIVenv transfected C1R.B*57:01 cells that matched the previously described motif (Supporting Information, Figure S1)[63] (Supporting Information Files SI–2). In addition to these endogenous peptides, seven HIVenv-derived peptides were identified in transfected cells that were absent in untransfected controls (Table 1 and Supporting Information, Table S1). Three of the peptides (RVKEYQHLWRGW) were from the signal sequence of HIVenv.
and shared a common core sequence. Other HIVenv peptides identified were from processed forms of HIVenv including surface gp120 (KAYDTEVHNVW and RIKQINMW) and gp41 (KSLEQIWNMTW, KSLEQIWND*MTW (deamidated at N9)). The majority of HIV peptides identified in this study map to previously described immunogenic regions of the HIVenv antigen (Table 1). The exception being KSLEQIWNMTW and its modified counterpart KSLEQIWND*MTW, which were previously unreported, along with a modified peptide from a previously identified immunogenic region represented by the RVKEKYQHL(Kyn) peptide. Due to the potential role of PTMs in modulating antiviral immunity we chose to focus on these PTM-bearing peptides.

3.2. Validation of HIV Peptides

Synthetic versions of all HIV peptides were made to verify the HLA peptides identified by LC–MS/MS. The retention time and fragmentation pattern of experimentally observed HLA-bound peptides matched these synthetic peptides confirming their experimental identification (Figure 1 and Supporting Information, Figures S2–S8).

3.3. What is the Source of the Kynurenine Containing Peptides?

The primary role of IDO1 is the conversion of free tryptophan to kynurenine.\(^{[62]}\) It is unclear whether IDO can modify tryptophan residues in peptides to kynurenine. To address this, the native RVK peptide was incubated with recombinant human IDO1 under various conditions to determine if the dioxygenase or the peroxidase activity\(^{[63]}\) of IDO1 could act on the RVKEKYQHLW (RVK) peptide and convert it into RVKEKYQHL(Kyn) (RVK(Kyn)) (tryptophan to kynurenine). The peptides were analyzed by LC–MS to identify any formation of the kynurenine-modified peptide. The conversion of free tryptophan controls to kynurenine was measured by HPLC using a standard assay.\(^{[63]}\) Analysis of the positive controls showed that \(\approx 30–35 \mu M\) free tryptophan (\(\approx 23\%\)) was oxidized under the peroxidase conditions and \(\approx 193–198 \mu M\) free tryptophan (\(\approx 100\%\)) was converted into kynurenine under the dioxygenase conditions by IDO1.

After treatment of the unmodified native peptides with IDO1 and \(H_2O_2\) (peroxidase condition), there was a significant amount (Figure 2B) of the kynurenine-modified peptide accounting for about 6–56% of the native peptide compared with peptides by themselves or when treated with IDO1 or \(H_2O_2\) alone. IDO1 was unable to modify native peptides under dioxygenase conditions. Importantly, addition of cyanide as a heme poison and inhibitor

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**Figure 1.** MS/MS fragmentation spectrum of a HLA-B*57:01 restricted HIV peptide RVKEKYQHL(Kyn) (A) KSLEQIWND*MTW (where the genetically encoded N9 is deamidated) (B). The fragmentation pattern and peptide identity were validated by comparing to a retrospectively synthesized peptide of the same amino acid sequence (negative polarity spectrum) with all major peaks including b- and y-ions mirrored. X-axis represents mass over charge ratio \((m/z)\), and y-axis refers to intensity represented as a percentage of maximum intensity. C) Table of peptide amino acid sequences and LC–MS search results for native and modified peptides. Retention time of the peptide and the synthesized peptide (in parenthesis) are within the limits of the LC system.
Figure 2. A) IDO1 in the presence of H2O2 is able to convert peptidyl-tryptophan into kynurenine in the three HIV peptides. TW10 and RVK when treated with IDO1 and H2O2 generated statistically significant amount of kynurenine modified peptide when compared to peptide only, IDO1 only or H2O2 only treatments (error bars represent mean ± SEM, n = 3, ANOVA, p-value adjusted (Tukey) for multiple comparisons). Although IW9 generated low amounts of kyn peptide, it failed to reach statistical significance. B) Stabilization of cellular HLA-B*57:01 molecules by synthetic HIIVenv peptides. All HIIVenv peptides tested, and the known HLA-B*57:01 ligand IALY, stabilized HLA-B*57:01 surface expression on T2-B*57:01 cells, while the negative control YY9 (non-HLA-B*57:01 restricted) peptide did not. The T2 cell line (with no HLA-B*57:01) showed no detectable HLA stabilization by any of the HLA-B*57:01-restricted peptides.

Figure 2A: Graph showing the % Kyn to native values for different treatments, where TW10 and RVK treatments under IDO1 and H2O2 conditions show statistically significant results compared to other treatments. Error bars represent mean ± SEM, n = 3, ANOVA, p-value adjusted (Tukey) for multiple comparisons.

Figure 2B: Graph showing the mean fluorescence intensity for different peptide concentrations (micromolar) across different peptides (RVK, RVK(Kyn), KSLE, KSLE(N9D), IALY, IAY, YY9). The stabilization effect on HLA-B*57:01 is observed for RVK and RVK(Kyn) treated samples compared to YY9 control, which did not show any stabilization.

3.4. HLA Binding of Native and Modified HIIVenv Peptides

To confirm that the HIIVenv-derived RVK, RVK(Kyn), KSLE, and KSLE(N9D) peptides identified could bind to HLA-B*57:01, we employed a cell-based epitope stabilization assay using the transporter associated with antigen processing (TAP) deficient T2 cell line. A HLA-B*57:01-specific antibody (3E12) was used to detect stable HLA-B*57:01 molecules at the cell surface and dose-dependent HLA-B*57:01 binding (Figure 2A). A well-documented HLA-B*57:01 binding peptide IALY (IA-LYLQW-NW) from the Epstein–Barr virus latent membrane protein was used as positive control and YY9 (YLNEKAVSY), a non-HLA-B*57:01-restricted peptide, as negative control. None of the test peptides showed detectable stabilization of endogenous HLA-A*02:01 (which is weakly recognized by 3E12) on the surface of the parental T2 cells confirming specific stabilization of HLA-B*57:01 on the T2.B*57:01 cell line. The RVK and RVK(Kyn) stabilized HLA-B*57:01 to a similar degree and to levels of around 50% of that stabilized by the control IALY peptide. Similarly, KSLE and KSLE(N9D) also equally stabilized HLA-B*57:01 molecules but in this case the stabilization was superior to the IALY control peptide. No stabilization was observed with the YY9 control peptide. These data confirm that the native and PTM RVK and KSLE peptides are capable of binding to HLA-B*57:01.

3.5. Evaluation of T-cell Responses toward HIIVenv RVK Peptides in Healthy Donors

To assess de novo T-cell responses toward the RVK peptides, healthy donors expressing HLA-B*57:01 (Supporting Information, Table S2) were recruited to evaluate HIV-specific T-cell responses after stimulation with the kynurenine modified (RVK(Kyn)) and the unmodified (RVK) peptides. As a positive control to demonstrate the in vitro expansion of Epstein–Barr virus-specific CD8+ T cells, either HLA-B*57:01-restricted IALY (HD001-005) and/or HLA-B*08:01-restricted RAKFKQHLL (RAK; HD004 only) peptides were used (Supporting Information, Table S3). Peripheral blood mononuclear cells (PBMCs) were stimulated with peptide-pulsed irradiated autologous APCs and cultured in vitro for 13 days. Activated peptide-specific T cells were identified, following restimulation with APCs (± peptide) for 6 h, based on their dual production of IFN-γ and TNF-α. Relevant controls were included in all assays with either the IALY or RAK peptides serving as the positive control and
media alone or DMSO as the negative control. On day 13, following the in vitro expansion, the positive controls demonstrated a robust T-cell response of $57.3 \pm 32.8\%$ (range 5.2–76.7%) for HLA-B*57:01-restricted IALY (HD001–HD005) and 21.1% for HLA-B*08:01-restricted RAK (HD004 only), as measured by quantitation of CD8+ T cells producing both IFN-γ and TNF-α (Supporting Information, Figure S10). Of the five healthy donors tested, one (HD004) responded to the modified RVK(Kyn) peptide with around 1.15% of cells expressing both TNF-α and IFN-γ (Figure 3).
3.6. Evaluation of T-cell Responses toward Novel HIVenv Peptides in HIV-Infected Subjects

To evaluate the immunogenicity of the HLA-B*57:01-restricted env epitopes identified above, 15 HLA-B*57:01+ HIV-infected patients (Supporting Information, Table S4) were recruited to evaluate T-cell responses to the peptides identified in this study and a number of previously characterized HLA-B*57:01-restricted HIV epitopes (Supporting Information, Table S3). We employed peptide-HLA (pHLA) tetramer staining of unstimulated PBMC to determine T-cell specificity toward HIV epitopes using cryopreserved specimens. The fluorescence minus one (FMO; containing all fluorochromes in panel except the one being measured) was used as negative control (see Supporting Information, Figure S11 for gating strategy). To identify HIV-specific CD8+ T cells, within the total T-cell population, pHLA tetramer complexes for previously reported epitopes (IW9, TW10, and KF11)[70] and the env epitopes identified above (RVK, RVK(Kyn), KSLE, and KSLE(N9D)) were used. Overall, the patient cohort revealed low frequencies of tetramer-specific T cells with 0.09–2.58% of KF11-specific CD8+ T cells, 0–0.1% of TW10-specific CD8+ T-cells, and 0.01–0.21% of IW9-specific CD8+ T cells (Supporting Information, Table S5; Figure 3B). The TW10 frequencies were not higher than 0.1% and no tetramer positive cells were identified in four of 15 patients. The negative control FMO was as expected. For the majority of patients examined RVK or RVK(Kyn) specific T cells could not be detected. A small population (≈0.14% of CD8+ T cells, Supporting Information, Figure S12) of RVK and RVK(Kyn) tetramer-specific CD8+ T cells was observed in one of the 15 patients (#3), reflecting weak but detectable responses to these novel epitopes.

3.7. Structural Analysis of HLA-B*57:01 Bound to Native and Kynurenine Containing RVK Peptides

The HLA-B*57:01-RVK and HLA-B*57:01-RVK(Kyn) structures were determined at a high resolution of 1.45 and 1.58 Å, respectively (Supporting Information, Table S6, and Figure 4A, B), allowing the clear distinction between the P10-W and P10-Kyn (Figure 4C, D). The central part of both peptides was highly mobile and partially built. The native RVK and RVK(Kyn) peptides reside in the binding groove of the HLA-B*57:01 molecule in similar conformations (root mean square deviation of 0.08Å) and make similar contacts with the HLA-B*57:01 molecule (Supporting Information, Table S7). The peptides bind in an extended conformation (Figure 4B) with specificity determining contacts of P2-V in the B pocket and P10-W or P10-Kyn in the F pocket (Supporting Information, Table S7). The P2-V contacts the HLA-B*57:01 heavy chain via hydrogen bonds to E63 and hydrophobic interactions with a network of Methionine and Tyrosine residue from the B pocket (Supporting Information, Table S7). The P10 residue interacts with a network of aromatic residues from the F pocket and the modification of W to Kyn at P10 does not significantly affect peptide binding (Supporting Information, Table S7). Both peptides interact with the Y123, which forms π–π stacking interactions with the benzimidazole ring of P10-W or P10-Kyn (Figure 4E). The imidazole ring of P10-W interacts with an amino group of N83 via hydrogen bond and N77 similarly bonds with the keto-group of kynurenine. S116 forms an additional hydrogen bond only with 2-amino phenyl moiety of kynurenine (Figure 4E).

The thermal stability of the RVK and RVK(Kyn) bound HLA-B*57:01 molecules, measured by a thermal melting assay, revealed a similar stability of both peptide-HLA complexes (Tm ≈73 °C), consistent with the maintenance of the majority of pHLA interactions in the two complexes. Thus, both native and RVK(Kyn) peptides bind to HLA-B*57:01 in a similar conformation and the majority of pHLA interactions are maintained. The kynurenine is accommodated in the F-pocket of HLA-B*57:01 with an additional hydrogen bond formed with the 2-amino phenyl moiety of kynurenine and S116. These minimal changes in peptide structure and interaction are consistent with the negligible changes in HLA-stabilization observed by differential scanning fluorimetry and cell surface binding assays.

4. Discussion

The association of HLA-B*57:01 with long-term slow-progression in HIV-1 infected individuals suggests that immune responses restricted toward this allele assist in providing long-term viral control. As such, examining naturally presented HIV antigens by HLA-B*57:01 provides options for vaccine design and monitoring immune responses toward the virus. In this study, we generated APCs that coexpressed HLA-B*57:01 and HIVenv protein, a prominent vaccine target. A large number of constitutively presented HLA-B*57:01-bound peptides (>8700) were identified in this study,[60] HIVenv derived peptides only contributed to a very small proportion (<0.1%) of the HLA-B*57:01-immunopeptidome with seven distinct peptides from two regions of HIVenv. Of note, three peptides from the signal sequence of HIVenv were detected including a 12mer and an overlapping 10mer peptide that was identified with the native sequence and also containing a C-terminal kynurenine residue. The N-terminal region of HIVenv has been previously implicated in antiviral immunity.[71] Two forms of a 12mer peptide from the gp41 region of env were also identified in this study, one bearing a deamidation of the asparagine residue at position 9 to an aspartic acid. This region of HIVenv has not been reported as immunogenic in the past. All seven peptides contained canonical HLA-B*57:01 P2 and P2 anchor residues substantiating their binding specificity.[60] HLA-B*57:01 binding assays demonstrated robust and specific binding of all identified peptides to HLA-B*57:01. The modified and native forms of both the peptides bound to HLA-B*57:01, however, the longer KSLE peptides (12 residues) showed higher binding affinity compared to the RVK and the control IALY peptides. Interestingly, the PTMs (W to Kyn or N to D) neither significantly affect binding nor the overall pHLA stability for the kynurenine modification. This contrasts significantly with many HLA class II bound PTM peptides where the modification is intimately involved in HLA allomorph specific binding. For instance, P4 citrullination is key to binding of joint autoantigens to rheumatoid arthritis associated HLA-DR4 allotypes[18] and deamidation of wheat proteins is critical for high-affinity interactions of the
antigenic peptides to HLA-DQ allomorphs associated with coeliac disease.\cite{20,72,73}

Tryptophan metabolism plays a significant role in HIV pathogenesis by contributing to neurocognitive disorders and immune suppression due to reduced free tryptophan and increased levels of metabolites like kynurenine.\cite{74} This is the first report of a kynurenine-modified epitope being naturally presented. There have been reports of kynurenine modified peptides in plants\cite{75} and while the role of kynurenine in neurocognitive disorders has been recognized,\cite{76,77} there have been no reports of it being incorporated into peptides for presentation to the immune system. It is still unclear how the kynurenine modified peptides form. It was possible to enzymatically convert a tryptophan residue of a peptide into kynurenine by treating with IDO1 in the presence of H2O2, however, incorporation of kynurenine metabolite during protein synthesis is also a possibility. There is evidence for incorporation of non-natural amino acids into proteins by native tRNA including selenocysteine, selenomethionine, and pyrrolysine.\cite{78,79} The ability of tryptophan aminoacyl-tRNA synthetase to incorporate 5-hydroxytryptophan into proteins has been exploited to probe protein–protein interactions\cite{80,81} and it is of interest to determine if it can incorporate kynurenine in a similar way.

To enable the evaluation of T-cell responses toward these modified and native peptides, healthy volunteers were initially examined, since immune responses in these individuals will not be influenced by antiviral therapy, followed by HIV-infected patients. In both cases the cultured PBMC from HLA-B*57:01+ healthy subjects (n = 5) and uncultured PBMC from HLA-B*57:01+ HIV+ individuals (n = 15) tested, HIV-specific T cells were low or not detectable. One healthy donor (HD004) had a distinct T-cell population that recognized both the native and modified RVK peptide after autologous stimulation (Figure 3). In HIV patients, there were weak responses to most of the peptides tested, including previously reported HLA-B*57:01-restricted CD8+ T-cell epitopes TW10, IW9, and IF9. Importantly, we detected small defined tetramer positive population of T cells specific for RVK and RVK(Kyn) in two of 15 HIV+ subjects examined (Figure 3B and data not shown). The paucity of detectable responses against RVK and RVK(Kyn) peptides could be due to differences in HIV viral sequences in patients. The region of HIVenv spanning the RVK peptide is not highly conserved aside from the tryptophan residue. In addition, most of the patients (11 of 15) were on effective long-term antiretroviral therapy, which has been shown to decrease the frequency of HIV-specific CTL precursors.\cite{82} It has also been noted that the magnitude of epitope-specific T-cell responses is reduced and the TCR repertoires of these epitope responses narrowed.\cite{83} This highlights the complex array of factors involved in mounting immune responses in HIV-infected patients where initial levels of virus, immunodominance hierarchy, and viral escape can all play roles in eliciting and maintaining immune cells. Thus, further analysis for responses to our novel env-specific modified HLA-B*57:01-restricted CD8+ T-cell responses in larger cohorts is warranted.

The structural analysis of pHLA-B*57:01 complexes demonstrated that the kynurenine modification bound the HLA molecule in a similar fashion to the tryptophan. Due to the high resolution of the structures, the electron density of P10-W and P10-Kyn was unambiguous and showed no significant difference in the interactions between the HLA-B*57:01 molecule and the peptides. The tryptophan to kynurenine modification of the P2 anchor residue is buried in the antigen binding cleft and well accommodated by the HLA-B*57:01 molecule, and this PTM did not alter the overall conformation of the pHLA complex. This explains the T-cell cross-reactivity observed in some individuals between the native and modified peptides. It would be of interest to study the effect of this modification if present in a surface accessible part of an antigen.
In summary, this study has probed the HLA-B*57:01-restricted immunopeptidome in depth with the identification of over 8700 ligands. We have identified several novel HIV-env-derived peptides also presented by HLA-B*57:01 including native and PTM forms of signal sequence and Gp41 derived peptides. Of particular interest, we demonstrate for the first time that peptides bearing a natively encoded C-terminal tryptophan residue can also be presented with a kynurenine residue at this location. Kynurenine is a major product of tryptophan catabolism and is highly abundant during inflammation and infection. Our study demonstrates the potential for kynurenine containing epitopes to be recognized by T cells. Given that such a modified peptide was naturally presented, it leads the way for other researchers to look for responses against such modified epitopes in their HIV cohorts as well as epitopes from other conditions where inflammation may drive the incorporation of kynurenine into other viral, tumor, or autoantigenic epitopes. It also raises the possibility that this modification may not always be invisible to the immune system, expanding the known peptidome, and may represent a novel way to modulate immunity.

**Abbreviations**

HLA, human leukocyte antigen; IDO, indoleamine 2,3-dioxygenase; KCN, potassium cyanide; Kyn, kynurenine; PTM, posttranslational modification

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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Raw LC-MS/MS data pertaining to the identification of the HIVenv-derived and endogenous HLA-B*57:01 peptides have been deposited to the ProteomeXchange Consortium via the PRIDE[84] partner repository with the dataset identifier PXD004471. Structural co-ordinates for the RVK and RVK(Kyn)+HLA-B*57:01 complexes have been deposited to the PDB databank under the accession codes 6BXQ and 6BXP, respectively.

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**Conflict of Interest**

The authors declare no conflict of interest.

**Keywords**

HLA-B*57:01, immunopeptidome, human immunodeficiency virus, kynurenine, posttranslational modification

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