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Title:
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Date:
2018-06-01

Citation:
Ramarathinam, S. H., Gras, S., Alcantara, S., Yeung, A. W. S., Mifsud, N. A., Sonza, S., Illing, P. T., Glaros, E. N., Center, R. J., Thomas, S. R., Kent, S. J., Ternette, N., Purcell, D. F. J., Rossjohn, J. & Purcell, A. W. (2018). Identification of Native and Posttranslationally Modified HLA-B*57:01-Restricted HIV Envelope Derived Epitopes Using Immunoproteomics. Proteomics, 18 (12), https://doi.org/10.1002/pmic.201700253.

Persistent Link:
https://hdl.handle.net/11343/268170

Identification of native and post-translationally modified HLA-B*57:01-restricted HIV envelope derived epitopes using immunoproteomics

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/pmic.201700253 .

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Abbreviations:

HLA - Human Leukocyte Antigen

Kyn - Kynurenine

IDO - indoleamine 2,3-dioxygenase

KCN - Potassium cyanide

Keywords: Immunopeptidome, kynurenine, HIV, PTM, HLA-B*57:01

Total number of words (including references as well as figure and table legends): 7921

Abstract (200words max):

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 endoplasmic reticulum (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 we identify naturally presented HLA-B*57:01 bound peptides derived from the envelope protein of the human immunodeficiency virus (HIVenv). HIVenv peptides were present at a very small percentage of the overall HLA-B*57:01 peptidome (<0.1%) and both native and post-translationally modified forms of two distinct HIV peptides were identified. Notably, a peptide bearing a natively encoded C-terminal tryptophan residue was 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. We examined the binding of these peptides at a molecular level and examine their immunogenicity in preliminary functional studies. Modest immune responses were observed to the modified HIVenv peptide, highlighting a potential role for kynurenine-modified peptides in the immune response to HIV and other viral infections.

Statement of significance of the study:

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This study shows that the viral specific peptidome of HIVenv 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.

1 Introduction

Post-translational 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) [1-10]. 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 the cellular proteome have also been correlated with disease states in humans and animal disease models (reviewed in [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

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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 deamination of islet-derived autoantigens [24, 25] and cysteine oxidation of disulfide-rich antigens in type 1 diabetes (T1D) [25, 26], and 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, cysteinylation, and S-glutathionylation of class I-restricted T cell epitopes frequently observed [29]. The molecular dissection the 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 whilst in other cases the PTM is directly required for interactions with the T cell receptor (TCR) (reviewed by Rossjohn *et al. [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 localised 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

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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 polyfunctional 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 amongst the known HLA-B*57:01-restricted epitopes. Out of a large constitutive immunopeptidome (>8700 non-redundant peptides), only 7 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 Materials and Methods

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 the high amounts of HIVenv protein were selected and maintained in RF10 containing 0.5 mg/mL G418 (Roche). These cells were then super transfected with a HLA-B*57:01 containing plasmid and maintained under hygromycin (0.3 mg/mL) 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

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dilution to obtain clones. Clonal cells were expanded in roller bottles at 37 °C and 5% CO_2, and the cell pellets (three replicates per plasmid) snap frozen in liquid nitrogen prior to analysis.

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2.2 Affinity purification of HLA-peptide complexes

HLA-peptide complexes were affinity purified from six C1R cell pellets (5 x 10^9 each) expressing HIVenv and HLA-B*57:01 as described previously [58]. Briefly, immunoaffinity captured (W6/32 anti-HLA pan class I antibody [57]) 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 x 50 or 100 mm long reversed-phase C18 endcapped HPLC column (Chromolith SpeedROD, Merck) using an ÄKTA 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 with separation based on a gradient of 2 to 15% in 1 min, 15 to 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 using a NanoUltra cHiPLC 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. 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 2400 V, curtain gas (CUR) at 25 L/min, ion source gas (GS1) at 10 L/min and an interface heater temperature (IHT) setting of 150 °C. MS/MS switch criteria

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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) [59] 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 [60]. 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 [60, 61]. The following search parameters were used: no cysteine alkylation, no enzyme digestion (considers all peptide bond cleavages), instrument-specific settings for TripleTOF 5600^{+} (MS tolerance 0.05 Da, MS/MS tolerance 0.1 Da, charge state +2 to +5), species *none*, biological modification probabilistic features on, thorough ID algorithm, detected protein threshold 0.05.

Please refer to supplementary methods for IDO1 dioxygenase and peroxidase activity assays (1.1), HLA-B*57:01 binding and stability assay (1.2), structure determination of HLA-B*57:01-peptide complexes (1.3) and evaluation of T-cell responses towards HIVenv peptides (1.4).

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 (Figure S-1) [60] (Supplementary File SI-2). In addition to these endogenous peptides, 7 HIVenv-derived peptides were identified in transfected cells that were absent in untransfected controls (Table 1 and Table S-

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1). Three of the peptides (RVKEKYQHLW, RVKEKYQHL(Kyn) and RVKEKYQHLWRW) 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 RIKQIINMW) and gp41 (KSLEQIWNNMTW, 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 KSLEQIWNNMTW 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 anti-viral 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 Figures S-2 to S-8).

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 analysed 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 ~30–35 μM free tryptophan (~23%) was oxidized under the peroxidase conditions and ~193–198 μM free tryptophan (~100%) was converted into kynurenine under the dioxygenase conditions by IDO1.

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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% to 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 of IDO1's peroxidase activity also blocked the IDO1-mediated increase in kynurenine-modified peptides (data not shown). IDO mediated modification of peptidyl-tryptophan residues was observed for all the peptides tested including previously described HIV epitopes IW9 and TW10 demonstrating that IDO1 in the presence of H_2O_2 enables conversion of tryptophan-containing peptides into kynurenine.

Analysis of the endogenous HLA-B*57:01 repertoire revealed that around 2% (207 of 8773 peptides) of the endogenous peptides contained the kynurenine modification. To identify if the kynurenine modification was an artefact of the peptide purification process, unmodified synthetic RVK peptide was subjected to same conditions as elution of peptides including acetic acid treatment, HPLC fractionation and subsequent analysis by mass spectrometry. Spontaneous conversion of tryptophan to kynurenine in this procedure was negligible (0.126% +/- 0.027; SEM, n=3) when compared to the experimentally observed RVK(Kyn) peptide that represents 3.8% of native RVK HIV peptide.

3.4 HLA binding of native and modified HIVenv peptides

To confirm that the HIVenv-derived RVK, RVK(Kyn), KSLE and KSLE(N9D) peptides identified could bind to HLA-B*57:01, we employed a cell-based epitope stabilisation assay using the transporter associated with antigen processing (TAP) deficient T2 cell line [64]. A HLA-B*57:01-specific antibody (3E12 [65]) 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 (IALYLQQNW) from the EBV latent membrane protein was used as positive control [66] and YY9 (YLNEKAVSY), a non HLA-B*57:01-restricted peptide, as negative control. None of the test peptides showed detectable stabilisation of endogenous HLA-A*02:01 (which is weakly recognized by 3E12) on the surface of the parental T2 cells confirming specific stabilisation of HLA-B*57:01 on the T2.B*57:01 cell line. The RVK and RVK(Kyn) stabilised HLA-B*57:01 to a similar

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degree and to levels of around 50% of that stabilised by the control IALY peptide. Similarly, KSLE and KSLE(N9D) also equally stabilised HLA-B*57:01 molecules but in this case the stabilisation was superior to the IALY control peptide. No stabilisation 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 towards HIVenv RVK peptides in healthy donors

To assess *de novo* T cell responses towards the RVK peptides, healthy donors expressing HLA-B*57:01 (Table S-2) 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 EBV-specific CD8+ T-cells, either HLA-B*57:01-restricted IALY (HD001-005) and/or HLA-B*08:01-restricted RAKFKQLL (RAK; HD004 only) peptides were used (Table S-3) [67-69]. 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% ± 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 (Figure S-10). Of the 5 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 towards 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 (Table S-4) were recruited to evaluate T-cell responses to the

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peptides identified in this study and a number of previously characterised HLA-B*57:01-restricted HIV epitopes (Table S-3). We employed peptide-HLA (pHLA) tetramer staining of unstimulated PBMC to determine T-cell specificity towards 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 Figure S-11 for gating strategy). To identify HIV-specific CD8+ T-cells, within the total T-cell population, pHLA tetrameric 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 (Table S-5; Figure 3B). The TW10 frequencies were not higher than 0.1% and no tetramer positive cells were identified in 4 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, Figure S-12) of RVK and RVK(Kyn) tetramer-specific CD8+ T-cells was observed in 1 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 Å and 1.58 Å, respectively (Table S-6, Figure 4A, 4B), allowing the clear distinction between the P10-W and P10-Kyn (Figure 4 C-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 (Table S-7). 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 (Table S-7). The P2-V contacts the HLA-B*57:01 heavy chain via

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hydrogen bonds to E63 and hydrophobic interactions with a network of Methionine and Tyrosine residue from the B pocket (Table S-7). 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 (Table S-7). Both peptides interact with the Y123, which forms pi-pi 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-aminophenyl 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-aminophenyl moiety of kynurenine and S116. These minimal changes in peptide structure and interaction are consistent with the negligible changes in HLA-stabilisation 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 towards 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 towards the virus. In this study, we generated APCs that co-expressed 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 (less than 0.1%) of

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the HLA-B*57:01-immunopeptidome with 7 distinct peptides from two regions of HIVenv. Of note, 3 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 anti-viral 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 7 peptides contained canonical HLA-B*57:01 P2 and PΩ 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) did not 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 [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 [74]. This is the first report of a kynurenine-modified epitope being naturally presented. There have been reports of kynurenine modified peptides in plants [75] and while the role of kynurenine in neurocognitive disorders has been recognized [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

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convert a tryptophan residue of a peptide into kynurenine by treating with IDO1 in the presence of H_2O_2, 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 [78, 79]. The ability of tryptophan aminoacyl-tRNA synthetase to incorporate 5-hydroxytryptophan into proteins has been exploited to probe protein-protein interactions [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 towards 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 2 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 anti-retroviral therapy (ART) which has been shown to decrease the frequency of HIV-specific CTL precursors [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 [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

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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 PΩ 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 8,700 ligands. We have identified several novel HIVenv-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, tumour 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.

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Supplementary Information

Supplementary file 1: Supplementary Figures (S-1 to S-12) and Tables (S-1 to S-7)

Supplementary file 2: HLA-B*57:01 endogenous peptide list

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.

Funding Sources

We thank the following funding sources for supporting this work; Australian National Health and Medical Research Council (NHMRC) project grants (APP1085018, APP1084283, APP1129320 and APP1052979); Australian Research Council (ARC) project grant (DP150104503); AWP is supported by a NHMRC Senior Research Fellowship (APP1044215), JR is supported by an ARC Laureate Fellowship; SG is supported by a Monash Senior Research Fellowship. SR was supported by an Australian Postgraduate Award. PI is supported by a NHMRC Early Career Fellowship (1072159).

ACKNOWLEDGMENTS

We would like to thank Dr. David van Bockel and Dr. Anthony Kelleher at the Kirby Institute, UNSW, for HLA-B*57:01-KF11 monomers for HLA tetramer preparation and Lyudmila Kostenko and James McCluskey for the 3E/12 antibody. We thank all the study participants and Ms. Julie Silvers and Helen Kent for assistance with recruitment.

Conflict of interest statement

The authors have declared no conflict of interest

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TABLES

Table 1. HLA-B*57:01 restricted HIVenv peptides identified in this study. 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 [85])

Peptide Sequence	HXB2 AA position in Protein	Sequence in Literature and HLA-restriction if known (HIV LANL DATABASE EPITOPE ID in parenthesis)	Reference
KAYDTEVHNVW	59-69	KAYDTEVHNVW (56054)	[86]
KSLEQIWNNMTW	617-628	Not known to be immunogenic	
KSLEQIWND*MTW (Deamidated N9)	617-628		
RIKQIINMW	419-427	RIKQIINMW (793) B57, *A*32:01*	[87]
RVKEKYQHLW	2-11	MRVKEKYQHLWRWGW (57674)	[88]
RVKEKYQHL(Kyn)	2-11		
RVKEKYQHLWRW	2-13	MRVKEKYQHLWRWGW (57674)	[71]

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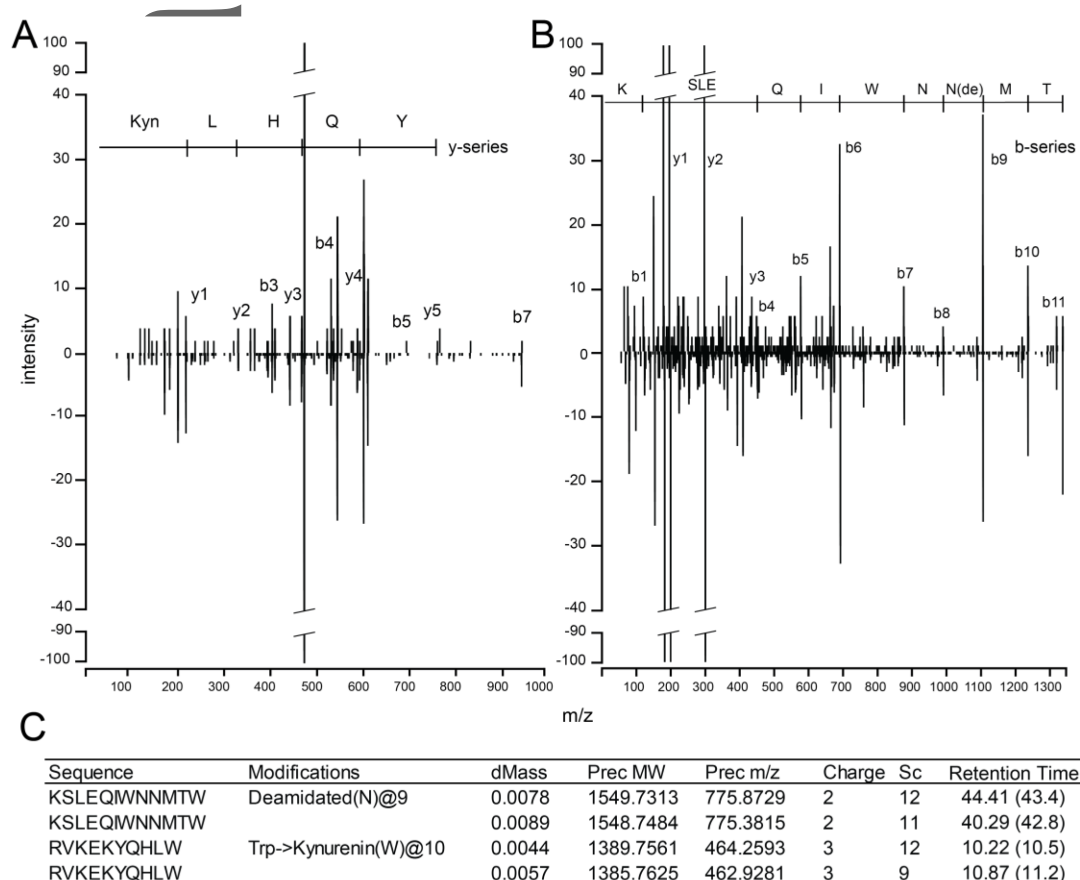
FIGURES

Figure 1: MS/MS fragmentation spectrum of a HLA-B*57:01 restricted HIV peptide RVKEKYQHL(Kyn) (A) KSLEQWND*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.



Sequence	Modifications	dMass	Prec MW	Prec m/z	Charge	Sc	Retention Time
KSLEQIWNNMTW	Deamidated(N)@9	0.0078	1549.7313	775.8729	2	12	44.41 (43.4)
KSLEQIWNNMTW		0.0089	1548.7484	775.3815	2	11	40.29 (42.8)
RVKEKYQHLW	Trp->Kynurenin(W)@10	0.0044	1389.7561	464.2593	3	12	10.22 (10.5)
RVKEKYQHLW		0.0057	1385.7625	462.9281	3	9	10.87 (11.2)

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Figure 2: (A) IDO1 in the presence of H_2O_2 is able to convert peptidyl-tryptophan into kynurenine in the three HIV peptides. TW10 and RVK when treated with IDO1 and H_2O_2 generated statistically significant amount of kynurenine modified peptide when compared to peptide only, IDO1 only or H_2O_2 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) Stabilisation of cellular HLA-B*57:01 molecules by synthetic HIVenv peptides. All HIVenv peptides tested, and the known HLA-B*57:01 ligand IALY, stabilised HLA-B*57:01 surface expression on T2-B*57:01 cells, whilst 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 stabilisation by any of the HLA-B*57:01-restricted peptides.

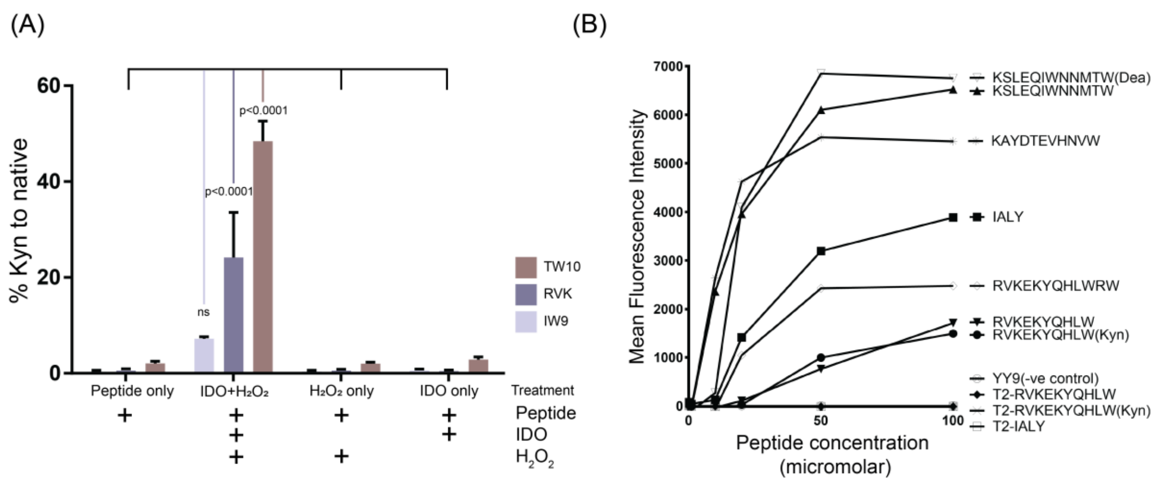

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Figure 3. (A) T-cell responses against antigenic peptides in healthy donor HD004. These plots show TNF-α and IFN-γ responses against the native RVK and RVK(Kyn) peptides. The control panel for parental C1R cells only (LHS panel), C1R cells pulsed with peptides, C1R-B*57:01 cells only, and T-cells only were negative as expected (see Figure S-10). When T cells originally primed with RVK(Kyn) were stimulated with peptide-pulsed C1R-B*57:01 cells or peptide alone, 1.15% of CD8+ cells expressed both TNF-α and IFN-γ (middle and RHS panels). No responses were seen in the RVK outgrown population, whilst robust responses were seen against RAK in the T-cell line originally primed with this peptide (Figure S-10). The gating strategy is shown in Figure S-9. (B) Representative plots from a HIV patient (HIV#3) showing CD8+ T-cell specificity for HLA-B*57:01/peptide tetramers specific for control KF11 and IW9 peptides as well as RVK and RVK(Kyn). The gating strategy is shown in Figure S-11.

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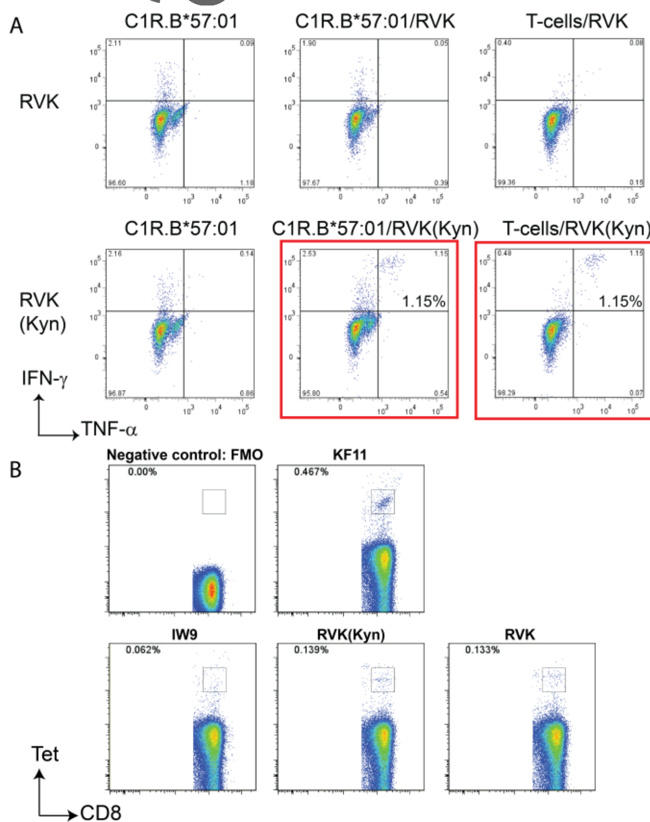

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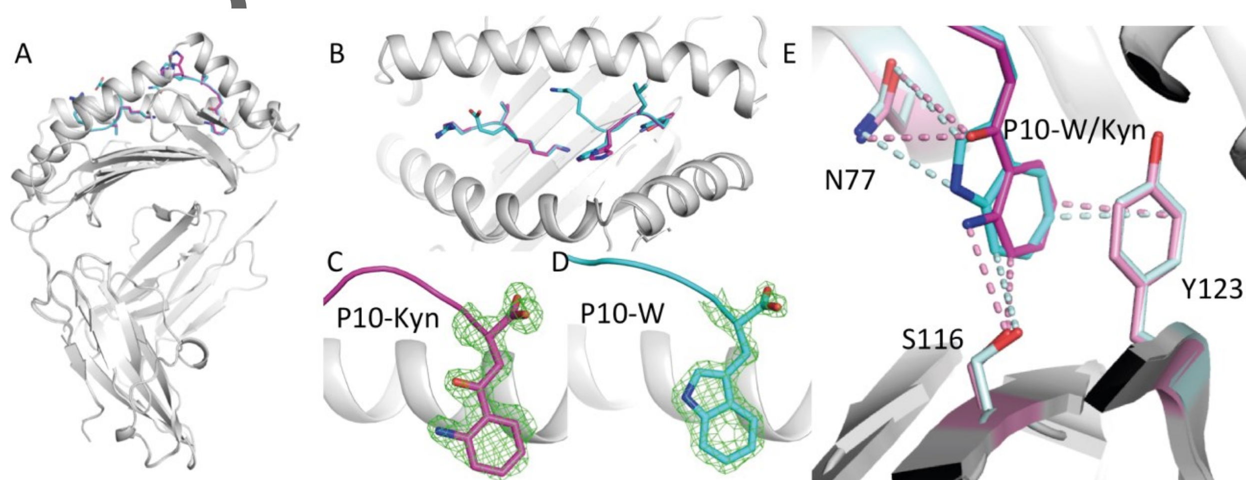
Figure 4: Structural analysis of HLA-B*57:01 in complex with the native and RVK(Kyn) peptide. (A) HLA-B*57:01 molecule with RVK peptide in the binding groove. (B) Superimposition of peptide binding clefts of HLA-B*57:01 in complex with RVK (cyan) or RVK(Kyn) (pink). Electron density omit map (mFo-Fc at 3σ) of the P10 residue from the RVK(Kyn) in pink (C) and of RVK in cyan (D) peptides is represented as a green mesh. (E) F-Pocket interactions of P10-W (cyan) and P10-Kyn (pink), with the dashed line representing the key interaction between HLA-B*57:01 residues (represented in stick) and each peptide.



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