Immunopeptidomic Analysis Reveals That Deamidated HLA-bound Peptides Arise Predominantly From Deglycosylated Precursors

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In Brief
A predominance of HLA-I bound Asn deamidated peptides have been found to be generated through the ERAD pathway by quantitative MS-based proteomics. The Asn deamidated peptides in the peptidome of HLA-I, but not in HLA-II peptidomes or shotgun proteolysis, bear a consensus the N-glycosylation motif. The results reported provide new insights into the generation deamidated peptide antigens and provides a strategy for predicting deamidated T cell epitopes derived from glycoproteins.

Graphical Abstract

Highlights
- A predominance of HLA-I bound deamidated peptides are generated through ERAD pathway.
- Deamidation of peptides with N-glycosylation motifs not in peptidome of HLA-II or proteolysis.
- Many precursors of ERAD generated deamidated peptides are glycoproteins.
Immunopeptidomic Analysis Reveals That Deamidated HLA-bound Peptides Arise Predominantly from Deglycosylated Precursors

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The presentation of post-translationally modified (PTM) peptides by cell surface HLA molecules has the potential to increase the diversity of targets for surveilling T cells. Although immunopeptidomics studies routinely identify thousands of HLA-bound peptides from cell lines and tissue samples, in-depth analyses of the proportion and nature of peptides bearing one or more PTMs remains challenging. Here we have analyzed HLA-bound peptides from a variety of allotypes and assessed the distribution of mass spectrometry-detected PTMs, finding deamidation of asparagine or glutamine to be highly prevalent. Given that asparagine deamidation may arise either spontaneously or through enzymatic reaction, we assessed allele-specific and global motifs flanking the modified residues. Notably, we found that the N-linked glycosylation motif NX(S/T) was highly abundant across asparagine-deamidated HLA-bound peptides. This finding, demonstrated previously for a handful of deamidated T cell epitopes, implicates a more global role for the retrograde transport of nascently N-glycosylated polypeptides from the ER and their subsequent degradation within the cytosol to form HLA-ligand precursors. Chemical inhibition of Peptide:N-Glycanase (PNGase), the endoglycosidase responsible for the removal of glycans from misfolded and retrotranslocated glycoproteins, greatly reduced presentation of this subset of deamidated HLA-bound peptides. Importantly, there was no impact of PNGase inhibition on peptides not containing a consensus NX(S/T) motif. This indicates that a large proportion of HLA-I bound asparagine deamidated peptides are generated from formerly glycosylated proteins that have undergone deglycosylation via the ER-associated protein degradation (ERAD) pathway. The information herein will help train deamidation prediction models for HLA-peptide repertoires and aid in the design of novel T cell therapeutic targets derived from glycoprotein antigens.

Post-translational modification (PTM) including phosphorylation (1), ubiquitinylation (2), acylation (3), deamidation (4) and other structural modifications (5) has the potential to vastly expand the ligand repertoire presented by cell surface human leukocyte antigen (HLA) class I molecules for recognition by T cells. The alteration of physiochemical properties following PTM may modulate binding affinity to a given HLA allotype (5) as well as eliciting or abrogating T cell responses compared with the native sequence (4, 6–10). For example, several citrullinated or phosphorylated peptides have higher binding affinity to HLA-A2 and can upregulate the response of CD8+ T cells compared with their unmodified forms in subjects with type 1 diabetes (4), and phosphorylation of tumor epitopes has been shown to stabilize the peptide–HLA complex (11). In addition, selective deamidation of HLA class II-restricted gliadin peptides enhances HLA-DQ2 and HLA-DQ8 binding with concomitant increases in immunogenicity (12). In contrast, the CD8+ T cell response to A-gliadin123–132 is abolished when the position 123 glutamine is deamidated, likely because of reduced HLA binding (10).

Our understanding of the human immunopeptidome—that is, the repertoire of peptides bound to and presented by HLA molecules—has grown rapidly because of increases in the speed and sensitivity of mass spectrometers and improvements in the specific isolation of peptide-HLA (pHLA) complexes from cells and tissues (13–19). These studies rely on computational algorithms to infer the peptide sequence from a reference protein database. Despite PTMs not being explicitly encoded in these databases, such algorithms have the capacity to search for and detect the appropriate mass shift in PTMs not being explicitly encoded in these databases, such algorithms have the capacity to search for and detect the appropriate mass shift associated with a large array of PTMs (20–22). However, except for S/T phosphorylation (23–25), there have been few systematic studies of PTM peptides in the immunopeptidome. Herein we describe the analysis of several comprehensive immunopeptidomics data sets (our data and those of others), with a focus on assessing the distribution of PTMs across different HLA allotypes. Other than oxidation of methionine deamidation of asparagine and glutamine was the next most prevalent type of modification across all HLA-I allotypes. Detailed analysis of the amino acid residues flanking the site of peptide ligand deamidation revealed a strong prevalence of...
the known N-linked glycosylation motif NX(S/T), where N is the deamidated asparagine residue and X is any amino acid except proline (26). Notably no motif was observed ﬂanking the site of glutamine deamidation or for asparagine deamida-
tion in peptides isolated from HLA class II molecules. Sub-
sequent blocking of PNGase activity conﬁrmed the prevalent
role of deglycosylation of NX(S/T)-bearing antigenic precursors
in the generation of asparagine-deamidated HLA ligands.
Although this mechanism is known for a handful of deami-
dated T cell epitopes (6, 7, 27–31), these data indicate that the
immunopeptidome is substantially enriched for peptides de-
derived from formerly glycosylated proteins and speciﬁcally
those that have been retro-translocated from the ER and
targeted for deglycosylation and degradation in the cyto-
plasm. These large data sets will help to train algorithms for
the prediction of deamidated peptide ligands and the discov-
ery of novel T cell targets. Moreover, our data highlights a
relatively unappreciated surveillance mechanism for glyco-
protein antigens via the HLA-I antigen presentation pathway.

Experimental Design and Statistical Rationale—In this study, pep-
tides from three sources were analyzed: (1) HLA-I immunopeptidome;
(2) HLA-II immunopeptidome; (3) shotgun proteomics. Each source
contains nine data sets. For data sets from HLA-I immunopeptidome,
three of them were identiﬁed from in-house experiments that involved
biological duplicates of 5 × 10^6 cells which were used for the isolation
of each HLA-I allotype (three in total) and analysis of their bound
peptides. The remaining data sets from HLA-I immunopeptidome
and other two sources come from publicly available data sets. For
PNGase inhibition experiments, quadruplicate biological replicates of
1 × 10^6 cells were tested, with equivalent numbers of control samples
(adding equivalent of DMSO instead of PNGase). t Test (32), one-way
ANOVA (33), and two-way ANOVA (19) were used in this study.

Cell Lines—The Epstein-Barr virus-transformed B-lymphoblastoid
cell line C1R, which expresses very low levels of endogenous
HLA-B35:03 and low levels HLA-C*04:01 (34, 35), was stably trans-
ferred with either HLA-A*01:01, HLA-A*02:01 or HLA-A*24:02 by
electroporation as previously described (36). Transfected cells were
grown in RPMI 1640 (Invitrogen) supplemented with 10 IU/ml penicil-
lin, 50 μg/ml streptomycin, 7.5 mM HepES (Sigma, St Louis, MO), 2
mm L-glutamine (MP Biomedical), 75 μM β-mercaptoethanolamine
(Sigma), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA)
and 10% fetal calf serum (FCS) (RF-10). 0.3 mg/ml hygromycin (Bio-
vitrogen) was added to select for stable expression of the transfected
plasm. These large data sets will help to train algorithms for
the prediction of deamidated peptide ligands and the discov-
ery of novel T cell targets. Moreover, our data highlights a
relatively unappreciated surveillance mechanism for glyco-
protein antigens via the HLA-I antigen presentation pathway.

PNGase Inhibition—The pan-caspase inhibitor Z-VAD-FMK
(ab120487, Abcam, Cambridge, UK) was used to block PNGase
activity, as described by Altrich-VanLith et al. (28). Briefly, 1 × 10^6 cells
were pelleted by centrifugation and resuspended in 10 ml of RF-10
containing either 50 μM Z-VAD-FMK or vehicle control (2.5 μl/ml
DMSO) and incubated for 30 min at 37 °C, 5% CO2. The cells were
then treated with an isotonic acid stripping buffer (0.066 M Na2HPO4
and 0.131 M citric acid, pH 3.3) to remove existing cell surface HLA
class I complexes (37, 38), resuspended in 10 ml of fresh RF-10
containing the 50 μM Z-VAD-FMK or vehicle alone and incubated at
37 °C, 5% CO2 for 5 h to allow re-expression of HLA class I mole-
cules. Cells were harvested, washed with PBS, snap frozen in liquid
nitrogen, and stored at −80 °C until processed.

Isolation of HLA-I Bound Peptides—was performed as previously
described (18, 39). Briefly, cell pellets were resuspended in lysis
buffer (0.5% IGE/PAL (Sigma), 50 mM Tris, pH 8 (Sigma), 150 mM NaCl
(Merck-Millipore, Darmstadt, Germany) and protease inhibitors (Com-
plete Protease Inhibitor Mixture Tablet [1 tablet per 50 ml solution];
Roche Molecular Biochemicals, Basel, Switzerland)) and incubated
for 45 min at 4 °C. Lysates were cleared by centrifugation at 16,000 ×
g for 40 min at 4 °C. pHLA complexes were immunoafﬁnity puriﬁed
from lysates using W6/32 monoclonal antibody (10 mg/10^6 cells)
crosslinked to protein A Sepharose (40). Bound complexes were
eulled with 10% acetic acid and fractionated by RP-HPLC as previ-
ously described (18). Briefly, the mixture of eluted peptides, class I
heavy chain and β2-microglobulin (β2m) was fractionated by RP-
HPLC using a 4.6 × 100 mm monolithic C18 column (Chromolith
Speed Rod, Merck-Millipore), an AKTAmicro™ HPLC system (GE
Healthcare, UK) and mobile phases consisting of buffer A (0.1%
trifluoroacetic acid (TFA) [Thermo Scientiﬁc, San Jose, CA]) and buffer
B (80% acetonitrile (ACN) [Fisher Scientiﬁc, Waltham, MA] and 0.1%
TFA). Fractions were combined, concentrated by vacuum centrifuga-
tion, and reconstituted in 2% v/v acetonitrile in 0.1% v/v aqueous
formic acid. Each pool contained 25 fmol/μl iRT peptides (Biognosys,
Schlieren, Switzerland (41)) as an internal retention time standard.

LC-MS/MS Acquisition—LC-MS/MS of HLA-I bound peptides was
carried out using a SCIEX TripleTOF™ 6600 equipped with an on-line
Eksigent Ekspert nanoLC 415 (SCIEX, Toronto, Canada). 10 μl of
each sample was directly loaded onto a trap column (ChromXP C18,
3 μm 120 Å, 350 μm × 0.5 mm [SCIEX]) maintained at an isocratic
ﬂow of buffer A (2% v/v acetonitrile in water supplemented with 0.1%
v/v formic acid) at 5 μl/min for 10 min and then separated using an
analytical column (ChromXP C18, 3 μm 120 Å, 75 μm × 15 cm
[SCIEX]) by increasing linear concentrations of buffer B (0.1% v/v
formic acid, 80% v/v acetonitrile) at a ﬂow rate of 300 nL/min for 75
min. Up to 20 MS/MS spectra were acquired per cycle using an IDA
strategy with accumulation times of 200 ms and 150 ms for MS1 and
MS2, respectively. The MS1 scan range was set to 300–1800 m/z
and MS2 set to 80–2000 m/z. To prevent multiple sequencing of the same
peptide, MS1 masses were excluded for sequencing after two occur-
rences for 30 s.

LC-Multiple Reaction Monitoring (MRM)—HLA-bound peptides pu-
rified from Z-VAD-FMK inhibition experiments (four biological repli-
cates for each condition; Tier 3 analysis) were analyzed using a
QTRAP® 5500+ (SCIEX) mass spectrometer equipped with an on-line
Eksigent Ekspert nanoLC 415 (SCIEX) using an identical trap-elute
schema to the LC-MS/MS experiments above. Data were acquired in
positive ion MRM mode (refer to supplemental Table S1 for full
transition list parameters) at unit resolution, with a triggered Enhanced
Product Ion scan (80–1000 m/z; dynamic fill time; rolling collision
energy) for any MRM transition exceeding 1000 counts per second.
Data were analyzed in Skyline (v19.1.0.193) and manually assessed
for any background or nonspeciﬁc interference, as well as ensuring all
dot-products were ≥0.8 when comparing against the spectral library
of pHLA derived from C1R-A1 cells. Peak areas for transitions were
summed and values normalized to levels of iRT peptides present in
each sample.

Peptide Sequence Identiﬁcation—MS/MS data were searched
against the human proteome [UniProt v2018_11] by PEAKS Studio
8.5 (Bioinformatics Solutions, Toronto, Canada) using the Homo
sapiens Uniprot database (71975 entries, dated 2018–11). Both
in-house generated MS data files and public MS data files from
PRIDE repository (42) (PRIDE accession: (HLA-I): PXD004894,
PXD000394, PXD005084, PXD004023, PXD008570, PXD008571,
was considered statistically significant. Analyses were calculated using GraphPad Prism 7, and a comparison was assessed by a one-way ANOVA multiple-comparison test. All modification sites among the peptides with NX(S/T) motif (Fig. 5A) were detected for each of the transfected alleles (range: 9000–11,000), with around 1800 peptides identified from the parental C1R cell, derived from HLA-C*04:01. This unbiased PTM search revealed ~21%, 22%, 17 and 38% of these peptides were post-translationally modified within the HLA-A*01:01, -A*02:01, -A*24:02 and -C*04:01 data sets, respectively (Fig. 1A). On assessment of the distribution of modification types, oxidation of Met accounted for the largest proportion (57–83%) of PTMs across all four allotypes (Fig. 1B), followed by deamidation of Asn or Gln (2.5–7%) (Fig. 1B). Phosphorylation (Ser, Thr or Tyr) and citrullination of Arg accounted for less than 3% of the detected PTM repertoire—all other modifications were individually lower in frequency, yet in total they represented up to 33% of the detectable PTM space.

Next, we investigated the properties of peptides bearing modified residues. Despite the high prevalence of oxidized Met, it is difficult to attribute this modification to bona fide biological origin over sample preparation artifacts (44, 45); as such, we chose to focus on deamidation, the next most prevalent PTM (Fig. 1). Both Asn and Gln deamidation were observed across each of the transfected alleles (Fig. 1B, Fig. 2A) and the endogenous HLA-C*04:01 allele expressed by the parental C1R cell line (46). Deamidation of Asn was observed to be more prevalent than for Gln across all allotypes (Fig. 2A). The length of deamidated peptides was predominantly 9–13 amino acids (Fig. 2B), consistent with the total peptide repertoire of the corresponding HLA-I allotypes (supplemental Fig. S1). To assess any positional bias, we normalized the position of the deamidated residue as a proportion of peptide length and expressed the results as a heatmap (Fig. 2C and 2D). This analysis showed some degree of positional bias for asparagine deamidation (Fig. 2C), with a higher proportion of peptides bearing a deamidated residue in the N-terminal half of the ligand. These biases reflected the individual HLA allotype preferences for aspartic acid residues at P3 for HLA-C*04:01 (46), P3 for HLA-A*01:01 (36) and to a lesser extent P4 for HLA-A*02:01 (for all binding motifs, see supplemental Fig. S2). For Gln deamidation, the spread of the modified residue

**RESULTS**

**PTM Profile of Peptides Presented by Various HLA Alleles**—We investigated the prevalence of PTMs presented by three common HLA-A allotypes using C1R cell lines expressing HLA-A*01:01, -A*02:01 or -A*24:02. We exploited the common expression of low levels of endogenous HLA-C*04:01 as an additional control. HLA-peptide complexes were purified by immunoaffinity capture, bound peptides eluted by mild acid treatment and sequenced by liquid chromatography coupled to high-resolution mass spectrometry. As part of this process, we used the PEAKS PTM algorithm (43) to detect the diversity of PTMs across the immunopeptidomes of these allotypes (Fig. 1). On average, 10,000 peptides were detected for each of the transfected alleles (range: 9000–11,000), with around 1800 peptides identified from the
position appears more random (Fig. 2D), with only a moderate change in density for HLA-A*01:01 and -A*02:01 consistent with the peptide binding motifs of these alleles favoring Glu residues at position 3 or 4 (supplemental Fig. S2).

Next, we assessed the amino acids flanking the deamidated Asn or Gln residues (Fig. 3). A motif analysis was performed for deamidated peptides by centralizing the deamidated residue and expanding the sequence to the seven naturally occurring amino acids either side of the modification (as a means to extend beyond either termini of the final bound HLA ligand). As shown in Fig. 3A, a preference of Thr or Ser at the +1 site was observed in Asn deamidation across each HLA allotype, whereas no obvious bias was observed for Gln deamidation. To verify that the +2 Ser/Thr bias was a feature of Asn deamidated sequences, we carried out the same type of centralized motif analysis on peptides bearing unmodified Asn residues (Fig. 3B). The results for these unmodified peptides do not demonstrate +2 Ser/Thr preference, but show clear dominance of the allele-specific anchor residues. Finally, to demonstrate the specific enrichment of the NX(S/T) motif in the deamidated peptide ligands, we carried out the same type of centralized motif analysis for Asp residues found across the peptide data sets (Fig. 3C) and once again no enrichment of a +2 Ser/Thr bias was observed.

The NX(S/T) motif is the canonical motif for N-linked glycosylation (47). The data from Fig. 3A show a clear enrichment of NX(S/T) for the four HLA class I data sets analyzed (on average, 59% of Asn deamidated peptides contain the NX(S/T) motif). We further extended our analysis to public immuno-peptidomics data sets (1, 16, 39, 48, 49). In total, this comprised 42 HLA-I allotypes and 153,066 peptides of length 8–20 amino acids, of which 2206 (≈1.4%) contained one or more deamidated Asn residues (supplemental Table S2). As shown in Fig. 4A, this combined data revealed and strengthened the same +2 Ser/Thr motif observed in our data. An additional significant preference for a Gly residue at the +1 position was observed (Fig. 4A), which was apparent to a lesser extent in our own data sets (see Fig. 3A). The presence of Gly at position +1 is of note because this motif is known to drive spontaneous deamidation of Asn (50, 51).

Organellar Profiling

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Fig. 2. Immunopeptidome profile of deamidated peptides presented by four HLA allotypes. A, The proportion of asparagine and glutamine deamidation in the total deamidation set of each allotype. B, The length distribution of asparagine and glutamine deamidation from each allotype. C and D, Heatmap of the normalized position of deamidated asparagine or glutamine across each allotype (data sets are from two biological replicates).
In order to investigate the division between H1 Gly and H2 Ser/Thr motifs, we initially analyzed data sets from either HLA-II immunopeptidomes (52, 53) (supplemental Table S3) or a selection of conventional shotgun proteomics data sets (19, 49, 54–57) (supplemental Table S4). In the former, HLA-II bound peptides will not derive from proteins degraded and processed via the ERAD pathway (58), whereas the latter will snapshot the intact protein content of a cell through specific enzymatic digestion (in this case, trypsin (49, 54–57) or elastase (19, 57)). Fig. 4B displays the motif flanking centralized deamidated Asn residues from pHLA-II, showing a clear enrichment of Gly at the H1 position. The global proteome analysis (Fig. 4C) shows an even stronger enrichment for deamidated Asn residues to bear a glycine at the H1 position. Neither data sets, however, revealed a bias for a H2 Ser/Thr motif.

Next, we segregated the deamidated Asn sequences from the HLA-I and global proteome analysis based on their motif types, separating into four categories: NGX, where X can be any amino acid except proline; NGx, where x can be any amino acid except Ser/Thr; NX(S/T);NU(S/T) where U can be any amino acid except Gly (Fig. 4D). This analysis shows that the proportion of deamidated Asn peptides with an NGX or NGx motif from the shotgun proteomics data sets are significantly higher than for peptides derived from the HLA-I presentation pathway (p < 0.0001). Conversely, we observe that ~48% of HLA-I bound deamidated peptides bear the NX(S/T) motif and only a small fraction (~7%) of these comprise the motif where X can be glycine (Fig. 4D). The results obtained here further indicate the enrichment of the NX(S/T) motif in the deamidated pHLA-I repertoire.

Analysis of N-linked Glycosylation Sites Across Deamidated Asparagine Peptides—We next compared the concordance of annotated N-linked glycosylation sites with peptides bearing this motif within immunopeptidome and proteome data sets (Fig. 5A). These data show a 30% concordance of asparagine-deamidated pHLA-I with known N-linked glycosylation sites, whereas only ~2% of non-deamidated peptides with the motif (number ~6516) overlapped with known glycosylation sites. It should be noted that around 130 known N-linked glycosylation sites are still found in non-deamidated peptides with the motif. In contrast only ~0.5% of NX(S/T)-bearing peptides (number ~1186) map to known glycosylation sites in the proteomics data sets (Fig. 5A).
Subcellular Distribution of Source Proteins of Peptides Bearing the NX(S/T) Motif—We next investigated the subcellular distribution of the source protein of each NX(S/T)-containing HLA-I bound peptide. As shown in Fig. 5A, a high proportion of deamidated HLA-I bound peptides contain a consensus N-linked glycosylation motif which is not reflected in peptides derived from trypsin/elastase digestion of cellular material. B, A comparison of the subcellular location of the source antigens for HLA-I bound peptides among unmodified and peptides with native or deamidated Asparagine residues that harbor the NX(S/T) glycosylation motif. C, A comparison of the subcellular location of the source proteins of deamidated peptides bearing the NX(S/T) motif shows significant differences between trypsin/elastase-digested proteomes and HLA-I bound peptides. Significance was determined by two-way analysis of variance (ANOVA) performed using Prism 7 (**, p = 0.0002, ***, p = 0.0021).

**Fig. 5.** Subcellular location of source proteins and the relationship between deamidation of peptides containing the NX(S/T) motif. A, A high proportion of deamidated HLA-I bound peptides contain a consensus N-linked glycosylation motif which is not reflected in peptides derived from trypsin/elastase digestion of cellular material. B, A comparison of the subcellular location of the source antigens for HLA-I bound peptides among unmodified and peptides with native or deamidated Asparagine residues that harbor the NX(S/T) glycosylation motif. C, A comparison of the subcellular location of the source proteins of deamidated peptides bearing the NX(S/T) motif shows significant differences between trypsin/elastase-digested proteomes and HLA-I bound peptides. Significance was determined by two-way analysis of variance (ANOVA) performed using Prism 7 (**, p = 0.0002, ***, p = 0.0021).
FIG. 6. **NX(S/T)-bearing pHLA are dependent on PNGase processing prior to presentation.** LC-MRM was used to quantify the relative presentation of a set of HLA-bound peptides on C1R-A*01:01 cells after incubation for 5 h in the presence or absence of the PNGase inhibitor Z-VAD-FMK. A, Representative LC-MRM traces from control (upper) or inhibitor-treated (lower) cells showing specific detection of peptides from three categories: (left) unmodified asparagine, or deamidated asparagine containing peptides with (middle) or without the **NX(S/T)** motif (right). Each LC-MRM trace consists of four transitions, with specific ions as indicated. B–C, LC-MRM quantitation of peptide detection in inhibitor-treated relative to control-treated cells (quantitation change on individual peptide (B), quantitation change on overall peptides from three categories (C)). Datapoints represent individual peptides from four biological replicates in (B) and the average of replicates in (C). Significance was determined using an ordinary one-way ANOVA multiple comparison test (****, \( p < 0.0001 \); ***, \( p = 0.0002 \)).
further corroborates the glycoprotein origins of the of NX(S/T) deamidated peptides bound to HLA-I. The Presentation of NX(S/T)-Containing pHLA is Dependent On Prior Enzymatic Removal of N-linked Glycosylation—It is well established that deamidation of the Asn residue of N-linked glycosylation sites occurs as a by-product of peptide: N-glycanase (PNGase)-mediated cleavage between the Asn and the innermost N-acetylglucosamine (GlcNAc) monosaccharide of glycosylated proteins (60–63). In addition, PNGase is primarily responsible for the removal of N-linked glycans following the dislocation of misfolded glycoproteins from the ER into the cytosol (64). Therefore, peptides bound to HLA-I with an NX(S/T) motif are expected to be dependent on this enzyme for their genesis, and indeed this has been shown for a HLA-I restricted epitope derived from tyrosinase (28). We used LC-MRM-MS to quantify the relative levels of a subset of peptides originally observed in our C1R-HLA-A*01:01 data set when purified from these cells cultured in the presence or absence of the potent PNGase inhibitor carbobenzyloxy-Val-Ala-Asp-\(\alpha\)-fluoromethylketone (Z-VADEMK) (61) (Fig. 6). Peptides were selected from the HLA-A*01:01/C*04:01 repertoire, consisting of eight control unmodified peptides (seven with unmodified and one without any asparagine residue), ten deamidated peptides with the NX(S/T) motif, and seven deamidated peptides with a NXx motif (X is any amino acid; x is any amino acid except Ser or Thr). Fig. 6A shows raw MRM traces of one representative peptide from each category, with each robustly detected in control-treated cells. However, PNGase inhibition showed marked reduction of the signal from peptide TFNHSGISV yet had no impact on the unmodified sequence WTDNRELTY nor the NGx motif deamidated peptide TSDNGKDGLAY. Fig. 6B shows levels of each peptide across the three categories from four biological replicates comparing inhibitor-treated versus control cells, with Fig. 6C showing that levels of NX(S/T)-containing peptides were significantly \(p < 0.0001\), one-way ANOVA multiple-comparison test) decreased compared with unmodified or NXx motif-bearing peptides.

**DISCUSSION**

Despite the potential importance of PTM of peptide antigens in the development of a range of human diseases (4, 65–77), few systematic studies of the nature and properties of PTM peptides in the immunopeptidome have been performed. In this study we have focused on deamidation of HLA-I bound peptides. Although prior studies have identified several deamidated asparagine-containing T cell epitopes generated following retrograde transport of glycoproteins from the ER to the cytosol and subsequent removal of the N-linked glycans, here we extend these studies to show that a large proportion of HLA-I bound Asparagine deamidated peptides are generated through this mechanism. Quantitative measurements of peptide presentation confirmed the selective reduction of deamidated sequences bearing the N-linked glycosylation motif when the enzyme responsible for catalyzing glycan removal and generation of neo-aspartic acid residues (PNGase) was inhibited. To our knowledge, this is the most detailed study of asparagine deamidation to date, including the analysis of deamidated peptides from the proteome as well as HLA-II immunopeptidomes for comparison. The significant lack of deamidated peptides with the N-linked glycosylation motif in related proteome and HLA-II immunopeptidome data sets further reinforces the enrichment of these peptides by the class I pathway. In contrast a much smaller (15%) fraction of asparagine deamidation in the HLA-I immunopeptidome could be attributed to spontaneous conversion of asparagine to aspartic acid. Spontaneous deamidation is associated with a NG motif allowing these peptides to be distinguished as potential sample preparation artifacts. As anticipated, the proportion of asparagine deamidated peptides with NG motif in proteome data sets was significantly higher than those in the HLA-I immunopeptidome.

Protein glycosylation plays important roles in cancers (78) and critical cellular pathways (79). We suggest that the enrichment of HLA-I bound asparagine deamidated peptides that are derived from glycosylated precursors provides a means for the immune system to survey the fidelity of N-glycosylation. This mechanism may allow CD8+ killer T cells to eradicate cells with perturbed glycosylation resulting from metabolic abnormalities infection or malignancy. Finally, we anticipate the NX(S/T) motif of asparagine deamidation in the HLA-I immunopeptidome as well as other features of deamidated peptides can be used to build models to predict HLA-I bound asparagine deamidated peptides for inclusion in vaccines and other immunotherapies that target glycosylated antigens.

**DATA AVAILABILITY**

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (80) partner repository with the data set identifier PXD014754, including raw .wiff files and peptide csv files exported by PEAKS Studio 8.5, as well as mzIdentML files. MRM data is available at http://www.peptideatlas.org/PASS/PASS01473.

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Conflict of interest—Authors declare no competing interests.

Abbreviations—The abbreviations used are: PTM, Post-translational modification; HLA, human leukocyte antigen; MRM, Multiple Reaction Monitoring; FDR, false discovery rate.
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