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Arginine (Di)methylated Human Leukocyte Antigen Class I Peptides Are Favorably Presented by HLA-B*07

Fabio Marino,[†,‡] Geert P. M. Mommen,[†,‡,§] Anita Jeko,[†,‡] Hugo D. Meiring,[§] Jacqueline A. M. van Gaans-van den Brink,[∥] Richard A. Scheltema,[†,‡] Cécile A. C. M. van Els,[∥] and Albert J. R. Heck*[,†,‡]

[†]Biomolecular Mass Spectrometry and Proteomics, Bijvoet Center for Biomolecular Research and Utrecht Institute for Pharmaceutical Sciences, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

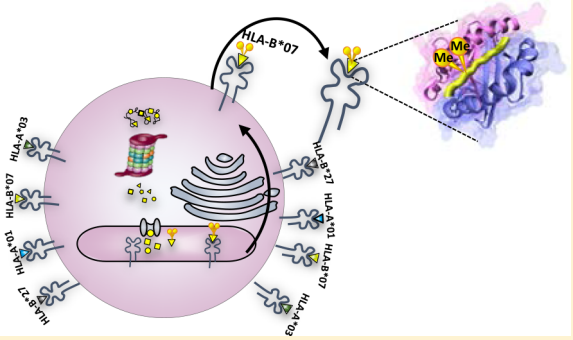
[‡]Netherlands Proteomics Centre, Padualaan 8, 3584 CH Utrecht, The Netherlands

[§]Institute for Translational Vaccinology, P.O. Box 450, 3720 AL Bilthoven, The Netherlands

[∥]Centre for Infectious Disease Control, National Institute for Public Health and the Environment, P.O. Box 1, 3720 BA Bilthoven, The Netherlands

Supporting Information

ABSTRACT: Alterations in protein post-translational modification (PTM) are recognized hallmarks of diseases. These modifications potentially provide a unique source of disease-related human leukocyte antigen (HLA) class I-presented peptides that can elicit specific immune responses. While phosphorylated HLA peptides have already received attention, arginine methylated HLA class I peptide presentation has not been characterized in detail. In a human B-cell line we detected 149 HLA class I peptides harboring mono- and/or dimethylated arginine residues by mass spectrometry. A striking preference was observed in the presentation of arginine (di)-methylated peptides for HLA-B*07 molecules, likely because the binding motifs of this allele resemble consensus sequences recognized by arginine methyl-transferases. Moreover, HLA-B*07-bound peptides preferentially harbored dimethylated groups at the P3 position, thus consecutively to the proline anchor residue. Such a proline-arginine sequence has been associated with the arginine methyl-transferases CARM1 and PRMT5. Making use of the specific neutral losses in fragmentation spectra, we found most of the peptides to be asymmetrically dimethylated, most likely by CARM1. These data expand our knowledge of the processing and presentation of arginine (di)methylated HLA class I peptides and demonstrate that these types of modified peptides can be presented for recognition by T-cells. HLA class I peptides with mono- and dimethylated arginine residues may therefore offer a novel target for immunotherapy.



KEYWORDS: *EThcD, HLA class I, arginine methylation, ADMA, SDMA, PRMT5, CARM1*

INTRODUCTION

Human leukocyte antigen (HLA) class I molecules present short peptides derived from the degradation of cellular proteins on the cell surface.[1] Recognition of pathogen- or disease-related HLA class I-presented peptides by CD8 T lymphocytes cells leads to the activation of a cytotoxic response and clearance of the affected cells. The repertoire of peptides presented by HLA class I molecules is dominated by self-peptides encompassing over 14,000 different species,[2,3] all with defined sequence motifs that determine binding to expressed class I alleles. The overwhelming majority of the naturally processed and presented HLA class I peptides are unmodified; however, a small fraction of peptides do harbor post-translational modifications (PTMs).[4] The importance of HLA class I presentation of peptides with PTMs and their specific recognition by T cells has been demonstrated in infectious diseases,[5] autoimmune diseases,[6,7] and cancer.[8] Protein arginine methylation is a common PTM catalyzed by a family of enzymes known as protein arginine methyl transferases (PRMTs), yielding monomethylated, symmetric (SDMA), or asymmetric (ADMA) dimethylated arginine residues (Figure 1A). Methylation of arginine is known to play a critical role in regulating gene expression[9] and has also been implicated in signal transduction and DNA repair.[10] Only a few HLA class I peptides carrying arginine methylated residues have previously been reported using mass spectrometry (MS)-based approaches.[11−13] Yagüe et al.[11] identified for the first time a naturally processed and HLA-B*39-presented dimethylated peptide derived from a RNA-binding nucleoprotein. Immuno-

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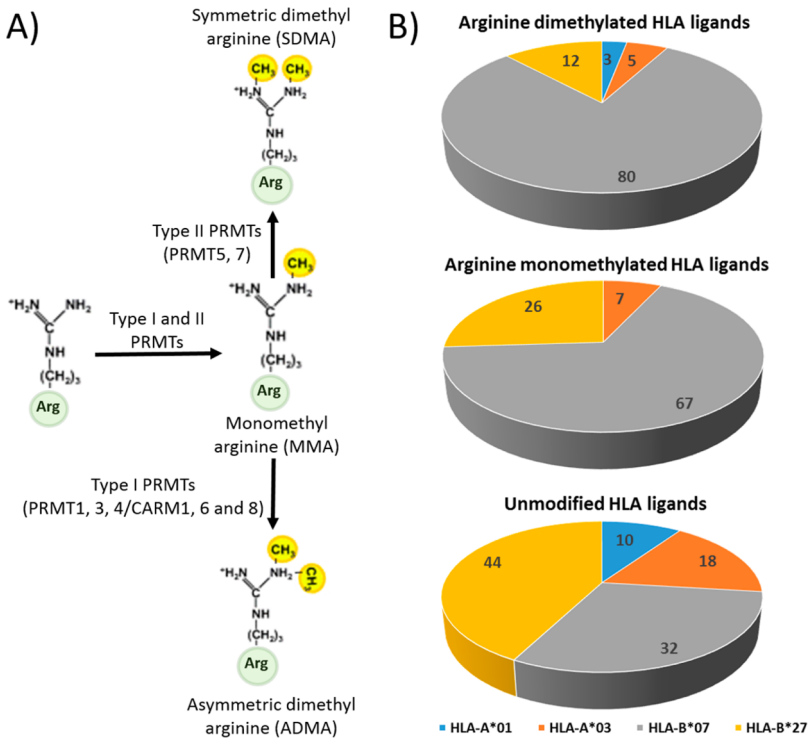

Figure 1. Overview of enzymatic arginine (di)methylation and the observed allele binding preference. (A) The family of protein arginine methylation transferases (PRMTs) mediate monomethylation and consecutive symmetric (SDMA) or asymmetric dimethylation (ADMA), the latter, by type II or I PRMTs, respectively. (B) Distribution of the detected HLA class I peptides over the four most prominent expressed alleles in the studied cell line. From top to bottom are displayed the distribution of dimethylated peptides, monomethylated peptides, and unmodified HLA peptides.

logical recognition of the side-chain modification of arginine residues was subsequently shown by Jarmalavicius et al.,[12] demonstrating that T cells of specific melanoma patients only reacted against the HLA-A*11-bound monomethylated peptide of the GPS-2 protein. Apart from these interesting findings, little is known about the nature of arginine methylated HLA class I peptides, their source protein origin, frequency, and rules of presentation. Here, we report 149 arginine (di)methylated HLA class I peptides, indicating that this PTM is frequently processed and presented for scrutiny by T cells. We found that arginine methylated peptides were presented with a marked allele-specific preference and a favorable P3 sequence position, thereby resembling the underlying substrate motif of specific arginine methyltransferases.

■ EXPERIMENTAL PROCEDURES

Cell Culturing, Isolation of HLA Class I-Associated Peptides, and LC-MS/MS Analysis

Cell culture conditions and isolation of HLA−peptide complexes was described previously.[14] Briefly, two biological replicates of the HLA-A*01:01, -A*03:01, -B*07:02, -B*27:05, -C*02:02, and -C*07:02-positive B-lymphoblastoid cell line GR were grown in RPMI-1640 medium to a total number of 9 × 10^9 cells. HLA class I-peptide complexes were immunoprecipitated from lysed GR cells, using the HLA-A-, -B-, and -C-specific mouse monoclonal IgG2a antibody W6/32. HLA class I−peptide complexes were eluted using 10% (v/v) acetic acid, and peptides were further purified by passage over a 10-kDa molecular weight cutoff membrane. HLA class I-eluted peptides were fractionated by strong cation exchange (SCX) chromatography. The system comprises a Hypercarb trapping column (5 × 0.2 mm i.d., 7 μm particle size; Thermo Fisher) and an SCX column (12 × 0.02 cm i.d. polysulfethyl aspartamide, 5 μm; Poly LC). The SCX fractions were analyzed directly by nanoscale LC-MS/MS using a Thermo Scientific EASY-nLC 1000 (Thermo Fisher Scientific) in combination with an ETD-enabled LTQ Orbitrap Elite (first biological replicate) or an Orbitrap Fusion (second biological replicate) mass spectrometer (Thermo Fisher Scientific). The LC system comprises a 20 × 0.1 mm i.d. trapping column (Reprosil C18, 3 μm; Dr. Maisch) and a 50 × 0.005 cm i.d. analytical column (Poroshell 120 EC-C18; 2.7 μm). For the Orbitrap Elite, full MS spectra were acquired in the Orbitrap at a resolution of 60,000 (FWHM at 400 m/z) while fragment ions were detected in the Orbitrap at a resolution of 15,000 (FWHM at 400 m/z). The 10 most abundant precursor ions were selected either for data-dependent EThcD, CID, ETD, or HCD as previously described.[14] The maximum ion accumulation time for MS scans was set to 200 ms and for MS/MS scans to 2,500 ms. For the Orbitrap Fusion, both full MS and MS/MS spectra were acquired in an Orbitrap with a resolution of 60,000 (FWHM at 400 m/z) and 15,000 (FWHM at 400 m/z), respectively. The Top Speed method was enabled for fragmentation where the most abundant precursor ions in a 3 s cycle were selected for data-dependent EThcD. The maximum ion accumulation time for MS and MS/MS scans was set to 50 and 250 ms, respectively.

Data Analysis

All RAW data files were analyzed using the Proteome Discoverer 1.4 software package (Thermo Fisher Scientific, Bremen, Germany). MS/MS scans were searched against the human Swissprot reviewed database (September 2015, 20203

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entries) with no enzyme specificity using the SEQUEST HT search engine. Precursor ion and MS/MS tolerances were set to 10 ppm and 0.05 Da. Methionine oxidation, arginine monomethylation, and arginine dimethylation were set as variable modifications. The peptides-to-spectrum matches were filtered for precursor tolerance 5 ppm, <1% FDR using Percolator,[15] XCorr >1.7, and peptide rank 1. Only peptides between 8 and 14 amino acid long were selected for further analysis. Precursor ion area detection node was enabled in order to obtain the area under the curve (AUC) of the LC elution profiles of monomethylated, dimethylated, and unmodified peptides. The AUCs of differently methylated peptides (none, mono-, di-) were only compared when detected in the same LC run. The NetMHC 3.4 algorithm[16] was used to predict the HLA−peptide binding affinities for each of the identified peptide sequences. The arginine residue was considered unmodified for binding affinity predictions of arginine (di)methylated peptides. Predictions were enabled for HLA-A*01:01, -A*03:01, -B*07:02, and -B*27:05, and peptides were assigned to bind a particular allele when IC50 < 1000 nM. Sequence logos were generated by the IceLogo[17] algorithm with the Uniprot-Swissprot protein database as reference set and a set p-value threshold of 0.05. Gene Ontology (GO) analysis of the source proteins of the (di)methylated HLA class I peptides was performed by PANTHER.[18] The mass spectrometry data have been deposited to the ProteomeXchange Consortium via the PRIDE[19] partner repository with the data set identifier PXD004233.

Distinction between Asymmetrical and Symmetrical Dimethyl Arginine

Asymmetric dimethyl arginine (ADMA) and symmetrical dimethyl arginine (SDMA) modified peptides are positional isomers, which can be distinguished by their specific neutral loss ions.[20] Fragmentation spectra of ADMA peptides contain specific losses of 45 Da corresponding to dimethylamine (DMA), whereas SMDA peptides display a neutral loss of 31 Da corresponding to monomethylamine loss (MMA).[21] Evaluation of symmetry was automated by annotating the neutral losses with a mass tolerance of 0.05 and calculating a likelihood of finding that many neutral losses in the background of annotated "normal" fragment ions, which acts as a p-value.[22] The specific neutral loss (DMA or MMA) with the best likelihood with a p-value below 0.05 was selected as the indicator for symmetry.

Synthesis of (Di)methylated Peptides and Their Unmodified Counter Parts

Peptide building blocks were purchased from Novabiochem, and appropriately functionalized resins were purchased from Applied Biosystems. Peptides were synthesized using standard Fmoc-based solid-phase peptide synthesis protocols and appropriately functionalized polyethylene glycol−polysterene Wang resins. Functionalized resins were subjected to coupling cycles, in which deprotection of the Fmoc group with piperidine/NMP (1:4 [v/v]) was followed by coupling with 4 equiv each of Fmoc-protected amino acid, di-isopropylethylamine, and (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate. Reactions were carried out in NMP at a volume of 1 mL/0.1 g resin. After the final coupling step, the Fmoc group was removed and peptides were fully deprotected and released from the resin by treatment with trifluoroacetic acid (TFA)/H_2O/triisopropylsilane (93:5:2 [v/v]) for 2.5 h. Alternatively, peptides were treated with 4 equiv each of acetic anhydride and di-isopropylethylamine for 40 min to acetylate the N terminus prior to deprotection and release from the resin.

RESULTS

Identification of Arginine Methylated Peptides Presented by HLA Class I Molecules

The repertoire of HLA class I peptides were isolated from two replicate cell cultures of the HLA-A*01:01, -A*03:01, -B*07:02, -B*27:05, -C*02:02, and -C*07:02-positive human B-cell line GR by immunopurifcation. Isolated HLA class I peptides were fractionated by strong cation exchange (SCX) and analyzed by LC-MS/MS using an LTQ Orbitrap Elite (replicate 1) and Orbitrap Fusion (replicate 2). By searching the MS/MS data files for side-chain modification of arginine residues, we detected, in the first biological replicate, 38 arginine monomethylated and 46 arginine dimethylated and 10 peptides with multiple (di)methylation sites (Supporting Information Table 1). In the second replicate, 25 arginine monomethylated and 44 arginine dimethylated and 12 peptides with multiple modification sites were detected. Cumulatively, a total of 55 unique arginine monomethylated, 74 unique arginine dimethylated, and 20 unique peptides with multiply arginine methylation or dimethylation sites were found (Supporting Information Table 1). Arginine (di)methylated peptides were mainly found with the canonical lengths of 9 and 10 amino acids. Compared to the number of unmodified HLA class I peptides that were detected in both data sets, the 149 arginine (di)methylated peptides represent in frequency approximately 1% of the total HLA class I ligandome.

For the accurate site assignment of the arginine (di)-methylated HLA class I peptides, we mainly relied on the dual fragmentation technique EThcD (Figure 2). EThcD generates complementary ion series of b/y-ions and c/z-ions, which have been shown to be beneficial for peptide sequence full coverage and therefore also accurate assignment of the PTM site.[23,24] The NetMHC algorithm[16] was used to predict the binding affinity and assign the identified peptides to the expressed HLA-A*01:01, -A*03:01, -B*07:02, or -B*27:05 alleles. The HLA-C*02:02 and -C*07:02 alleles were excluded from this analysis because of the low surface expression and lack in accuracy in binding affinity predictions.[14] This analysis revealed that approximately 80% of the identified arginine (di)-methylated peptides were predicted to bind to one of the four most prominent expressed alleles (Supporting Information Table 1). To further validate the peptide assignments and their site of modification, we compared our data with previously identified arginine methylation sites deposited in the PhosphoSite.org database, one of the largest publicly available inventories of protein modifications. Notably, 13 arginine monomethylation and 18 arginine dimethylation sites have been previously reported (Supporting Information Table 1). Furthermore, we verified the identification and PTM site localization of 12 endogenous peptides (monomethylated, dimethylated, and unmodified) using synthetic peptide analogues. Supporting Information Figures 3−14 display the excellent match between the annotated MS/MS spectra of the endogenous peptides and their synthetic counterparts.

The arginine (di)methylated HLA class I-associated peptides originated from 105 nonredundant source proteins. Gene ontology (GO) annotation revealed enrichment of RNA/DNA

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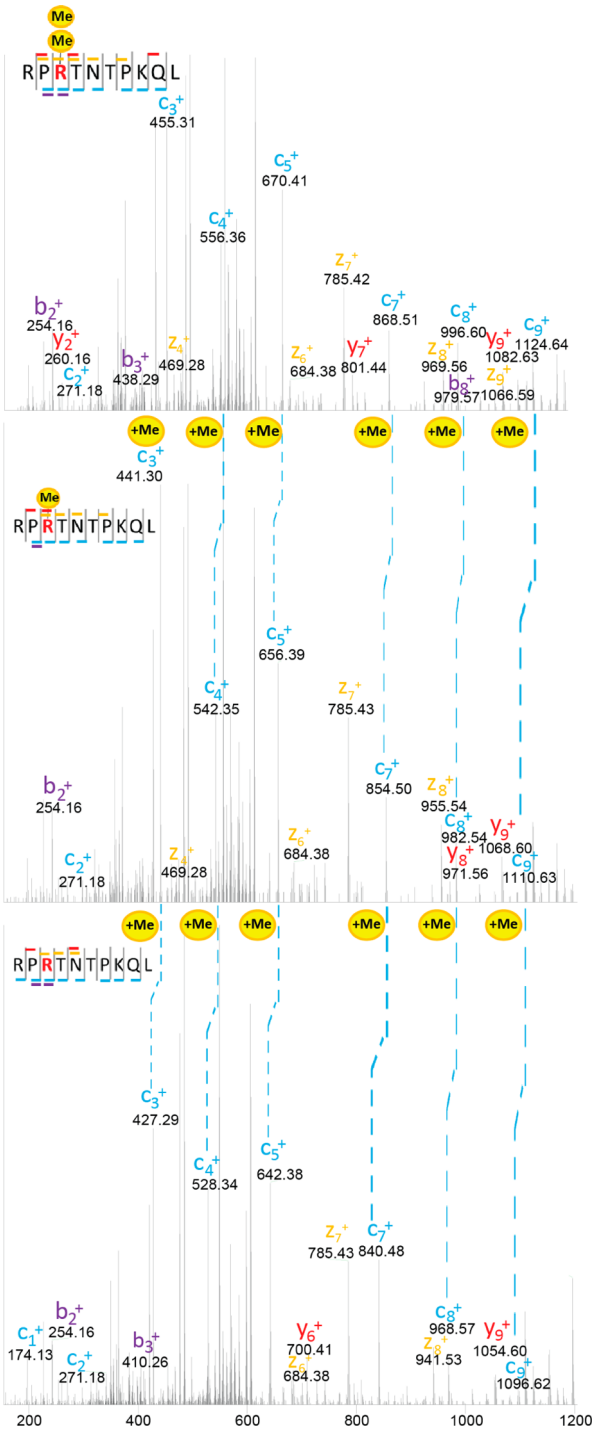

Figure 2. Exemplary EThcD fragmentation spectra of arginine (di)methylated and unmodified peptide. Exemplary EThcD peptide fragmentation spectra of the dimethylated, monomethylated, and unmodified forms of the peptide RPRTNTPQL, originating from the protein NCoA-3. EThcD enabled unambiguous PTM site localization of methylation at P3 on the peptide sequence (blue dashed line).

processing and binding, and regulation of transcription. A complete overview of the here identified source proteins is given in Figure 3. In addition to those previously mentioned, we also observed enrichment of proteins implicated in cellular functions such as RNA-export (i.e., TPR is a component of the nuclear pore complex), endosomal trafficking, and cytoskeletal rearrangements.

Allele-Specific Presentation of Arginine (Di)methylated Peptides

Most of the arginine (di)methylated peptides could be successfully associated with the expressed HLA-A*01:01, -A*03:01, -B*07:02, and -B*27:05 alleles. However, we found that in frequency of occurrence the HLA-B alleles present a larger number of (di)methylated peptides than the HLA-A alleles. Figure 1B shows the frequency distribution of the identified arginine dimethylated, the arginine monomethylated, and the unmodified peptides over the four above-mentioned HLA alleles. In comparison to the set of unmodified peptides, both arginine monomethylated and arginine dimethylated HLA class I peptides exhibited a remarkable preference for HLA-B*07, with respectively 67% and 80% of the predicted binders being associated with HLA-B*07. The favorable presentation of arginine (di)methylated peptides by HLA-B*07 could be partly explained by the higher frequency of arginine residues in the consensus peptide sequence for this allele. Sequence logos (Supporting Information Figure 1) indicated that arginine residues are favored at P1, P3, P5, and P6 in the set of unmodified HLA-B*07-associated peptides. HLA-A*03 and HLA-B*27 prefer an arginine residue at their N-termini, while HLA-A*01 does not seem to favor an arginine residue in any part of the peptide sequence.

To further investigate the seemingly enhanced presentation of (di)methylated peptides by HLA-B*07, we evaluated the positional (di)methylation occurrence on peptide sequences and compared these with the positional frequency of nonmodified arginine residues in the total set of identified HLA class I peptides (Figure 4). This analysis revealed that in the set of unmodified peptides (black bars) there was a preference for arginine at position P1 and P2, which corresponds to the previously discussed consensus sequence motifs for HLA-A*03:01, HLA-B*07:02, and HLA-B*27:05 (Supporting Information Figure 1). For both arginine monomethylated (orange bars) and arginine dimethylated peptides (blue bars), however, we found a high preference for the modification site on position P3: frequency distributions of 36% and 48%, respectively (Figure 4). Notably, the arginine (di)methylated peptides modified at position P3 were exclusively associated with the HLA-B*07 allele (Supporting Information Table 1). This enhanced modification frequency at position P3 could either reflect structural features specifically dictated by HLA-B*07 allele or represent specific motifs required for the recognition and modification of the protein substrate by arginine methyltransferases, as discussed below. In addition, a moderate preference was detected for (mono-) methylation at position P2 (Figure 4, 21%). The P2 arginine monomethylated HLA peptides were exclusively associated all with HLA-B*27. This is a curious finding, as P2 is an anchoring site for this allele. For HLA-B*27, nearly no dimethylated sites were detected, suggesting that one but likely not two methylations are tolerated at the anchor position (Supporting Information Figure 1).

Arginine Dimethylated Peptides Are More Frequently and More Abundantly Presented by the HLA-B*07 Allele

To further characterize the preferential presentation of arginine (di)methylated peptides by HLA-B*07, we first performed a qualitative comparison analysis with a recent proteomics study.[25] Using antibody enrichment and LC-MS/MS analysis, Guo et al. identified 1500 arginine (di)methylation sites in a human cell line. Evaluating this data set revealed that 66% of

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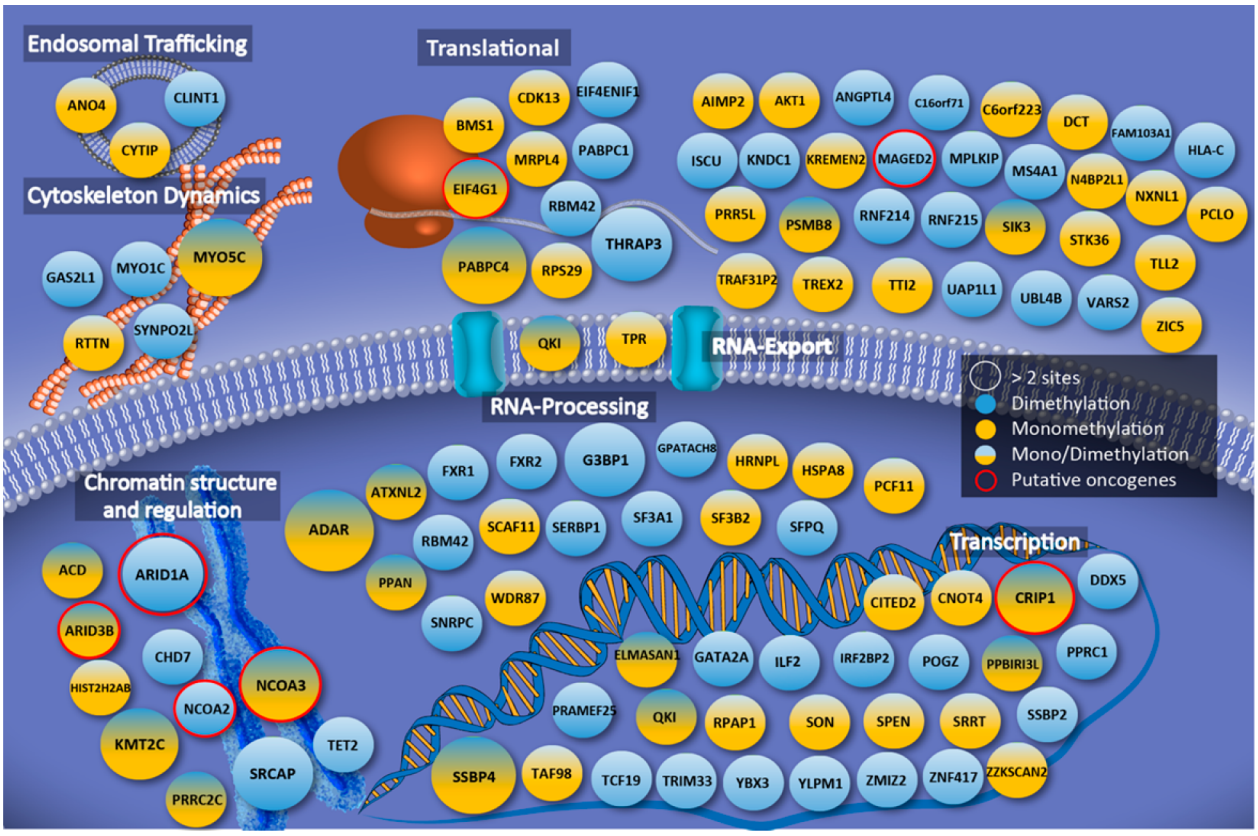

Figure 3. Overview of the source proteins of here observed arginine (di)methylated HLA class I peptides. Proteins are grouped according to their annotated molecular function (based on the UniprotKB database and Panther).[18] Proteins are color coded yellow, blue, and yellow/blue when HLA class I peptides respectively harbored monomethylation, dimethylation, or both. Proteins for which more than two arginine (di)methylated HLA peptides were detected are annotated with larger circles, while the ones annotated with red circles are from acknowledged oncogenes.

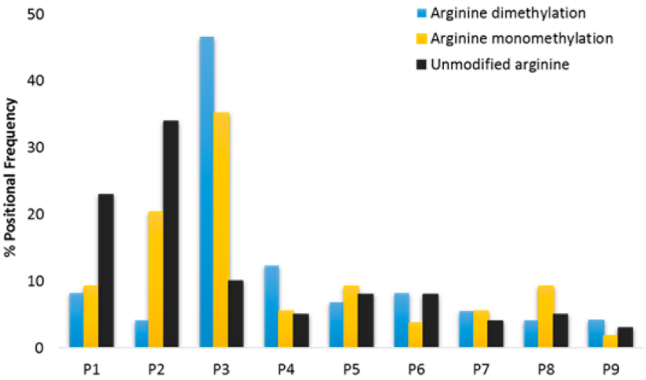

Figure 4. Positional (di)methylation occurrence on peptide sequences. The positional frequency distributions (%) for monomethylation (orange bars) and dimethylation (blue bars) harbored on HLA class I peptides were compared to the distribution of unmodified arginine residues in the total data set of HLA class I peptides (black bars).

the identified protein modification sites were found to be arginine monomethylated and 33% to be arginine dimethylated. In contrast, in our study a higher number of arginine dimethylated HLA class I peptides were identified compared to arginine monomethylated peptides (57% versus 43%). This could indicate that the processing and presentation of arginine-modified proteins by the HLA class I pathway is somewhat biased for arginine dimethylation. To test this hypothesis, we quantified the abundance of the arginine monomethylated and dimethylated HLA class I peptides in comparison to their corresponding unmodified counterparts. Relative abundances were obtained from 22 nonmodified peptides with their corresponding arginine monomethylated peptides, 21 nonmodified with their corresponding dimethylated peptides, and a further 14 peptides with mono- and dimethylation sites (Supporting Information Table 2). The abundance varied considerably from peptide to peptide, but when we further clustered the data with respect to the preferred arginine modification at the position P3 (see Figure 5A–B), clear trends could be observed. On average, the nonmodified peptides seem to be presented at higher abundance compared to their arginine (di)methylated variants (Figure 5A, blue box plots). This is also the case in the comparison of nonmodified and dimethylated peptides (Figure 5A, blue box plot), although a large fraction of the peptides carrying a modification at position P3 displayed considerably higher abundances compared to their nonmodified counterparts (Figure 5A, left red box plot, and 5B). The same trend was not observed for P3 monomethylated ligands (Figure 5A, right red box plot, and 5B). Altogether, the qualitative and quantitative results clearly show a preferential bias in the presentation of arginine dimethylated peptides at P3.

To test whether the observed preference at P3 could be imposed by an underlying substrate motif of PRMTs, we first extracted all reported human protein (di)methylated sites from the PhosphoSite.org database. Next we used this data set to generate *in silico* the set of potential 9-mer HLA class I peptides predicted from the source sequences, with a window of 9 residues around the protein arginine (di)methylation sites. The NetMHC algorithm[16] was used to predict whether this large set

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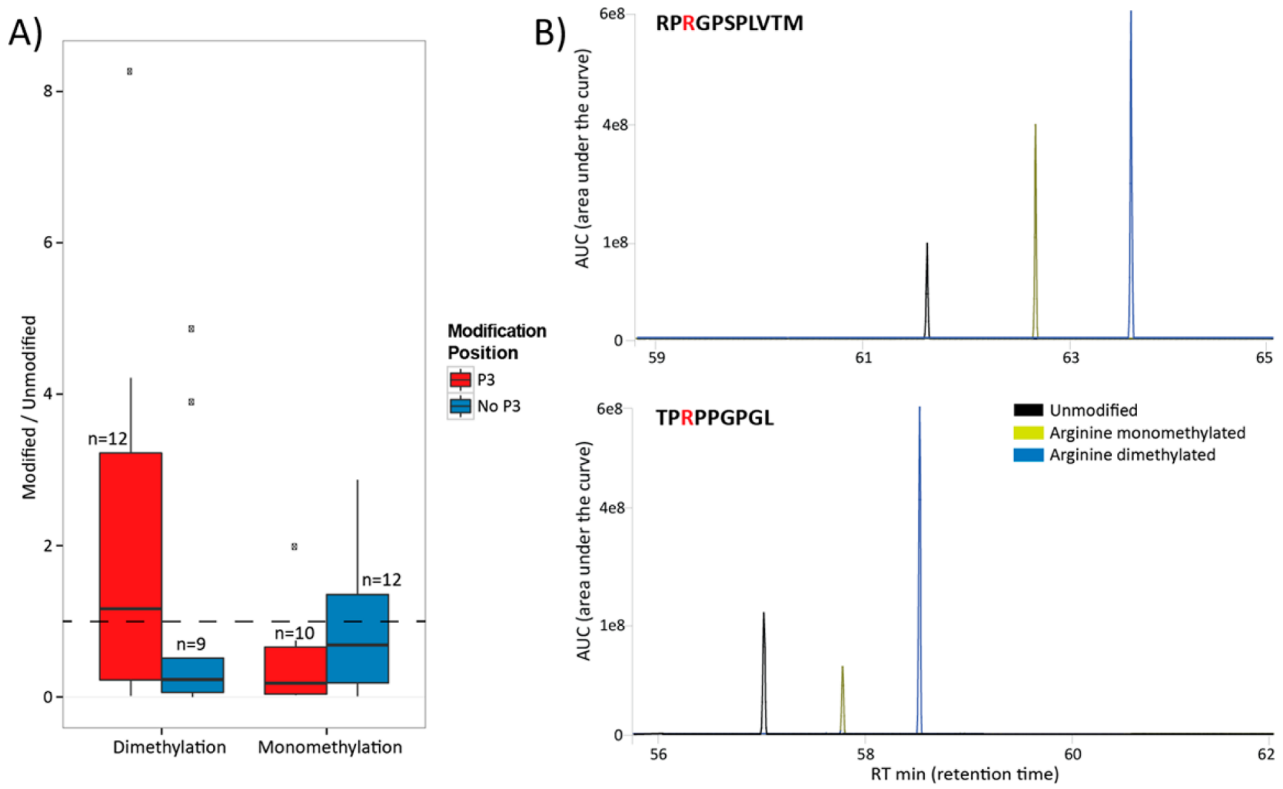

Figure 5. Relative abundance of arginine (di)methylated HLA class I peptides compared to their unmodified counterparts. (A) The ratio of extracted area under the curves (AUCs) of monomethylated (right boxes) and dimethylated ligands (left boxes) was calculated compared to their unmodified counterparts. The trends were studied separately for peptides harboring (di)methylation on P3 positions (red boxes) and all other (di)methylated modified positions (blue boxes). The dashed line represents when modified and unmodified peptides had equal AUCs. (B) Exemplary extracted chromatograms of two highly abundant dimethyl P3 modified HLA-B*07 binders also found in both unmodified (black trace) and P3 monomethylated (yellow trace) forms.

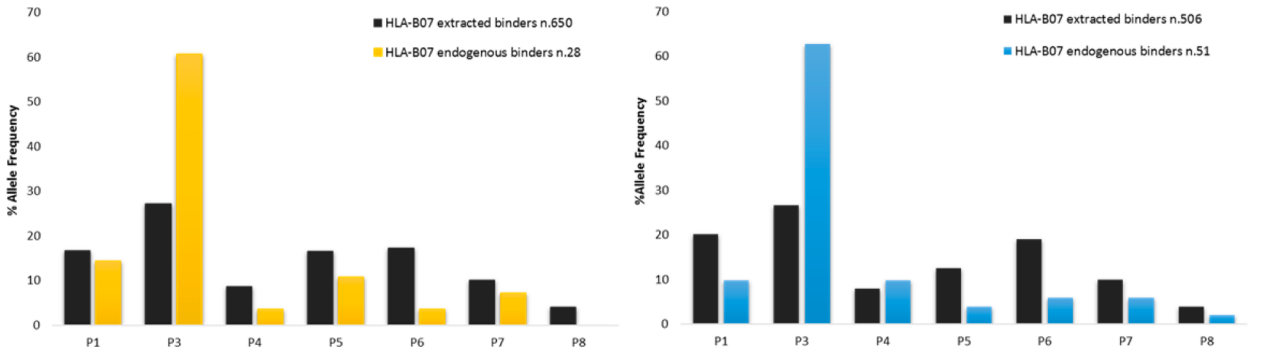

Figure 6. Frequency distribution of arginine (di)methylation sites among the identified and extracted HLA-B*07 peptides. The frequency of distribution of monomethylation endogenous ligands is given in orange (left graph), while the dimethylation one is given in blue (right graph). The positional mono- (left graph) and dimethylation (right graph) frequency of the *in silico* predicted binders extracted from PhosphoSite.org is represented by the black bars. Potential HLA-B*07-binding peptides (9-mers) in silico extracted from the PhosphoSite.org database were predicted using the NetMHC algorithm.[61]

of *in silico* extracted (di)methylation could be presented by HLA-B*07. From this data set, 650 monomethylated and 506 dimethylated arginine 9-mers were predicted to bind to HLA-B*07 allele (Figure 6). The positional frequency of the modified sites from the *in silico* extracted set of HLA-B*07 binders was compared against our identified set of arginine-modified HLA-B*07:02 peptides. In this analysis, we excluded the position P2 and C-terminal anchor residues because no endogenous HLA-B*07 peptides were detected modified at these positions. The extracted set of predicted HLA-B*07 binders (black bars) showed a moderate preference for arginine (di)methylation at P1, P3, P5, and P6, which corresponds nicely to enrichment of arginine residues at these positions in the consensus binding motif of this allele (Supporting Information Figure 1). More importantly, preference for the arginine (di)methylation site at position P3 (<30%) was found for the extracted date set, but only at a moderate enrichment in comparison to the identified set of endogenous peptides in the HLA-B*07 ligandome (>60%). These results indicate that the enhanced presentation of arginine (di)methylated peptides by HLA-B*07 could be partially derived from underlying PRMTs substrate motifs resembling the HLA-B*07 binding motif. Still,

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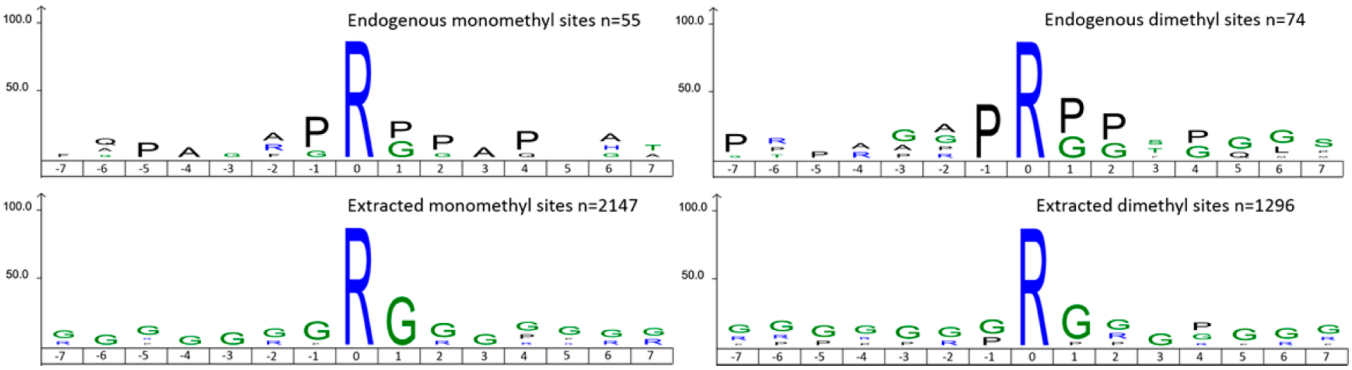

Figure 7. Sequence motif analysis of the monomethylated and dimethylated sites. Motif analysis of preferred amino acids flanking the arginine monomethylation and dimethylation sites identified in the HLA class I ligandome (top panels). For comparison, the bottom panels shown the motifs of all arginine monomethylated and dimethylated sites annotated in the PhoSphosite.org database.

such a potential substrate motif does not fully explain the more frequent and more abundant presentation of arginine dimethylated peptides, suggesting that additional structural features favor a dimethyl group at P3 in the HLA-B*07 binding fold.

Arginine (Di)methylated HLA Class I Ligands Exhibit Substrate Motifs Linked to Specific PRMTs

We next examined the sequence motifs flanking the identified modified sites to investigate whether the modifications had been performed by specific PRMTs. The aligned sequences of the HLA class I arginine monomethylated and dimethylated ligands (Figure 7, upper panels) were submitted to IceLogo[17] to calculate the over-representation of specific amino acids flanking the modified arginines.

For comparison, we extracted from PhophoSite.org all deposited protein arginine monomethylated and dimethylated sites and subjected them to an alike analysis (Figure 7, bottom panels). The sites extracted from this latter analysis revealed that arginine methylation is primarily occurring at glycine–arginine-rich (GAR) stretches.[26] In our HLA class I ligandome, both monomethylated and dimethylated data sets displayed a distinctive preference, in PRP- and PRG-rich motifs. This was expected, as this PRP and PRG substrate motif reflected the occurrence of the proline anchor residue at position 2 and the favored (di)methylated arginine residue at position P3 in HLA-B*07-associated peptides. It has been established that most PRMTs methylate on glycine- and arginine-rich (GAR) motifs, except PRMT4/CARM1 and PRMT5, which both seem to be able to methylate also proline-rich motifs.[27] Distinctively, PRMT4/CARM1 asymmetrically dimethylates arginine residues (ADMA), whereas PRMT5 symmetrically dimethylates arginine (SDMA) residues (Figure 1A). To investigate which of these two PRMTs are specifically involved in the arginine modification of the identified HLA class I ligands, we inspected their MS/MS fragmentation spectra for neutral loss patterns as described in the Experimental Procedures section. Making use of distinct patterns in neutral losses,[28] we were able to annotate 30 ADMA peptides and 4 SDMA peptides in our HLA class I ligandome (Supporting Information Table 1 and Figures 15–48). The verified 30 ADMA peptides were mostly predicted to be HLA-B*07 binders (83%) (Supporting Information Figure 2). The majority of the verified ADMA peptides displayed a "-RP" motif, thus most likely being modified by CARM1.[29] The 4 SDMA verified peptides, which are likely substrates of PRMT5, contained both "-RG" and "-RP" rich motifs.

A Subset of (Di)methylated HLA Ligands Is Derived from Source Proteins Associated with Cancer

As depicted in Figure 3, quite a few of the source proteins, especially the (di)methylated HLA peptides in GR cells, included proteins that have been associated with cancer (Figure 3, red circles). Illustrative examples include the proteins ARID1A, ARID3B, and CRIP1, which were detected in the HLA class I ligandome with both arginine monomethylated and dimethylated peptides. These proteins have roles as tumor suppressors[30] and to increase tumor growth in ovarian cancer, respectively.[31] CRIP1 is overexpressed in several tumors,[32−35] while the melanoma-associated antigen, MAGED2, as for other MAGE proteins, is reported to act as an antitumoral immune target.[36,37] Furthermore, NCoA-3[38] is known to be deregulated in cancer and was found in our data set multiple times, as both dimethylated and monomethylated HLA-binding peptides.

■ DISCUSSION

Post-translationally modified HLA class I peptide antigens can be specifically recognized by the immune system.[39−41] As certain PTMs (e.g., phosphorylation) represent hallmarks of human diseases,[37,42−46] these modified HLA class I peptides have received significant attention as putative candidates for immunotherapy or vaccination. Here, we report the identification of 55 unique arginine monomethylated, 74 arginine dimethylated, and 20 HLA class I peptides with multiple (di)methylation sites from two replicate ligandome studies on a heterozygous B cell line. The total number of 149 arginine (di)methylated peptide antigens represent in frequency approximately 1% of the total repertoire of unique HLA class I peptides. This frequency of presentation is on par with that of other identified PTMs, such as phosphorylation,[47] glycosylation,[13] and asparagine deamidation.[14] Although the number of identified arginine (di)methylated peptides is relatively small, these results still indicate that this type of modification is frequently presented by HLA class I for recognition by T cells. The arginine methylated peptide ligands originated from 105 source proteins. GO analysis revealed that the source proteins were highly enriched in translation, RNA-processing, transcription, and chromatin regulation, which is in close agreement with the known main regulatory functions of arginine methylation.[48]

A comparison of the number of arginine methylated peptides predicted to be bound to the expressed HLA-A and -B alleles revealed enhanced presentation of peptides by HLA-B molecules, in particular to HLA-B*07. Interestingly, in our

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data set we also found a striking prevalence for dimethylated HLA-B*07 binders modified at P3. In order to verify these findings, we reanalyzed the raw data of a HLA class I ligandome study using 6 cell lines,[49] of which 2 cell lines were HLA-B*07:02 positive and 4 cell lines cell lines were HLA-B*07 negative. This analysis revealed allele-specific enrichment and positional P3 preference in arginine (di)methylated peptides only for the cell lines that express HLA-B*07:02, in agreement with our results (Supporting Information Table 3). Interestingly, a total of 16 (di)methylated ligands were detected both in our study and by reanalysis of raw data of the HLA-B*07 positive cell lines, providing further evidence that these arginine (di)methylated peptides are associated with HLA-B*07 ligandomes. We argue that this enhanced presentation is related to the similarity between the binding motif of HLA-B*07 and the consensus sequences on substrates targeted by PRMTs. The consensus binding motif of HLA-B*07 is highly enriched in proline residues at position P2 and also displays a moderate preference for arginine at position P3.[50] Thus, with hindsight, it is perhaps not surprising that many of the detected position P3 arginine (di)methylated peptides exhibit so-called proline−arginine-rich domains of PRMTs.[48,51] Such a proline−arginine-rich motif is not the most common motif observed for PRMTs (that is the glycine−arginine-rich motif), but it has been linked to a few less common, albeit selective PRMTs. It has been implied that presentation of peptides having a position P2 Pro from either terminus may be favored because of limitations of processing proteases that have degraded the original proteins.[52,53] Thus, we cannot rule out that the preferential presentation of (di)methylated peptides with a proline−arginine motif observed in this study might be related partially to the effect of Pro on P2 on processing the proteases involved.

However, the underlying proline−arginine-rich motif cannot alone fully explain the high selectivity of HLA-B*07 in presenting arginine dimethylated ligands. First, a higher number of arginine dimethylated than arginine monomethylated peptide ligands were found, which is in disagreement with the overall frequency of protein arginine (di)methylation sites found in in-depth studies of the cellular proteome.[25] Second, the positional preference for P3 arginine (di)methylation was only partially observed in a large set of *in silico* extracted 9-mer HLA-B*07-associated peptides harboring previously identified modification sites (PhosphoSite.org database). Third, quantitative comparison of peptide pairs with differentially modified methylation sites revealed that arginine dimethylated peptides were presented at higher abundances compared to their arginine monomethylated and unmodified peptide counterparts only when the modification was at P3. Hence, we argue that additional structural features in the HLA-B*07-binding groove could play a role in the selective presentation of arginine dimethylated peptides when the modification is harbored at P3.

Large scale proteomics studies have revealed that the majority of protein arginine methylations are mediated on glycine- and arginine-rich motifs (GAR motifs),[26] although a few motifs beyond the "RGG" paradigm have been recognized.[54] In the study performed by Yagüe et al., the identified 10-mer was found methylated in the context of HLA-A*39 and also contained the canonical "RGG motif". In our analysis of the enriched amino acids flanking the modified monomethylated and dimethylated sites in the HLA class I peptide data set, we found distinct motifs beyond the "RGG" paradigm, including the enrichment of proline- and arginine-rich motifs relating to the consensus binding motif of HLA-B*07. Specific PRMTs are known to target proline-enriched motifs, notably CARM1 and PRMT5. CARM1 preferentially modifies protein substrates containing proline-, glycine-, and methionine-rich motifs (PGM motifs),[29] while PRMT5 can also modify substrates with PGM motifs,[51] At first glance, we therefore hypothesized that PRMT5 and CARM1 are the likely enzymes that have modified the majority of the here reported HLA class I peptides.

Type I PRMT enzymes catalyze protein monomethylation (MMA) and asymmetrical dimethylation (ADMA), while type II PRMT enzymes catalyze monomethylation and symmetrical dimethylation (SDMA) (Figure 1A). It has been shown that neutral losses occurring in the MS/MS spectra of arginine dimethylated peptides can be used to assign ADMA- and SMDA-modified peptide.[20] We employed this approach to our data and classified 30 of the dimethylated HLA class I peptides to harbor ADMA and 4 to harbor SMDA. Since CARM1 leads to ADMA, whereas PRMT5 produces SMDA, most of the here detected dimethylated HLA class I peptides are thus putative CARM1 substrates,[29] while the 4 confirmed SDMA peptides, displaying mixed "-RG" and "-RP" motifs, are putative PRMT5 substrates.

Protein arginine (di)methylation has been linked to carcinogenesis and metastasis.[55] For instance, the transcriptional coactivator NCoA-3[38] is known to be deregulated in cancer and was found in our data set with representative arginine monomethylated and arginine dimethylated HLA class I peptide antigens (Figure 3). Notably, aggressive breast tumors overexpressing CARM1 also have high levels of the oncogenic coactivator NCoA-3,[56] and it has been hypothesized that the oncogenic properties of NCoA-3 may be attenuated by inhibiting CARM1 activity.[55] A further notable example is ARID1A (a tumor suppressor), for which decreased levels have been linked to poor prognosis.[30,57−60] We observed both arginine monomethylated and arginine dimethylated HLA class I peptides originating from ARID1A in our data.

Evidently the here reported arginine (di)methylated peptides are self-antigens, presented on GR B lymphoblasts. Although the GR cell line does not represent a prototypic cancer cell line, it has an immortalized phenotype after transformation with the Epstein−Barr virus. Therefore, we argue that GR-derived methylated peptides could represent interesting therapeutic antigens, since their proteins of origin are often overexpressed in cancer tissues, combined with the fact that methylation or dimethylation may affect the protein turnover and transcription. Translation of these findings would, however, first require the demonstration that there is a T cell repertoire recognizing the HLA class I-presented methylated epitopes involved. An encouraging precedent for this has been demonstrated by Jarmalavicus et al.[12] More generally, the rules and selectivities of (di)methylated peptide antigen presentation extracted from our data will be of use to predict and identify disease-related HLA class I antigens.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00528.

Sequence motifs of the identified unmodified HLA class I peptides. Sequence motif of asymmetrically dimethylated

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sites in the HLA ligandome. Comparison between EThcD assigned spectra of synthetic and endogenous unmodified, monomethylated, and dimethylated HLA class I peptides. Evaluation of MS/MS spectra of symmetrically and asymmetrically dimethylated ligands for specific neutral loss patterns (PDF)

List of identified (di)methylated unique peptides and their features, in each biological replicate and cumulatively for the two experiments. The table contains also a summary of the findings (XLSX)

Label free quantitative comparison between peptide pairs of monomethylated/unmodified, dimethylated/unmodified, and dimethylated/monomethylated variants (XLSX)

Characteristics of (di)methylated ligands identified in our study and by reanalysis of the raw data of six publicly available HLA class I ligandome data sets (XLSX)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: a.j.r.heck@uu.nl.

Notes

The authors declare no competing financial interest.

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