



# Structural Basis for the Presentation of Tumor-Associated MHC Class II-Restricted Phosphopeptides to CD4<sup>+</sup> T Cells

Yili Li<sup>1,2†</sup>, Florence R. Depontieu<sup>3†</sup>, John Sidney<sup>4</sup>, Theresa M. Salay<sup>3</sup>, Victor H. Engelhard<sup>5</sup>, Donald F. Hunt<sup>6,7</sup>, Alessandro Sette<sup>4</sup>, Suzanne L. Topalian<sup>3\*</sup> and Roy A. Mariuzza<sup>1,2\*</sup>

<sup>1</sup>University of Maryland Institute for Bioscience and Biotechnology Research, W. M. Keck Laboratory for Structural Biology, Rockville, MD 20850, USA

<sup>2</sup>Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD 20742, USA

<sup>3</sup>Department of Surgery and the Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA

<sup>4</sup>La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037, USA

<sup>5</sup>Carter Immunology Center and Department of Microbiology, University of Virginia, Charlottesville, VA 22908, USA

<sup>6</sup>Department of Chemistry, University of Virginia, Charlottesville, VA 22901, USA

<sup>7</sup>Department of Pathology, University of Virginia, Charlottesville, VA 22901, USA

Dysregulated protein phosphorylation is a hallmark of malignant transformation. Transformation can generate major histocompatibility complex (MHC)-bound phosphopeptides that are differentially displayed on tumor cells for specific recognition by T cells. To understand how phosphorylation alters the antigenic identity of self-peptides and how MHC class II molecules present phosphopeptides for CD4<sup>+</sup> T-cell recognition, we determined the crystal structure of a phosphopeptide derived from melanoma antigen recognized by T cells-1 (pMART-1), selectively expressed by human melanomas, in complex with HLA-DR1. The structure revealed that the phosphate moiety attached to the serine residue at position P5 of pMART-1 is available for direct interactions with T-cell receptor (TCR) and that the peptide N-terminus adopts an unusual conformation orienting it toward TCR. This structure, combined with measurements of peptide affinity for HLA-DR1 and of peptide–MHC recognition by pMART-1-specific T cells, suggests that TCR recognition is focused on the N-terminal portion of pMART-1. This recognition mode appears to be distinct from that of foreign antigen complexes but is remarkably reminiscent of the way autoreactive TCRs engage self- or altered self-peptides, consistent with the tolerogenic nature of tumor–host immune interactions.

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\*Corresponding authors. R. A. Mariuzza is to be contacted at the University of Maryland Institute for Bioscience and Biotechnology Research, W. M. Keck Laboratory for Structural Biology, Rockville, MD 20850, USA. S. L. Topalian is to be contacted at the Department of Surgery and the Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD 21231, USA. E-mail addresses: [stopali1@jhmi.edu](mailto:stopali1@jhmi.edu); [mariuzza@carb.nist.gov](mailto:mariuzza@carb.nist.gov).

† Y.L. and F.R.D. contributed equally to this work.

Abbreviations used: MHC, major histocompatibility complex; MART-1, melanoma antigen recognized by T cells-1; pMART-1, MART-1 phosphopeptide; TCR, T-cell receptor; EBV, Epstein–Barr virus; GM-CSF, granulocyte–macrophage colony-stimulating factor; APC, antigen-presenting cell; IFN $\gamma$ , interferon  $\gamma$ .

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## Introduction

A variety of posttranslational modifications of naturally processed peptides displayed by major histocompatibility complex (MHC) class I or II molecules have now been described, including glycosylation, deamidation, cysteinylolation, and phosphorylation.<sup>1</sup> Furthermore, peptides bearing these posttranslational modifications can be discriminated from their unmodified homologs by T cells. Among the posttranslationally modified peptides identified to date, phosphopeptides<sup>2–4</sup> are of particular interest, since dysregulated phosphorylation is one of the hallmarks of malignant transformation and contributes directly to oncogenic signaling cascades involved in cell growth, differentiation, and survival.<sup>5,6</sup> Indeed, phosphopeptides directly isolated from the human MHC class I molecule HLA-A2 include those derived from proteins involved in oncogenic signaling and cell cycle regulation.<sup>2,3</sup> Because many of these phosphopeptides are differentially displayed on cancer cells,<sup>3</sup> they provide a new cohort of targets for cancer immunotherapy.<sup>1,7</sup>

Until very recently, it was unknown whether MHC class II molecules could present phosphopeptides for specific recognition by CD4<sup>+</sup> T cells, whose activation and recruitment are critical for the development of effective and long-lasting antitumor immunity.<sup>8,9</sup> In one study, mass spectrometric sequencing was used to demonstrate the existence of HLA-DR-associated phosphopeptides on two pairs of autologous human melanoma and Epstein–Barr virus (EBV)-transformed B lymphoblastoid lines.<sup>4</sup> The 150 unique phosphopeptides identified derived from 53 different source proteins representing all cellular compartments. As characteristic of nonphosphorylated MHC class II-restricted epitopes, most of the phosphopeptides occurred in nested sets, and their average length was 16 amino acids (range, 8–28). Significantly, the majority of source proteins support vital cellular functions, such as metabolism, cell cycle regulation, and signal transduction.<sup>4</sup> Similar results were reported in a separate study of human MHC class II-restricted phosphopeptides derived from one melanoma and one B lymphoblastoid cell line,<sup>10</sup> suggesting the generality of phosphopeptide presentation by MHC class II molecules.

The ability of human CD4<sup>+</sup> T cells to specifically recognize MHC class II-restricted phosphopeptides was first demonstrated using as an example an HLA-DR1-restricted phospho-MART-1 (melanoma antigen recognized by T cells-1; also known as Melan-A)

peptide (pMART-1<sub>100–111</sub>; APPAYEKLP<sub>SAEQ</sub>, where pS is phosphoserine) that was isolated from a cultured melanoma line.<sup>4</sup> MART-1 is of special interest because its selective expression by cells of the melanocytic lineage has made it a prime target for immunotherapeutic approaches to melanoma, including vaccines and adoptive T-cell transfer.<sup>11,12</sup> CD4<sup>+</sup> T cells recognizing pMART-1 presented by HLA-DR1 were highly specific for the phosphate moiety of the peptide. More importantly, these T cells recognized intact melanoma cells expressing MART-1 and HLA-DR1, indicating the presence of sufficient quantities of pMART-1 peptide–MHC complexes at the cell surface to trigger T-cell signaling.

Establishing the molecular basis for phosphopeptide presentation and recognition will enable the rational design of new cancer immunotherapies

**Table 1.** Data collection and refinement statistics

	pMART-1 <sub>100–114</sub> –HLA-DR1
<i>Data collection statistics</i>	
Space group	P2 <sub>1</sub> 2 <sub>1</sub> 2
Unit cell (Å)	<i>a</i> = 91.2, <i>b</i> = 135.5, <i>c</i> = 40.9
Resolution (Å)	30–2.1
Observations	430,714
Unique reflections	30,332
Completeness (%) <sup>a</sup>	100 (100)
Mean <i>I</i> /σ( <i>I</i> ) <sup>a</sup>	51.3 (5.4)
<i>R</i> <sub>sym</sub> (%) <sup>a,b</sup>	7.4 (46.0)
<i>Refinement statistics</i>	
Resolution range (Å)	30–2.1
<i>R</i> <sub>work</sub> (%) <sup>c</sup>	21.1
<i>R</i> <sub>free</sub> (%) <sup>c</sup>	24.8
Protein atoms	3134
Water molecules	198
Average <i>B</i> values (Å <sup>2</sup> )	
Protein main chain	34.3
Protein side chain	35.3
Water molecules	37.6
R.M.S.D.s from ideality	
Bond lengths (Å)	0.010
Bond angles (°)	1.58
<i>Ramachandran plot statistics</i>	
Most favored (%)	92.5
Additionally allowed (%)	7.2
Generously allowed (%)	0.0
Disallowed	0.3

<sup>a</sup> Values in parentheses are statistics for the highest-resolution shells.

<sup>b</sup>  $R_{\text{sym}} = \sum |I_j - \langle I \rangle| / \sum I_j$ , where  $I_j$  is the intensity of an individual reflection and  $\langle I \rangle$  is the average intensity of that reflection.

<sup>c</sup>  $R_{\text{work}} = \sum ||F_o| - |F_c|| / \sum |F_o|$ , where  $F_c$  is the calculated structure factor.  $R_{\text{free}}$  is as for  $R_{\text{work}}$  but calculated for a randomly selected 10.0% of reflections not included in the refinement.

targeting this category of tumor-derived epitopes. Recent crystal structures of several phosphopeptide–HLA-A2 complexes showed that the phosphate moiety formed an integral part of these structures, stabilizing interactions with the MHC class I molecule.<sup>13,14</sup> Here we report the first structure of a phosphopeptide–MHC class II complex involving pMART-1<sub>100–114</sub> bound to HLA-DR1. This structure, in conjunction with measurements of peptide–MHC affinity and T-cell recognition of truncated and substituted pMART-1 peptides, reveals the basis for presentation of tumor-associated MHC class II-restricted phosphopeptides to CD4<sup>+</sup> T cells.

## Results and Discussion

### Structure of phosphorylated MART-1 peptide bound to HLA-DR1

To understand how phosphorylation influences antigenic identity, we determined the crystal structure of pMART-1<sub>100–114</sub> (APPAYEKLpSAEQSP) in complex with HLA-DR1 to 2.1 Å resolution (Table 1 and

Fig. 1a). This peptide belongs to a nested set of four phosphopeptides derived from the C-terminus of MART-1 that range in length from 12 to 17 residues, all starting at Ala100. The nested peptides were eluted from HLA-DR–peptide complexes isolated from a human melanoma line expressing a single DR molecule (HLA-DRβ1\*0101), thereby assuring unambiguous HLA allele restriction, as well as from a second melanoma line expressing HLA-DRβ1\*0101, β1\*0404, and β4\*0103.<sup>4</sup> Phospho-MART-1 peptides were not found on autologous EBV-transformed B cells, consistent with the selective expression of MART-1 in melanocytic lineage cells. The choice of pMART-1<sub>100–114</sub> for structural studies was based on its higher affinity for HLA-DR1 (IC<sub>50</sub>=102 nM) compared to three other naturally processed pMART-1 peptides: pMART-1<sub>100–111</sub> (459 nM), pMART-1<sub>100–115</sub> (142 nM), and pMART-1<sub>100–116</sub> (327 nM). This facilitated assembly of stable pMART-1–HLA-DR1 complexes for crystallization. The affinities of pMART-1 peptides for HLA-DR1 are considered to be in the intermediate range, as defined by the IC<sub>50</sub> values of nonphosphorylated peptides for HLA-DR molecules.<sup>22</sup>

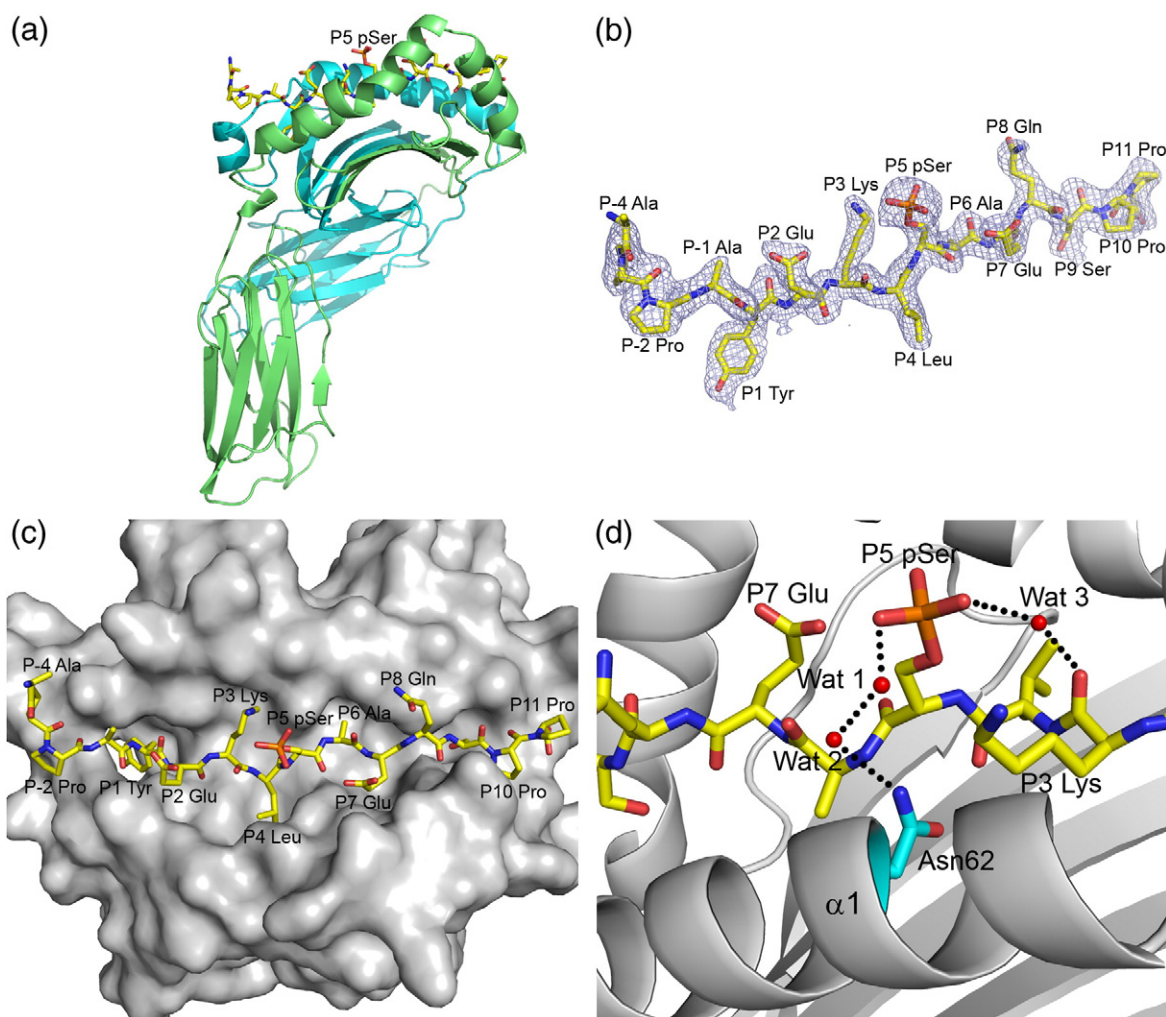
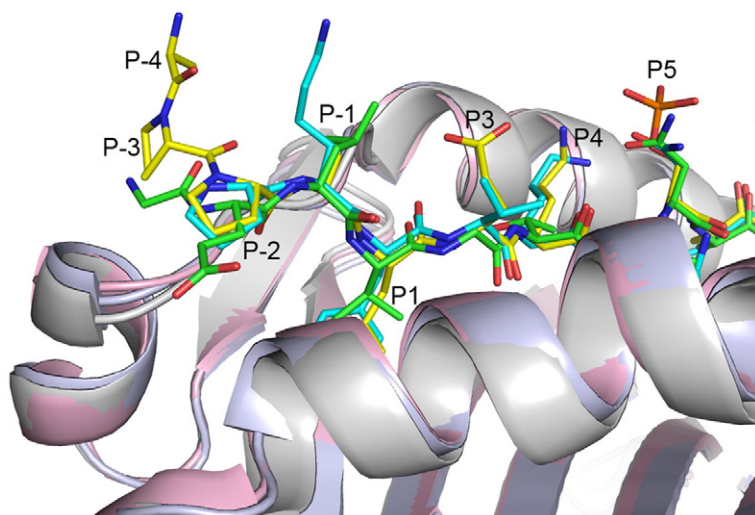


Fig. 1 (legend on next page)





**Fig. 2.** Superposition of peptides bound to human MHC class II molecules. Phospho-MART-1 peptide bound to HLA-DR1 is yellow (carbon atoms). Hemagglutinin peptide bound to HLA-DR1 is cyan (PDB 1DLH).<sup>23</sup> Triose phosphate isomerase peptide bound to HLA-DR1 is green (PDB code 1KLU).<sup>27</sup> The peptide backbones superpose well from P-1 to P-5, but diverge at P-2.

Continuous and unambiguous electron density was observed for the entire 15-residue pMART-1<sub>100–114</sub> peptide, including the N- and C-termini and the phosphate moiety attached to Ser108 (Fig. 1b). The phosphopeptide adopts an extended conformation in the HLA-DR1 binding groove resembling that of nonphosphorylated peptides bound to MHC class II molecules (Fig. 1c). The primary anchor residues are Tyr104 (P1) and Ala109 (P6), in agreement with the requirement for an aromatic or large hydrophobic residue at P1 and a small uncharged residue at P6 for

efficient binding to HLA-DR1.<sup>22,23</sup> The secondary anchors are Leu107 (P4), Glu110 (P7), and Ser112 (P9). This binding register is likely to be maintained in nonphosphorylated counterparts of pMART-1<sub>100–114</sub>, since the side chain of the phosphorylated Ser108 residue at P5 projects into solvent, away from the peptide-binding groove of HLA-DR1 (Fig. 1a and c).

The conformation of pMART-1<sub>100–114</sub> was directly compared with those of peptides from other peptide–MHC class II complexes<sup>23–27</sup> by superposition of the  $\alpha 1/\beta 1$  domains of the class II molecules

**Fig. 1.** Structure of the pMART-1<sub>100–114</sub>–HLA-DR1 complex. (a) Side view of the pMART-1<sub>100–114</sub>–HLA-DR1 complex. MHC  $\alpha$  chain is cyan and  $\beta$  chain is green. The peptide is drawn in stick representation with carbon atoms in yellow, oxygen atoms in red, nitrogen atoms in blue, and the phosphate atom in orange. (b) Electron density for the bound pMART-1<sub>100–114</sub> peptide. The  $2F_o - F_c$  map at 2.1 Å resolution is contoured at  $1\sigma$ . (c) Top view of the pMART-1<sub>100–114</sub>–HLA-DR1 complex. (d) Interactions of P5 pSer with P3 Lys and the HLA-DR1  $\alpha$  chain. Bound water molecules (Wat 1–3) are drawn as red spheres. Hydrogen bonds are indicated by broken black lines. The pMART-1–HLA-DR1 complex was assembled in two steps to maximize the yield of recombinant protein. First, HLA-DR1 bearing CLIP<sub>87–101</sub> peptide (PVSKMRMATPLIMQA) was prepared by *in vitro* folding from bacterial inclusion bodies. Second, pMART-1<sub>100–114</sub> phosphopeptide (APPAYEKLpSAEQSP) was loaded into CLIP–HLA-DR1 using the peptide-exchange catalyst HLA-DM. Briefly, the extracellular portions of the HLA-DR1  $\alpha$  and  $\beta$  chains (residues 1–181 and 1–192, respectively) were expressed separately as inclusion bodies in *Escherichia coli* BL21(DE3) cells (Novagen). Inclusion bodies were dissolved in 8 M urea, 50 mM Tris–HCl (pH 8.0), and 10 mM DTT, followed by purification on a Poros HQ20 anion-exchange column (Perspective Biosystems) in 50 mM Tris–HCl, 8 M urea, and 1 mM DTT at pH 8.0 (DR $\alpha$ ) or pH 8.5 (DR $\beta$ ), using a linear NaCl gradient.<sup>15</sup> For *in vitro* folding, the purified subunits were diluted to a final concentration of 40  $\mu$ g/ml each in a folding solution containing 50 mM Tris–HCl, 30% (w/v) glycerol, 0.5 mM EDTA (ethylenediaminetetraacetic acid), 3 mM reduced glutathione, and 0.9 mM oxidized glutathione (pH 8.0). CLIP peptide (GenScript) was added to a final concentration of 5  $\mu$ M, and the folding mixture was kept for 2 weeks at 4 °C. The final folding solution was concentrated and dialyzed against 50 mM Mes (pH 6.0). Purification was carried out with sequential Superdex S-200 and Mono Q FPLC columns (GE Healthcare). The CLIP–HLA-DR1 complex was concentrated to 0.8 mg/ml and loaded with pMART-1<sub>100–114</sub> (Pi Proteomics) by overnight incubation at 37 °C in 100 mM sodium citrate–HCl (pH 5.8) containing 200  $\mu$ M phosphopeptide and soluble HLA-DM (0.2 mg/ml). The HLA-DR1–pMART-1 complex was crystallized at room temperature in hanging drops by mixing equal volumes of the protein solution at 5 mg/ml and a reservoir solution of 20% (w/v) PEG (polyethylene glycol) 8000 and 0.1 M sodium cacodylate (pH 6.5). For data collection, crystals were transferred to a cryoprotectant solution [mother liquor containing 30% (w/v) PEG 8000] prior to flash-cooling. X-ray diffraction data to 2.1 Å resolution were recorded at beamline X29 of the Brookhaven National Synchrotron Light Source with an ADSC Quantum-315 CCD detector. All data were indexed, integrated, and scaled with the program HKL2000.<sup>16</sup> Data collection statistics are shown in Table 1. The structure was solved by molecular replacement with the program Phaser<sup>17</sup> using MIG1–HLA-DR1 [Protein Data Bank (PDB) accession code 1T5W],<sup>18</sup> with all the peptide residues truncated to alanine as a search model. The DR1 molecule was located by automatic rotation and translation searches with a Z score of 40.0; initial rigid-body refinement gave  $R_{\text{free}}$  of 37.2% and  $R_{\text{work}}$  of 37.7%. Refinement was carried out with CNS1.2,<sup>19</sup> including iterative cycles of simulated annealing, positional refinement, and B-factor refinement, interspersed with model rebuilding into  $\sigma_A$ -weighted  $F_o - F_c$  and  $2F_o - F_c$  electron density maps with XtalView.<sup>20</sup> Stereochemical parameters were evaluated with PROCHECK.<sup>21</sup> Refinement statistics are summarized in Table 1.

(Fig. 2). An unusual feature of the phospho-MART-1 peptide is that N-terminal residues P-4 and P-3, which lie outside the binding groove, are well ordered in the structure (Fig. 1b), despite the absence of crystal contacts to the N-terminus. In other peptide-MHC class II complexes, by contrast, these residues (when present) are generally not clearly defined in the electron density, indicating flexibility. Another notable feature of pMART-1 is that its main chain makes a nearly 90° turn at P-2 Pro102, causing P-4 Ala100 and P-3 Pro101 to point upward from the  $\alpha 1/\beta 1$  platform of HLA-DR1 (Fig. 2). This orientation suggests the possibility of T-cell receptor (TCR) contacts to the ordered N-terminus of the peptide (Fig. 1b), as discussed later.

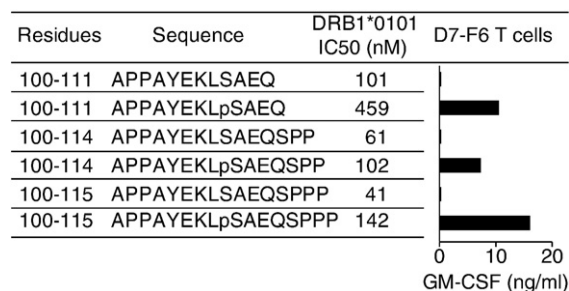
### Phosphate-mediated interactions between phosphopeptide and HLA-DR1

No direct contacts were observed between the phosphate moiety of P5 pSer108 and the phospho-MART-1 peptide or HLA-DR1. However, P5 pSer108 is stabilized by ordered water molecules that form bridging hydrogen-bonding interactions (Fig. 1d). In particular, the P5 pSer108 O2P atom forms a water-mediated hydrogen bond with the main-chain oxygen atom of P3 Lys106. Similarly, the P5 pSer108 O1P atom interacts with the N $\delta 2$  atom of HLA-DR1 Asn62 $\alpha$  via two bridging water molecules. By contrast, the reported phosphopeptide-HLA-A2 complexes include multiple direct contacts between the phosphate group and the  $\alpha 1$  and  $\alpha 2$  helices of the MHC class I molecule, in addition to water-mediated interactions.<sup>13,14</sup>

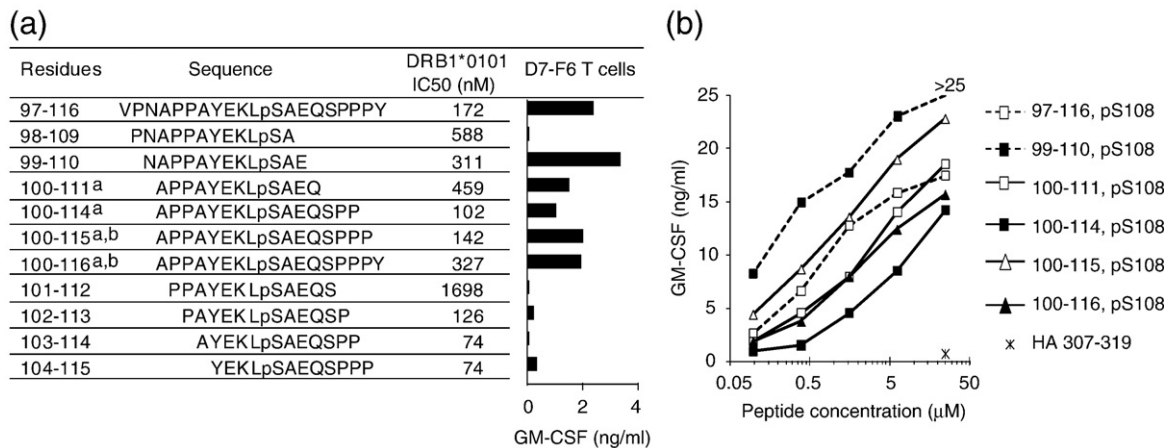
Phosphorylated MART-1 peptides bind HLA-DR1 with two- to fourfold lower affinity than their nonphosphorylated counterparts (Fig. 3). This modest reduction may be attributed to electrostatic repulsion between the negatively charged phosphate group of P5 pSer108 and the nearby carboxylate group of P7 Glu110 (Fig. 1d). In phosphopeptide-HLA-A2 complexes, by comparison, phosphorylation was sometimes (but not always) found to increase affinity for MHC class I,<sup>13,14</sup> suggesting that certain MHC class II-restricted phosphopeptides might also display phosphorylation-dependent binding to MHC.

### Recognition of phospho-MART-1 peptides by CD4<sup>+</sup> T cells

The phosphate group of P5 pSer is fully exposed to solvent and therefore potentially available for direct interactions with TCR. Indeed, the phosphate is a critical determinant for TCR recognition, as demonstrated using a CD4<sup>+</sup> T-cell clone (D7-F6) specific for pMART-1 (Fig. 3). These cells secreted the cytokine granulocyte-macrophage colony-stimulating factor (GM-CSF) in response to phosphorylated MART-1 peptides pulsed onto DR1-expressing antigen-presenting cells (APCs), but not to their nonphosphorylated homologs.



**Fig. 3.** Specific recognition of phosphorylated *versus* nonphosphorylated MART-1 peptides by CD4<sup>+</sup> T cells. The phospho-MART-1-specific T-cell clone D7-F6 was co-incubated overnight with HLA-DR1<sup>+</sup> 2048-EBV cells prepulsed with peptides (25  $\mu$ M). GM-CSF secretion was measured by ELISA. Background GM-CSF secretion from T cells + APC + HA<sub>307-319</sub> control peptide was <15 pg/ml. Results are representative of four separate experiments with D7-F6 and the parent T-cell line D7 showing specific T-cell recognition of phosphopeptides but not nonphosphorylated peptides, despite the comparatively lower MHC affinities (higher IC<sub>50</sub> values) of the phosphorylated MART-1 peptides. The oligoclonal pMART-1-specific CD4<sup>+</sup> T-cell line designated "D7" was raised by repetitive *in vitro* stimulation of peripheral blood mononuclear cells (PBMCs) from a melanoma patient expressing HLA-DR $\beta$ 1\*0101. Briefly, T cells were grown under microculture conditions and stimulated every 10–14 days with irradiated autologous PBMCs or HLA-DR1<sup>+</sup> allogeneic EBV-B cells pulsed with pMART-1<sub>100-111</sub> (APPAYEKLPsAEQ). Long-term CD4<sup>+</sup> T-cell cultures were maintained in RPMI 1640 + 10% heat-inactivated human AB serum, IL-2 (120 IU/ml), and IL-7 and IL-15 (25 ng/ml each). The T-cell clone designated "D7-F6" was subcultured from D7 T cells under limiting dilution conditions in microtiter plates and stimulated repetitively with pMART-1<sub>100-115</sub> (APPAYEKLPsAEQSPPP). To assess T-cell recognition of peptides, 1.2–5  $\times 10^4$  T cells per well were co-cultured overnight in flat-bottom 96-well plates with 1  $\times 10^5$  HLA-DR1<sup>+</sup> EBV-B cells that had been prepulsed with peptides. The medium consisted of RPMI 1640 + 10% human AB serum with IL-2 (120 IU/ml). Culture supernatants were harvested, and GM-CSF and interferon  $\gamma$  (IFN- $\gamma$ ) secretion by activated T cells was measured with commercially available ELISA kits (R&D Systems). Competition assays to quantitatively measure peptide binding to HLA-DR $\beta$ 1\*0101 were based on inhibition of binding of a high-affinity radiolabeled peptide to purified MHC molecules.<sup>28</sup> HLA class II molecules were purified from the EBV-transformed homozygous B lymphoblastoid cell line LG2. Peptide binding assays were performed by incubating purified human class II molecules (5–500 nM) with various concentrations of unlabeled peptide inhibitors and 0.1–1 nM <sup>125</sup>I-radiolabeled probe peptide for 48 h in phosphate-buffered saline containing 0.05–0.15% Nonidet P-40 and a protease inhibitor cocktail.<sup>22,28</sup> MHC binding of the radiolabeled peptide (HA<sub>307-319</sub>; PKYVKQNTLKLAT) was determined by capturing peptide-MHC complexes on L243 (anti-HLA-DRA) antibody-coated Lumitrac 600 plates (Greiner Bio-one) and measuring bound counts per minute. The concentration of peptide yielding 50% inhibition of the binding of the radiolabeled probe peptide (IC<sub>50</sub>) was then calculated. Peptides were typically tested at six different concentrations covering a 100,000-fold dose range and in three or more independent assays. Under the conditions utilized, where [label] < [MHC] and IC<sub>50</sub>  $\geq$  [MHC], the measured IC<sub>50</sub> values are reasonable approximations of the K<sub>D</sub> values.<sup>22</sup>



**Fig. 4.** Binding of phospho-MART-1 peptides to HLA-DR1 and recognition by CD4<sup>+</sup> T cells. (a) Affinities of overlapping phosphorylated MART-1 peptides for HLA-DR1, as measured by 50% inhibitory concentrations (IC<sub>50</sub>), are shown. Synthetic phosphopeptides include four naturally expressed sequences isolated from cultured melanoma cells: <sup>a</sup>1363-mel and <sup>b</sup>2048-mel. The bar graph demonstrates specific recognition of phospho-MART-1 peptides (15  $\mu$ M) by the CD4<sup>+</sup> T-cell clone D7-F6. GM-CSF secretion from T cells was measured by ELISA; background secretion from T cells + APC + HA<sub>307-319</sub> control peptide was <0.02 ng/ml. Similar results were obtained with IFN- $\gamma$  secretion and using the parent T-cell line D7. Results are representative of four separate experiments. (b) Recognition of titrated overlapping phospho-MART-1 peptides (0.1–25  $\mu$ M) by the specific CD4<sup>+</sup> T-cell line D7. Background GM-CSF secretion from T cells + APC + HA<sub>307-319</sub> control peptide (25  $\mu$ M) was 0.7 ng/ml. Results are representative of three separate experiments. Similar results were observed with IFN- $\gamma$  secretion.

Having established that phosphorylation is essential for TCR recognition, we next examined recognition of a series of synthetic overlapping phospho-MART-1 peptides to identify other determinants of T-cell reactivity (Fig. 4a and b). Similar to pMART-1<sub>100-116</sub>, a series of naturally occurring C-terminally truncated phosphopeptides (pMART-1<sub>100-115</sub>, pMART-1<sub>100-114</sub>, and pMART-1<sub>100-111</sub>) and the synthetic peptide pMART-1<sub>99-110</sub> were also capable of stimulating T-cell reactivity, indicating that residues Gln111 (P8) through Tyr116 (P13) are not involved in critical interactions with TCR. In the pMART-1<sub>100-114</sub>-HLA-DR1 structure, P8 Gln111 and P11 Pro114 (like P5 pSer108) are ~70% accessible to solvent and therefore potentially available for contacts to TCR, whereas P9 Ser112 and P10 Pro113 are ~80% buried (P9 Ser112 is a secondary anchor residue) and unlikely to contact TCR directly. Indeed, removal of P9 Ser112 and P10 Pro113 (compare pMART-1<sub>100-114</sub> with pMART-1<sub>100-111</sub>) resulted in a fourfold loss of affinity for HLA-DR1 but a paradoxical increase in T-cell activation (Fig. 4b). Residues P12 Pro115 and P13 Tyr116, although absent from the pMART-1<sub>100-114</sub>-HLA-DR1 structure, are expected to extend beyond the peptide-binding groove.

In sharp contrast to C-terminal residues P8 to P13, N-terminal residues P-4 to P-7 contain important determinants for T-cell recognition, besides the critical P5 pSer108 residue. Thus, removal of P-7 Glu110 (compare pMART-1<sub>99-110</sub> with pMART-1<sub>98-109</sub>) abrogated T-cell stimulation, even though this truncation reduced affinity for HLA-DR1 by less than twofold (Fig. 4a). In the pMART-1<sub>100-114</sub>-HLA-DR1 structure (Fig. 1c), the side chain of P-7 Glu110 projects from the surface of the MHC molecule and could interact with

TCR. Removal of N-terminal residues P-4 Ala100, P-3 Pro101, P-2 Pro102, and P-1 Ala103 (hereinafter referred to as the APPA motif) effectively abolished TCR recognition, as evident from comparison of T-cell stimulation by pMART-1<sub>100-114</sub> versus pMART-1<sub>103-114</sub>, and pMART-1<sub>100-115</sub> versus pMART-1<sub>104-115</sub> (Fig. 4a). Since truncation of residues P-4 to P-1 did not reduce binding to HLA-DR1 (indeed, a significant increase in MHC binding affinity was noted with removal of P-4 Ala100), these four N-terminal residues must include structural determinants required for TCR recognition.

To assess the contribution of individual residues of the APPA motif to TCR recognition, P-4 Ala100 and P-1 Ala103 of pMART-1<sub>100-114</sub> were each replaced by isoleucine, while P-3 Pro101 and P-2 Pro102 were replaced by alanine. Except for the replacement at P-4, these substitutions significantly reduced T-cell stimulatory capacity compared to that of wild-type pMART-1<sub>100-114</sub>, suggesting interactions with TCR (Supplementary Fig. 1). Most pronounced was the effect of substituting P-3 Pro101, which contributes directly to the unusual conformation of the APPA motif, whereby the peptide main chain makes a 90° turn at P-2 Pro102 directing N-terminal residues P-4 Ala100 and P-3 Pro101 toward the TCR (Fig. 2). In addition, although replacement of P-4 Ala100 by the chemically similar isoleucine residue did not significantly affect T-cell recognition, removal of P-4 Ala100 caused a total loss of T-cell reactivity (Fig. 4a).

The marked focus on the N-terminal portion of pMART-1<sub>100-114</sub> by TCR D7-F6 closely resembles peptide recognition by autoreactive TCRs specific for self-antigens, including tumor antigens, presented by MHC class II molecules.<sup>29</sup> In particular, X-



ray crystallographic studies of autoimmune TCRs specific for myelin basic protein associated with multiple sclerosis,<sup>30,31</sup> and of a tumor-specific TCR that recognizes a somatically mutated human melanoma antigen,<sup>32</sup> have revealed substantial alterations in the topology of TCR binding to peptide–MHC compared to that of anti-foreign TCRs.<sup>29</sup> In the self-reactive TCR–peptide–MHC complexes, the TCR is skewed toward the peptide N-terminus and the MHC class II  $\beta$  chain helix relative to its central position in anti-foreign TCR–peptide–MHC complexes, resulting in suboptimal TCR binding that may have enabled escape from negative thymic selection. The concentration on the N-terminal half of pMART-1 is therefore highly reminiscent of the way autoreactive TCRs engage self- or altered self-peptides.<sup>29</sup>

MART-1 protein plays an essential role in regulating mammalian pigmentation<sup>33</sup> but was not known to be phosphorylated prior to the discovery of phospho-MART-1 peptides complexed with HLA-DR1.<sup>4</sup> Tissue expression patterns of phospho-MART-1 protein *versus* its nonphosphorylated counterpart have yet to be determined. If MART-1 is expressed in the thymus and phosphorylation occurs after thymic development, then this post-translational modification will create a neo-epitope, most likely by increasing TCR affinity for MART-1–HLA-DR1 ligands beyond the threshold required for efficient T-cell activation in the periphery. Alternatively, if pMART-1 is expressed in the thymus during T-cell development, then surviving reactive TCRs are predicted to have suboptimal peptide–MHC binding characteristics. Future efforts to determine the structure of TCR D7-F6 bound to pMART-1<sub>100–114</sub>–HLA-DR1 and to measure the biophysical properties of this complex should provide relevant insights.

#### PDB accession number

Atomic coordinates and structure factors for the pMART-1<sub>100–114</sub>–HLA-DR1 complex have been deposited in the PDB under accession code 3L6F.

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#### Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2010.04.037](https://doi.org/10.1016/j.jmb.2010.04.037)

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