

## Research Article

Theme: Controlling Unwanted Immunogenicity to Biotherapeutics

Guest Editors: Alexandra Joseph, An Song, Shibani Mitra-Kaushik and Stephan Kontos

# Role of HLA-DP in the Presentation of Epitopes from the Truncated Bacterial PE38 Immunotoxin

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**Abstract.** Identification of helper T-cell epitopes is important in many fields of medicine. We previously used an experimental approach to identify T-cell epitopes in PE38, a truncated bacterial toxin used in immunotoxins. Here, we evaluated the ability of antibodies to DR, DP, or DQ to block T-cell responses to PE38 epitopes in 36 PBMC samples. We predicted the binding affinities of peptides to DR, DP, and DQ alleles using computational tools and analyzed their ability to predict the T-cell epitopes. We found that HLA-DR is responsible for 65% of the responses, DP 24%, and DQ 4%. One epitope that is presented in 20% of the samples (10/50) is entirely DP restricted and was not predicted to bind to DR or DP reference alleles using binding algorithms. We conclude that DP has an important role in helper T-cell response to PE38.

**KEY WORDS:** DP; epitope prediction; HLA class II; *in silico*; MHC class II; T-cell epitopes.

## INTRODUCTION

Identification of helper T-cell epitopes is very important in several fields of medicine, which include vaccine development, allergy and autoimmunity, and immunogenicity of protein-based therapeutics. It is well established that presentation of peptide antigens by major histocompatibility complex (MHC) class II is a necessary though not sufficient step in helper T-cell activation and in mounting an effective and specific immune response (1).

HLA class II proteins are heterodimeric cell surface glycoproteins expressed on the membrane of professional antigen presenting cells (APCs). They consist of an alpha and beta chain encoded in one of three loci: HLA-DR, HLA-DP, and HLA-DQ (2). HLA class II molecules are extremely polymorphic with thousands of allelic variants, which affect the structure of their binding groove and, as a result, their binding specificity to peptides (3).

HLA binding prediction algorithms have been developed to calculate affinity of a specific peptide binding to a specific binding core. Such prediction algorithms were shown to be

productive in the identification of high affinity binders for vaccine therapies (4–7). While eliciting an immune response is desirable in the development of a vaccine, the development of antidrug antibodies to a protein therapeutic compromises the safety and efficacy of the drug product. Thus, these algorithms have been used to identify strong and promiscuous peptide binders. Then protein engineering can be used to construct mutant proteins that are poor binders and do not induce antibody responses (8–10).

T-cell epitope prediction using HLA binding prediction focuses mainly on DR molecules and rarely on DP or DQ (8–16). The focus on DR for immunogenicity management is not limited to the use of HLA binding algorithms; DP and DQ alleles are usually not considered in the assembly of representative human cohorts. Furthermore, recombinant or purified DP molecules or tetramers for functional and binding assays are uncommon. This trend is due, in part, to an assumption that DP molecules are less important for immune responses than DR (17) and because cell surface expression of DP and DQ was reported to be lower than that of DR (18–20). Also *in vitro* stimulation of T cells was shown to induce DR expression in more T cells than DP and DQ (21). This lack of emphasis on DP and DQ alleles has resulted in smaller datasets, which affect the accuracy of predictions of peptide binding affinities for these alleles.

We previously sought to identify the T-cell epitopes in a truncated bacterial toxin PE38 that is used as the cytotoxic moiety in recombinant immunotoxins (RITs). These therapeutic proteins have high specificity and low toxicity and were shown to be highly effective in tumor eradication in

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hematological patients (22,23). However, RITs are also very immunogenic due to the bacterial origin of the toxin and all patients formed neutralizing antibodies after one or two cycles of treatment (24). Using an experimental approach, we identified eight major epitopes (25) that were common on naive donors and previously treated patients; only four of these were predicted to bind at high affinity to multiple HLA molecules by commonly used HLA binding prediction programs (26). In addition, the programs predicted the presence of several high affinity binders that were not identified as epitopes in our experimental approach. A possible explanation for the DR binding analysis not predicting the binding of some epitopes is that some could be DP or DQ restricted. In this study, we examined the HLA class II restriction of the T-cell epitopes in PE38 and evaluated the effect of non-DR-restricted epitopes on successful identification by HLA binding algorithms. We found that DP has an important role in T-cell immunity to this protein and that this epitope is not predicted using DR, DP, or DQ binding prediction algorithms.

## MATERIALS AND METHODS

### Human Donor PBMC Samples

Peripheral blood mononuclear cells (PBMCs) from healthy volunteer donors at the National Institutes of Health (NIH<sup>3</sup>) blood bank were collected under research protocols approved by the NIH Institutional Review Board (99-CC-0168) using apheresis. Also apheresis samples were obtained from patients treated with moxetumomab pasudotox or SS1P after informed consent in clinical trials (protocols 08-C-0026 and 06-CC-0150). A total of 36 PBMC samples were selected based on previously determined responses to PE38 peptides (25). Samples were qualified for the study if it demonstrated a response to one of the peptides described in Table I with all the following criteria:  $\geq 85$  spot forming cells (SFC)/ $10^6$  cells,  $\geq 3$ -fold the response in the no peptide control, and  $\geq 3\%$  of all SFC.

PBMCs were isolated and cryopreserved as previously described (27). Briefly, buffy coat was collected after Ficoll-Hypaque (GE Healthcare) density-gradient separation. Cells were washed three times with Dulbecco's phosphate buffered saline (PBS) without Ca and Mg and cryopreserved in liquid nitrogen at a concentration of  $1\text{--}3 \times 10^7$  cells/ml in RPMI media (Lonza) supplemented with 10% heat-inactivated human AB serum (Gemini) and 7.5% DMSO (Cellgro).

### HLA Typing of PBMC Samples

Genomic DNA was isolated from each PBMC sample using quick-gDNA MiniPrep (Zymo Research) and sent for HLA typing. *HLA-DRB* genes were typed using polymerase chain reaction (PCR) sequence-specific-based tissue typing by the HLA typing unit at the NIH. *HLA-DPB* and *HLA-DQB* genes were typed by the Texas BioGene molecular typing laboratory.

### Peptide Synthesis

All peptides were synthesized by American peptides with the exception of the CEFT peptide pool control

(Axxura). Peptides were purified to  $>95\%$  and were analyzed for homogeneity by HPLC. All custom peptide sequences are shown in Table I. Upon arrival, the peptides were resuspended in DMSO to a concentration of 2 mg/ml. After resuspension, the peptide stocks were stored at  $-20^\circ\text{C}$ . Peptides NNYGSTIEGLLD and PMDEPTLLYVLFVVF were designed to be used as a control that specifically binds to DQ and DP molecules based on a search of the Immune Epitope Database ([www.iedb.org](http://www.iedb.org)) (28–30).

### Specific *In Vitro* Expansion of PBMC

PBMCs were expanded and stimulated as previously described (27,31). Briefly, cells were thawed in assay media that contains RPMI, 5% heat-inactivated AB serum, and P/S antibiotics. Cells were stimulated with 5  $\mu\text{g}/\text{ml}$  of a PE38 containing immunotoxin (LMB-9) (32). On days 4, 7, and 10, cells were supplemented with fresh assay medium containing 20 units of interleukin 2 (IL-2) (Millipore). On day 14, cells were harvested, washed, and used for enzyme-linked immunoSpot (ELISpot) assays.

### HLA Restriction by ELISpot Assay

White polyvinylidene fluoride (PVDF) filter plates (Mabtech) were coated with anti-IL-2 antibody (clone MT2A91/2C95 Mabtech) according to the manufacturer's protocol and incubated at  $4^\circ\text{C}$  for 18 h. Plates were washed with PBS and *in vitro* expanded cells were placed in a concentration of  $1 \times 10^5/\text{well}$ . Next, the following inhibiting antibodies were added to the cells at a final concentration of 15  $\mu\text{g}/\text{ml}$ : anti-DR (clone G45-6, BD), anti-DP (clone B7/21, Leinco), anti-DQ (clone SPVL3, Beckman Coulter), Pan class I (clone W6/32, BD), or combination of anti-DR, anti-DP, and anti-DQ antibodies. After 30 min incubation at  $37^\circ\text{C}$ , PE38-derived peptides (listed in Table I) that were previously shown to activate the selected PBMC samples were added. Cells were incubated at  $37^\circ\text{C}$  for an additional 18 h. Additional negative controls included cells with no peptide stimulation, cells with a peptide but no antibodies and in 5/36 samples, a negative control of an irrelevant peptide and antibodies that resulted in no T-cell response. Phytohemagglutinin (Sigma) was used as a technical positive control. The secretion of IL-2 following stimulation with peptides was detected using a secondary biotinylated anti-IL-2 antibody (clone MT8G10, Mabtech) followed with streptavidin, alkaline phosphatase conjugate (SA-ALP) (Mabtech), and nitro blue tetrazolium and 5-bromo-4-chloro-3'-indolyl phosphate (BCIP/NBT) substrate (KPL).

The ability of the anti-HLA antibodies to specifically inhibit DR, DP, or DQ presented T-cell activation was controlled by routinely stimulating specific samples using peptides with known restriction: CEFT (Axxura) for DR, NNYGSTIEGLLD for DQ, and a combination of TRHRQPRGWEQLEQC and PMDEPTLLYVLFVVF for DP to confirm the effectiveness of the inhibiting antibodies.

Spots were counted by computer-assisted image analysis (Immunospot 5.0; Cellular Technology Limited). Each assay was performed in quadruplicate and repeated at least twice.

Table I. Peptides Used in the Study

Peptide description	Amino acid sequence	Length	Origin	Ref
Peptides 13–15	GYPVQRLVALYLAARLSWNQV	21	PE38	This work
Peptide 74	PDARGRIRNGALLRV	15	PE38	(27)
Peptide 75	RGRIRNGALLRVYVP	15	PE38	(27)
Peptide 76	IRNGALLRVYVPRSS	15	PE38	(27)
Peptide 77	GALLRVYVPRSSLPG	15	PE38	(27)
Peptide 78	LRVYVPRSSLPGFYR	15	PE38	(27)
Peptides 8–9	TRHRQPRGWEQLEQCGYP	18	PE38	This work
Peptide 5	ACHLPLETFTRHRQP	15	PE38	(27)
Peptide 6	LPLETFTRHRQPRGW	15	PE38	(27)
Peptide 67	WRGFYIAGDPALAYG	15	PE38	(27)
Peptide 68	FYIAGDPALAYGYAQ	15	PE38	(27)
Peptide 93	GPEEEGGRLETILGW	15	PE38	(27)
Peptide 94	EEGGRLETILGWPLA	15	PE38	(27)
Peptide 95	GRLETILGWPLAERT	15	PE38	(27)
Peptide 96	ETILGWPLAERTVVI	15	PE38	(27)
Peptide 51	TVERLLQAHRQLEER	15	PE38	(27)
Peptide 52	RLLQAHRQLEERGYV	15	PE38	(27)
Peptide 56	GYVFGYHGTFLCAA	15	PE38	(27)
Peptide 57	FVGYHGTFLCAAQSI	15	PE38	(27)
Peptide 58	YHGTFLCAAQSIVFG	15	PE38	(27)
Peptide 59	TFLEAAQSIVFGGVR	15	PE38	(27)
DQ control	NNYGSTIEGLLD	12	Human herpesvirus 2	(28,29)
DP control	PMDEPTLLYVLFEEV	15	Human mastadenovirus C	(29,30)

### Determination of Restriction Element

To determine the restricting element, the spots for each sample and epitope combinations were normalized; 0% represents no antibody control (no inhibition) and 100% represents the combination of DR, DP, and DQ (maximal inhibition). Responses were considered restricted to a specific class II molecule if they exhibit a normalized inhibition of  $\geq 70\%$ . Responses were considered to have co-presentation of two or three class II molecules if either more than one antibody was required for  $\geq 70\%$  inhibition or when more than one molecule had  $\geq 70\%$  inhibition. Assays that were not included in the restriction analysis include (1) signal in “no antibody” wells  $< 1.0 \times 10^2$  SFC, (2) signal in “no antibody”  $< 2$ -fold background (no peptide control), and (3) three samples were excluded because the inhibition of all three class II together molecules was  $< 70\%$  of the combination.

### Determination of DR, DP, and DQ Expression on PBMC Using Flow Cytometry

PBMC, at  $2 \times 10^6$  cells/tube, were blocked with mouse immunoglobulin G (IgG) for 10 min, then stained with a panel of antibodies identifying lymphocyte and antigen presenting cell subsets that target the following cellular markers: BDCA1, BDCA2, CD56, CD14, CD3, CD4, CD8, and CD19 and HLA-DR, DP, or DQ or isotope controls (Supplementary Table S1). Cells were incubated for 20 min at  $4^\circ\text{C}$  in the dark, washed, and resuspended in fluorescence activated cell sorting (FACS) buffer (calcium- and magnesium-free HBSS with 0.2% BSA and 0.1%  $\text{NaN}_3$ ). Data were collected on a Gallios™ Flow Cytometer

(Beckman Coulter) and analyzed with FlowJo 9.6 (Treestar). The geometric mean fluorescence intensity of HLA antigens was determined for each cell population.

### Predicted Binding for HLA-DR, DP, and DQ Alleles

The IEDB-recommended method (33) was used to predict the binding affinity of 24 PE38-derived peptides. The binding affinity to 15 HLA-DR alleles, 6 HLA-DP alleles, and 6 HLA-DQ alleles was submitted to the IEDB MHC-II binding predictions. The percentile rank of each peptide to each allele was recorded: the smaller the rank, the stronger the predicted binding. The binding threshold of 20% was imposed to calculate the number of alleles among the reference alleles that have predicted binding to each epitope peptide identified experimentally. A peptide was considered an epitope if it was predicted to bind to a third of the HLA molecules (*i.e.*,  $\geq 5/15$  for DR,  $\geq 2/6$  for DP and DQ, and  $\geq 9/27$  for all HLA class II combined). These criteria were previously shown to give the best correlation between HLA-DR prediction analysis and T-cell epitopes (26).

## RESULTS

### HLA Restriction of T-Cell Responses to PE38-Derived Peptides

We previously screened PBMC samples from 50 naive donors and 16 immunotoxin-treated patients and identified eight major T-cell epitopes in PE38 (25). To identify the class II subtype that is responsible for presentation of PE38-derived antigenic peptides, we expanded some of those PBMC samples for 14 days with LMB-9 (a RIT containing

PE38) and IL-2 and restimulated with peptides containing the cognate epitopes. The activation of T cells was assessed by IL-2 ELISpot, and the restriction was determined by the ability of antibodies to HLA-DR, DP, or DQ to inhibit the response. Figure 1 shows representative responses of PBMC samples from four different donors.

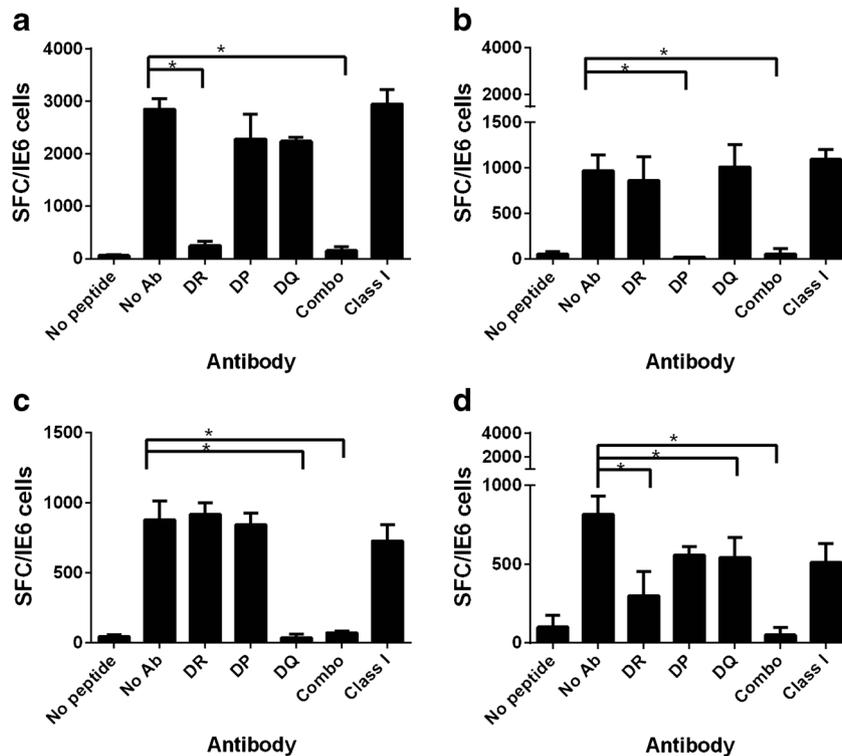
Figure 1a shows a response that is entirely DR restricted. PBMCs were stimulated with peptides 13–15 that represent epitope 1. Cells that were not treated with inhibitory antibodies had a strong stimulation ( $2.8 \times 10^3$  SFC/ $1 \times 10^6$  cells); similarly, cells treated with anti-DP, DQ, or class I pan had a strong stimulation with a range of  $2.2 \times 10^3$ – $2.9 \times 10^3$  SFC/ $1 \times 10^6$  cells, indicating that DP and DQ are not involved in the presentation of this peptide. However, cells that were incubated with antibodies against DR or a combination of antibodies against DR, DP, and DQ (“combo”) had a significantly lower response than the cells with no antibody ( $2.5 \times 10^2$  and  $1.5 \times 10^2$  SFC/ $1 \times 10^6$  cells, respectively) ( $p < 0.05$  in the Mann-Whitney test). This result indicates that these PBMCs use DR molecules exclusively for the presentation of epitope 1.

Figure 1b shows a response that is entirely DP restricted. PBMCs were stimulated with peptides 8–9 that represent epitope 3. Cells that were not treated with inhibitory antibodies had a strong stimulation ( $9.7 \times 10^2$  SFC/ $1 \times 10^6$  cells); similarly, cells treated with anti-DR, DQ, or class I pan had a strong stimulation with a range of  $8.6 \times 10^2$ – $1.1 \times 10^3$  SFC/ $1 \times 10^6$  cells, indicating that DR and DQ are not involved

in the presentation of this peptide. However, cells that were incubated with antibodies against DP or a combination of the antibodies had a significantly lower response ( $2.0 \times 10$  and  $5.3 \times 10^1$  SFC/ $1 \times 10^6$  cells, respectively) ( $p < 0.05$  in the Mann-Whitney test). This result indicates that these PBMCs use DP molecules exclusively for the presentation of epitope 3.

Figure 1c shows a response that is entirely DQ restricted. PBMCs were stimulated with a combination of peptides 93 and 94 that represents epitope 6. Cells that were not treated with inhibitory antibodies had a strong stimulation ( $8.8 \times 10^2$  SFC/ $1 \times 10^6$  cells); cells treated with anti-DR, DP, or class I pan also had a strong stimulation with a range of  $7.3 \times 10^2$ – $9.2 \times 10^2$  SFC/ $1 \times 10^6$  cells, indicating that DR and DP are not involved in the presentation of this peptide. However, cells that were incubated with antibodies against DQ or a combination of antibodies against DR, DP, and DQ had a significantly lower response than the cells with no antibody ( $6.4 \times 10$  and  $8.8 \times 10$  SFC/ $1 \times 10^6$  cells, respectively) ( $p < 0.05$  in the Mann-Whitney test). This indicates that these PBMCs use DQ molecules exclusively for presentation of epitope 6.

Figure 1d shows an example of co-presentation on DR, DP, and DQ. PBMCs were stimulated with peptides 13–15 that represent epitope 1. Cells that were not treated with inhibitory antibodies had a strong stimulation ( $8.2 \times 10^2$  SFC/ $1 \times 10^6$  cells); however, cells that were treated with either DR or DQ antibodies had a significantly lower response ( $p < 0.05$  in the Mann-Whitney test). While the combination of the three antibodies produced



**Fig. 1.** Representative inhibition pattern of antibodies against DR, DP, DQ, and class I pan in IL-2 ELISpot. PBMCs were stimulated with PE38 for 14 days and restimulated with various specific peptides that induce T-cell activation and IL-2 secretion. **a** PBMC stimulated with peptides 13–15 and inhibited by DR antibody. **b** PBMC stimulated with peptides 8–9 and inhibited by DP antibody. **c** PBMC stimulated with peptides 93–94 and inhibited by DQ antibody. **d** PBMC stimulated with peptides 13–15 and partial inhibition by DR, DQ, and DP antibodies

inhibition with a near background response of  $5.0 \times 10^6$  SFC/ $1 \times 10^6$ , none of the individual antibodies was able to produce complete inhibition. Normalization of the inhibition between the “no antibody” (0%) and “combination” (100%) showed that the antibody against DR inhibits 66% of the response, DQ 34%, and DP 31%. The sum of the three responses (inhibition by anti-DR, anti-DP, or anti-DQ) is not necessarily expected to be precisely 100% because the responses were normalized to the combo control wells that included all three antibodies together.

*Epitope Restriction Analysis*

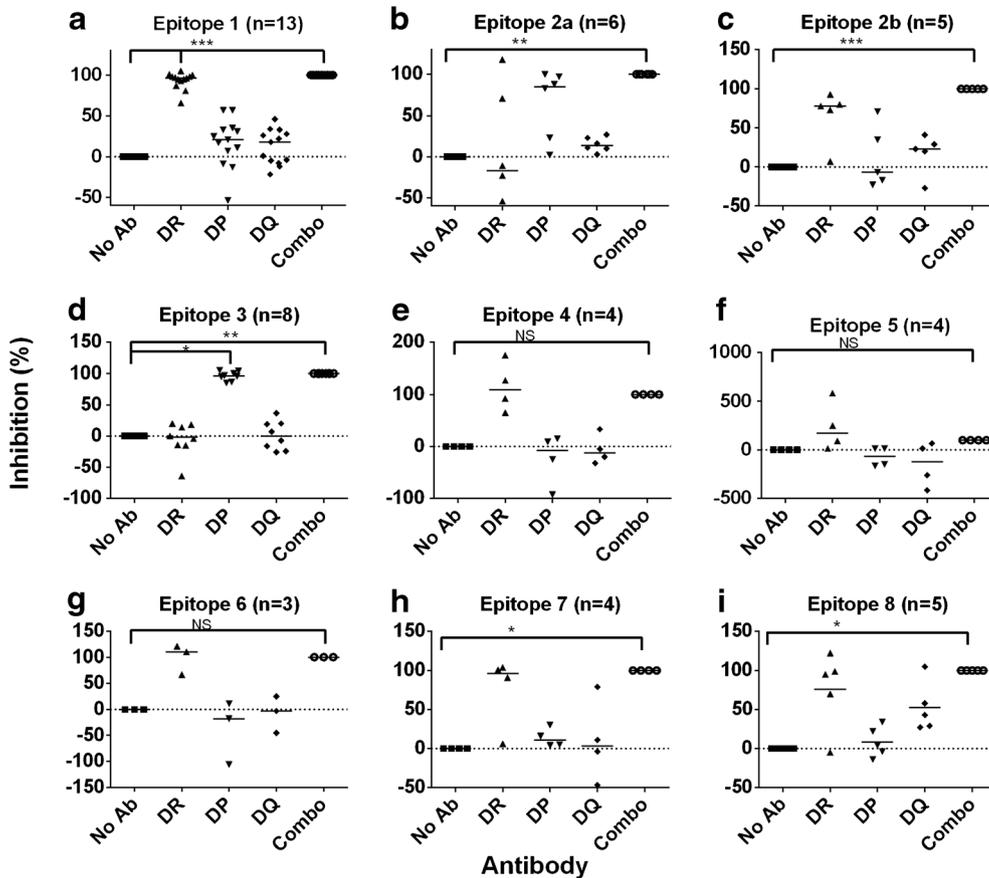
We used the antibody inhibition approach to analyze the restriction of peptides from all eight epitopes. To do this, we analyzed 54 PBMC/epitope combinations. Three responses were excluded because the cumulative inhibition of the three class II molecules was <70%. The results for the remaining 51 samples are summarized in Fig. 2 and described below. In order to determine HLA class II subtype restriction, only the PBMCs from donors that responded to a particular epitope were used for that epitope.

To identify the restriction to epitope 1, PBMC samples were stimulated with a 21 mer peptide spanning amino acids 288–308 in PE38 (peptides 13–15) (Supplementary Table S1). Epitope 1 was significantly restricted by DR ( $p = 0.0001$  in the Friedman test with Dunn’s multiple comparisons) with a

median of 91% inhibition (Fig. 2a). One PBMC sample showed co-presentation of this epitope with 66% inhibition by DR, 34% by DQ, and 31% by DP.

Epitope 2 is a complex epitope because the responses to this epitope spread over five overlapping peptides (peptides 74–78), which indicate more than a single 9 mer core. We previously divided this epitope into two subepitopes: epitope 2A spans peptides 74–76 and epitope 2B spans peptides 76–78. To identify the restriction to epitope 2A, six PBMC samples were stimulated with peptides 74, 75, and 76. We found that 4/6 donors that responded to epitope 2A were restricted by DP with a median of 86% inhibition and 2/6 were restricted to DR (Fig. 2b). To identify the restriction to epitope 2B, five PBMC samples were stimulated with peptides 77 and 78; four of those samples were unique and one sample was also included in the analysis shown in 2A and 2B. We found that 4/5 donors that responded to epitope 2B were presented by DR with a median inhibition of 78% (Fig. 2c). The sample that was common with 2A was presented by DP. This result corroborates the complexity of this epitope and demonstrates that PBMCs from different individuals can present an epitope with different class II presentation molecules.

To identify the restriction to epitope 3, eight PBMC samples were stimulated with an 18 mer peptide spanning amino acids of



**Fig. 2.** Inhibition of T-cell responses to PE38-derived epitopes. **a** Epitope 1, peptides 13–15 ( $n = 13$ ). **b** Epitope 2A, peptides 75 and 76 ( $n = 6$ ). **c** Epitope 2B, peptides 76–77 ( $n = 5$ ). **d** Epitope 3, peptides 8–9 ( $n = 8$ ). **e** Epitope 4, peptides 5 and 6 ( $n = 4$ ). **f** Epitope 5, peptides 67 and 68 ( $n = 4$ ). **g** Epitope 6, peptides 94 and 95 ( $n = 3$ ). **h** Epitope 7, peptides 51 and 52 ( $n = 4$ ). **i** Epitope 8, peptides 57 and 58 ( $n = 5$ )

peptides 8 and 9. We found that all eight responses were inhibited by an antibody to DP but not with other antibodies with a median of 97% inhibition ( $p=0.018$  in the Friedman test with Dunn's multiple comparisons) (Fig. 2d).

To identify the restriction to epitope 4, PBMC samples were stimulated with peptides 5 and 6. Epitope 4 was presented by DR in 3/4 samples with a median of 110% inhibition (Fig. 2e). An inhibition >100% can occur when the spots in the anti-DR wells have lower activation than the combination of anti-DR, DP, and DQ. One PBMC sample showed co-presentation of this epitope with 65% inhibition by DR and 33% by DQ.

For epitope 5, PBMC samples were stimulated with peptides 67 and 68. Epitope 5 was presented by DR in 3/4 samples with a median of 171% inhibition (Fig. 2f). One PBMC sample showed co-presentation of this epitope with 67% inhibition by DQ, 14% by DR, and 14% by DP.

For epitope 6, three PBMC samples were stimulated with peptides 94 and 95. Epitope 6 was presented by DR in 2/3 samples with a median of 110% inhibition (Fig. 2g). One PBMC sample showed co-presentation of this epitope with 67% inhibition by DR and 25% by DQ.

For epitope 7, four PBMC samples were stimulated with peptides 51 and 52. Epitope 7 was presented by DR in 3/4 samples with a median of 96% inhibition (Fig. 2h) and 1/4 samples was exclusively presented by DQ.

Finally, to identify the restriction to epitope 8, five PBMC samples were stimulated with peptides 57 and 58. Epitope 8 was presented by DR in 4/5 samples with a median of 95% inhibition (Fig. 2i) and 1/5 samples was exclusively presented by DQ. All five donors had moderate inhibition by DQ antibodies with a median of 43% inhibition.

The complete restriction for each epitope is summarized in Table II. Overall, we found that of 51 qualifying T-cell responses to PE38-derived epitopes, 33/51 (65%) of the responses were exclusively restricted to DR, 12/51 (24%) to DP, and 2/51 (4%) to DQ. Four out of 51 (8%) of the responses were presented by more than a single presenting molecule, 2/51 were co-presented by DR+DQ, and 2/51 by all three DR+DP+DQ.

### HLA Class II Molecule Density on the Surface of APCs

To measure HLA class II expression on PBMCs, we used multicolor flow cytometry. As expected, we found that B cells (CD19+) and myeloid-derived dendritic cells (BDCA1+, BDCA2-) have a high expression of all three class II molecules (DR, DP, and DQ). Natural killer (CD56+) cells have very low expression and T cells (CD3+CD4+ or CD3+CD8+) have low but variable expression, which may reflect higher expression of HLA II molecules on activated T-cell populations (21). We did not investigate activation markers but focused our analysis on B cells and myeloid-derived dendritic cells. To alleviate bias that could be introduced by laser compensation, the DR, DP, and DQ antibodies share the same FITC label and were added in separate tubes.

To examine whether the presentation of the PE38 epitopes on different MHCs is related to differential expression of DR, DP, or DQ, we separated the PBMC samples into two groups and analyzed for MHC expression levels using antibodies to DR or DP. Group 1 contained PBMCs that presented peptides on DP ( $n=9$ ). Group 2 contained PBMCs that presented peptides on DR ( $n=6$ ). We found that T cells of donors in group 1 do not have a significant difference in DP expression compared with donors in group 2, which did not present on DP, with average geometric means of  $11.7 \pm 2.5$  and  $12.0 \pm 2.0$ , respectively (Fig. 3a). Similarly, no significant difference in expression was observed in myeloid-derived dendritic cells with median geometric means of  $11.3 \pm 3.0$  and  $12.0 \pm 3.0$  in groups 1 and 2, respectively (Fig. 3b). Furthermore, no significant differences were observed in the expression of DR in the two donor groups on B cells or myeloid-derived dendritic cells (Fig. 3c, d). We also found that no difference in DQ expression was observed (Fig. 3e, f). In summary, we did not find any significant differences between donor samples in group 1 and group 2 in their expression of HLA-DR, HLA-DP, or HLA-DQ on B cells and myeloid-derived dendritic cells.

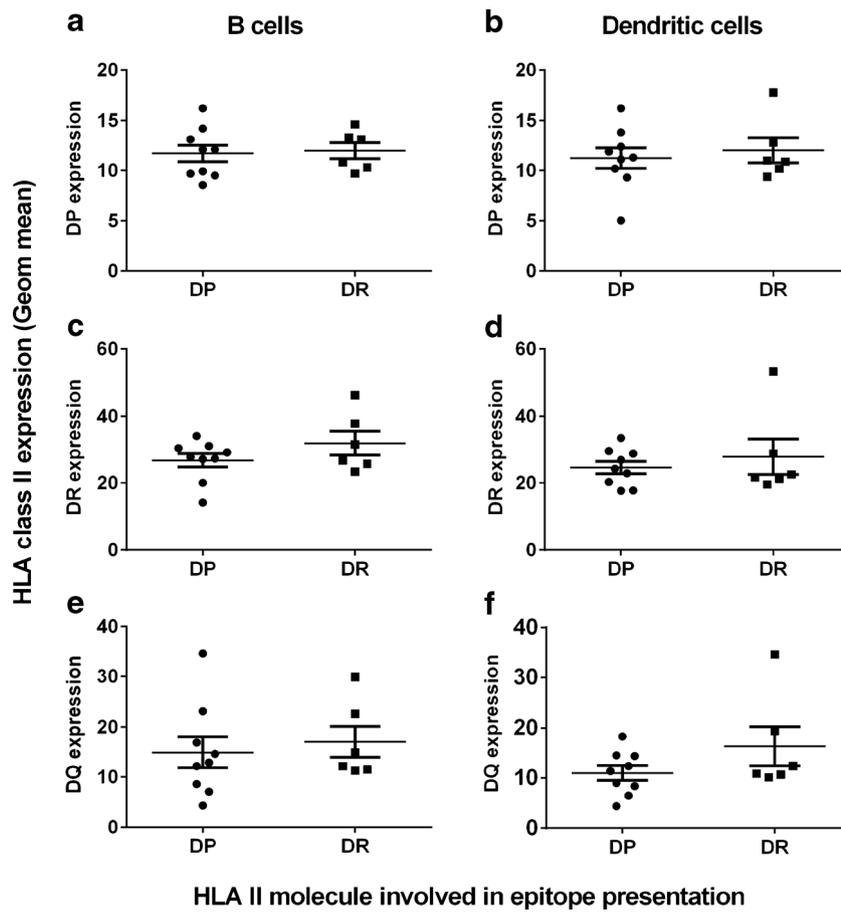
**Table II.** HLA Class II Restriction of PE38 Epitopes

Epitope no.	Peptides	Peptide sequence	Restriction <sup>a</sup>					Total
			DR	DP	DQ	DR+DQ <sup>b</sup>	DR+DP+DQ	
1	13–15	GYVQRLVALYLAARLSWNQV	12	0	0	0	1	13
2A+B	74–78	PDARGRIRNGALLRVYVPRSSLPGFYR	6	4	0	0	0	10
3	8–9	TRHRQPRGWEQLEQCGYP	0	8	0	0	0	8
4	5–6	ACHLPLETFTRHRQPRGW	3	0	0	1	0	4
5	67–68	WRGFYIAGDPALAYGYAQ	3	0	0	0	1	4
6 A+B	93–96	GPEEEGGRLETILGWPLAERTVVI	2	0	0	1	0	3
7	51–52	TVERLLQAHRQLEERGYV	3	0	1	0	0	4
8 A+B	56–59	GYVFGYHGTFLEAAQSIVFGGVR	4	0	1	0	0	5
Total			33	12	2	2	2	51

Three samples were excluded because the inhibition of all three class II molecules was <70%

<sup>a</sup> Responses were considered restricted to a specific class II molecule if it exhibits a normalized inhibition >70%

<sup>b</sup> Co-restriction was defined when two alleles or more were required for >70% inhibition or when more than one molecule had >70% inhibition



**Fig. 3.** Density of DR, DP, and DQ in cells from DR- and DP-restricted donors on B cells and myeloid-derived dendritic cells. PBMCs from 15 donors were placed in two groups for DP- and DR-restricted responders and labeled with fluorescently labeled antibodies for cellular markers CD19, BDCA1, and BDCA2, as well as FITC-labeled antibodies targeting DP, DR, and DQ. Expression of DP on CD19+ lymphocytes (B cells) is shown in **a**, DR is shown in **c**, and DQ is shown in **e**. Expression of DP on BDCA1+, BDCA2- myeloid-derived dendritic cells is shown in **b**, DR (**d**) and DP (**f**)

#### HLA Types Within the Epitope Responder Groups

To determine if the restricted responses occurred in donors with different HLA alleles, we analyzed the DNA of 36 samples for HLA DRB1, DR3/4/5, DQB1, and DPB1 (Table III).

To identify a common HLA allele in epitope 1, we examined the different DR alleles in 13 samples that responded to this epitope and were mostly presented by DR. We found that all 13 samples had DRB5\*01 or DRB5\*02 in common and that 12/13 samples also had DRB1\*15 (\*1501, \*1502, or \*1503) (Table III).

To identify common HLA molecule in epitope 2 (A and B), we examined the DR and DP alleles in 10 samples. We found that the DR-restricted samples were mixed with three donors expressing DRB1\*1501, two donors expressing DRB1\*0804, two expressing DRB1\*1103/\*1104, and a few expressing unique alleles like DRB1\*0301 and DRB1\*0401. DRB3/4/5 was heterogeneously expressed as well. While the donors restricted to DR do not have common DRB1 alleles, three of the four DP-restricted samples have DPB1\*04 in common (Table III).

For epitope 3, we examined the different DP alleles in seven samples that responded to epitope 3 and were all

presented by DP. We found that all samples have variable DPB1 alleles including DPB1\*101, DPB1\*401, and other alleles. A single common allele for all donors was not observed. This indicates a possible shared DP motif (Table III).

For epitope 4, we examined the different DR alleles in four samples that responded to this epitope and were mostly presented by DR. We found that 3/4 samples had DRB1\*13 in common (1301 or 1302) (Table III).

To identify a common HLA allele in epitope 5, we examined the different DR alleles in three samples that responded to this epitope and were presented by DR. All three samples share a common DR3\*02 allele (Table III).

For epitope 6, we examined the different DR alleles in three samples that responded to this epitope and were presented by DR or a combination of (DR+DQ). All three samples share a common DRB1\*15 (1501 or 1503) (Table III).

For epitope 7, we examined the different DR alleles in four samples that responded to this epitope and were presented by either DR ( $n = 3$ ) or DQ ( $n = 1$ ). We found that the three DR-restricted samples did not share a common DR allele (Table III).

Finally, to identify a common HLA allele in epitope 8, we examined the different DR alleles in five samples that

**Table III.** HLA Class II Alleles of Donors and Patients Used in the Study

ID	HLA class II allele								Epitope	Presenting molecule
	DRB1*	DRB3/4/5*		DQB1*		DPB1*				
Donor 1	8:04	15:01	5*0101		03:01	06:02	04:01	17:01	1, 2B	DR, DR
Donor 2	04:07	15:02	4*0103	5*0102	03	06:01			1	DR
Donor 3	13:01	15:03	3*0101	5*0101	06	06			1, 6	DR (DR+DQ)
Donor 4	04:01	15:01	4*01	5*01	03:01	06:02	04:01	16:01	1, 3	DR, DP
Donor 5	03:02	15:03	3*0101	5*0101	04:02	06:02			1	DR
Donor 6	07:01	15:01	4*01	5*00	03:03	06:02			1	DR
Donor 7	08:04	15:03	5*0101		06:02	06:02	10101	4001	1	DR
Donor 8	15:01	15:02	5*0102	5*02	05:03	06:02			1	DR
Donor 9	12:01	16:02	3*02	5*0101	05:01	05:02	09:02	85:01	1	DR
Donor 10	14:01	15:01	3*0202	5*0101	05:03	06:02	40101	2301	1	DR
Donor 11 <sup>a</sup>	08:03	15:02	5*01		05:01	06:01	36:01	13:01	1, 3, 4	DR, DP, DR
Donor 12	08:06	15:01	5*01		05:01	06:02	03:01	04:01	1, 6	DR, DR
Donor 13	15:01	15:03			06:02	06:02	04:01	40:01	2A	DP
Donor 14	03:01	11:04	3*0101	3*0202	02:01	03:01	04:02	04:02	2A, 2B	DP
Donor 15	09:01	13:01	3*02	4*01	02:02	03:03	01:01	105: 01	2A	DP
Donor 16	04:01	04:04	4*0103		03:02	03:02	02:01	18:01	2B	DR
Donor 17	08:04	13	3*0101		03:01	04:02			2B	DR
Donor 18	03:01	04:01	3*01	04*0103	02:01	03:01			3	DP
Donor 19	03:01	03:02	3*0101	3*0202	02:01	04:02	01:01	105:01	3, 5	DP, DR
Donor 20	01:01	03:01	3*0101		02:01	05:01	01:01	03:01	3	DP
Donor 21	04:01	13:02	3*0301	4*01	03:01	06:09	04:01	05:01	3	DP
Donor 22	10:01	13:02	3*0202	3*0303	03:01	05:01	01:01	02:01	3	DP
Donor 23	07:01	07:01	4*0103		02:02		01:01	02:01	3	DP
Donor 24	04:01	13	3*0202	4*0103	03:02	06:03	03:01	04:02	4	DR
Donor 25 <sup>b</sup>	12:01	13:01			05:01	06: 03			4	DR
Donor 26	08:04	13:02	3*0301		3	5	01:01	02:01	4	(DR+DQ)
Donor 27	03:01	07:01	3*02	4*01	02:01	02:02	01:01	85:01	5, 8	DR, DR
Donor 28	11:01	13:02	03*0202	03*0301	3	05:01	01:01	02:01	5	(DR+DP+DQ)
Donor 29	15:01	15:02	05*01		06:01	06:02	04:01	104:01	7	DQ
Donor 30	04:01	13:03			03:01	03:02	29:01	105:01	7	DR
Donor 31	07:01	08:04	04*01		02:02	03:19	01:01	11:01	7, 8	DR, DR
Patient 1	07:01	15:01			02:02	06:02	02:01	04:01	1, 2A, 6, 8	DR, DR, DR, DR
Patient 2	15:01	15:01			06:02	06:02	04:01	04:02	2B	DR
Patient 3	04:01	12:01			03:01	03:01	04:01	05:01	2A	DP
Patient 4 <sup>c</sup>	01:03	03:01			02:01	03:01	25:01	20:01	8	DR
Patient 5 <sup>d</sup>	11:03	13:03			03:01	03:01	2:01	23:01	2A, 5, 7, 8	DR, DR, DR, DQ

<sup>a</sup> Could not rule out DPB1\*135:01 and 107: 01, 173: 01

<sup>b</sup> Could not rule out DRB1\*12:06, 12:10, 12:17, DRB1\*13:117, DQB1\*105:07, 05: 11, 05:12 and DQB1\*06:28, 06:31, 06:41, 06:44

<sup>c</sup> Could not rule out DPB1\*75:01, 79:01, 132:01, 164:01, 271:01, 36:801 and DPB1\*50:01, 57:01, 59:01, 68:01, 80:01, 108:01

<sup>d</sup> Could not rule out DPB1\*71:01 and DPB1\*105:01

were presented by either DR ( $n = 4$ ) or DQ ( $n = 1$ ). We found that the four DR-restricted samples did not share a common DR allele (Table III).

Interestingly, we observed that the two donors that had complete inhibition by DQ antibodies (in epitopes 7 and 8) were both homozygous (DQB1\*06 for epitope 7 and DQB1\*0301 in epitope 8). Overall, we found that several alleles and loci are involved in the presentation of the eight epitopes in PE38.

#### Can Multiple HLA Class II Molecules Be Used to Present Different Epitopes?

To examine if donors that responded to more than one epitope in PE38 use a single type of presenting molecule or different presentation molecules, we examined responses

from nine PBMC samples that had a robust response to more than one epitope. Table IV shows the epitopes and restriction results for each of the samples. Five of the individuals had all their responses presented exclusively by DR, three individuals had a mixed restriction pattern in which one epitope was presented by DR and another by DP, and two individuals had mixed presentation on DR and DQ. These data show that an individual can use more than one HLA molecule to present different epitopes.

#### Prediction of PE38 Epitopes Using DR, DP, and DQ Binding Algorithm

Because we found that DP and DQ molecules are involved in the presentation of PE38 peptides, we used the IEDB prediction algorithm to analyze the six most common

**Table IV.** T-Cell Restriction in Individuals with more than One Epitope

ID	Epitopes	Presenting molecule
D1	1, 2B	DR, DR
D2	1, 6	DR (DR+DQ)
D3	1, 3	DR, DP
D4	1, 3, 4	DR, DP, DR
D5	1, 6	DR, DR
D6	2A, 2B	DP, DP
D7	3, 5	DP, DR
D8	5, 8	DR, DR
D9	7, 8	DR, DR
P1	1, 2A, 6, 8	DR, DR, DR, DR
P2	2A, 5, 7, 8	DR, DR, DR, DQ

DP and DQ alleles in the worldwide population. These alleles were selected because they cover 95 and 82% of DP and DQ phenotype frequency in major ethnicities worldwide (34). Table V shows the number of alleles predicted to bind to experimentally determined PE38 peptides using a cutoff of 20% percentile rank. The 20% cutoff was chosen because it is frequently used for T-cell epitope prediction (35–37) and because we previously found in DR binding prediction analysis that this threshold accepts many peptides as positive and has a relatively low rate of false negative predictions (false negative rate was 30% and 55/111 peptides were considered positive in this threshold in DR predictions) (26). We define epitopes as peptides that are predicted to bind to a third of the reference alleles or more. Thus,  $\geq 9/27$  HLA class II alleles would be considered as an epitope by prediction. For analysis of DP alleles,  $\geq 2/6$  DP alleles were considered as an epitope, and similarly  $2/6$  alleles for DQ binding. These predictions are in italics in Table V.

We found that none of the HLA-DP reference alleles were predicted to bind the peptides in epitope region 3 (peptides 8–9). This was surprising because this epitope is restricted to DP and is a strong epitope. We considered the possibility that DP alleles may require a different cutoff. We found that peptide 9 would have been predicted as a binder with a cutoff of 47% and peptide 8 with a cutoff of 67%. These thresholds would consider 68 and 82 of the 111 peptides as positive binders.

Overall, inclusion of DP and DQ in the HLA class II analysis did not improve the accuracy of the overall prediction. Seven of the eight epitopes had at least one peptide predicted correctly in the analysis of 15 DR reference alleles; however, only six out of the eight epitopes were predicted in the analysis using 27 HLA class II alleles.

## DISCUSSION

The role of non-DR class II presentation in immunogenicity of vaccines and protein therapeutics has been undervalued in the past few years (8–16,38). To understand the relationship between T-cell epitopes, their presentation by HLA class II molecules, and their potential to be predicted based on commonly used *in silico* binding algorithm, we experimentally determined which HLA class II molecules

were responsible for their presentation. We found that DR restricted 65% of the responses and that DP and DQ restricted the other 35% of the responses. In addition, we found one important epitope that was only DP restricted. This epitope was undetected using DR, DQ, or DP high affinity binding predictions.

Our results indicate an important role for DP in the presentation of PE38 epitopes; 24% were DP restricted and 4% were DQ restricted. Non-DR-restricted T-cell epitopes in foreign and nonforeign proteins have been observed in several other proteins including influenza A Puerto Rico/8/34 matrix protein, Epstein-Barr virus (EBV)-encoded nuclear antigen 1, adenovirus hexon, dengue virus, streptococcal M12 protein, tetanus toxin, dimorphic fungus, and the NY-ESO-1 tumor antigen (39–45). Also the susceptibility to several diseases, including infectious diseases, is associated with DP- and DQ-restricted epitopes (46–49).

The identification of non-DR epitopes has a high translational value in the development of vaccines and for the therapy of allergy because HLA-DQ and HLA-DP subtypes are more conserved across populations than HLA-DR subtypes. For example, HLA-DPB\*0401 and HLA-DPB\*0402 alleles are present together in ~50% of the Caucasian population (50). Therefore, the role of DP epitopes in the immune response should always be taken into account. The distribution of restriction elements in our study (35% non-DR) is in agreement with Mellins *et al.* that found that approximately 40% of the T-cell proliferation responses to purified protein derivative (PPD) and tetanus toxoid were restricted by non-DR class II molecules (51).

We found that two of the donors that responded to epitope 3, which is entirely DP restricted, share the same DPB1 allele DPB1\*0201; however, two additional donors that did not respond to this epitope also share the same DPB1 allele (Table III). This finding indicates that the alleles for DP beta chain are not sufficient to determine whether a specific peptide will activate T cells. Other factors that may affect the restriction include the DPA chain and the availability of T cells. Similarly, not all donors with a specific DR allele respond to the same epitopes; a donor with a DR-restricted T-cell response to epitope 2B is homozygote to DRB1\*1501 allele (Table III). Based on the haplotype of the DR molecules involved in the response in conjunction with the result that the epitope is presented by DR, one would expect that all donors that have DRB1\*1501 will have a positive response to epitope 2B. However, this is not the case. Several donors with a DRB1\*1501 allele did not respond to epitope 2, including primed patients. This finding points out that HLA binding to DRB1 molecules is required but not sufficient to drive T-cell activation, for example DRB3/4/5 could be presenting the epitope as well.

We previously reported the identification and restriction of epitope 1 (27). Here, the restriction analysis for this epitope was repeated and included in the analysis of all eight epitopes in PE38. Minor changes were made in the methodology including stimulation with a 21 mer peptide (spanning peptides 13, 14, and 15) and not peptide 15 alone which is 15 mer. In addition, different PBMC samples were used for the most part because many of the PBMC samples that were used in 2012 were no longer available. In the restriction analysis in 2012, we found that 9/9 samples were restricted to DR. Similarly, the restriction

**Table V.** HLA Binding Prediction for DR, DP, and DQ PE38 Peptides

Epitope	Peptide ID	Experimental responses	Number of predicted alleles			HLA class II
			DR	DP	DQ	
1	13	6	<i>14</i>	<i>6</i>	<i>3</i>	<i>23</i>
	14	19	<i>14</i>	<i>6</i>	<i>5</i>	<i>25</i>
	15	22	<i>12</i>	<i>6</i>	<i>3</i>	<i>21</i>
2	74	3	<i>10</i>	<i>1</i>	<i>0</i>	<i>11</i>
	75	4	<i>10</i>	<i>1</i>	<i>2</i>	<i>13</i>
	76	9	<i>13</i>	<i>1</i>	<i>1</i>	<i>15</i>
	77	12	<i>13</i>	<i>1</i>	<i>0</i>	<i>14</i>
	78	11	<i>10</i>	<i>0</i>	<i>0</i>	<i>10</i>
3	8	10	0	0	0	0
	9	8	0	0	0	0
4	5	7	<i>4</i>	<i>2</i>	<i>0</i>	<i>6</i>
	6	8	<i>6</i>	<i>1</i>	<i>0</i>	<i>7</i>
5	67	7	<i>13</i>	<i>3</i>	<i>4</i>	<i>20</i>
	68	8	<i>9</i>	<i>1</i>	<i>4</i>	<i>14</i>
6	93	4	0	<i>1</i>	<i>0</i>	<i>1</i>
	94	5	<i>6</i>	<i>1</i>	<i>0</i>	<i>7</i>
	95	5	<i>10</i>	<i>4</i>	<i>2</i>	<i>16</i>
	96	2	<i>5</i>	<i>2</i>	<i>2</i>	<i>9</i>
7	51	4	<i>6</i>	<i>3</i>	<i>1</i>	<i>10</i>
	52	3	<i>3</i>	<i>1</i>	<i>0</i>	<i>4</i>
8	56	3	<i>10</i>	<i>5</i>	<i>6</i>	<i>21</i>
	57	4	<i>9</i>	<i>5</i>	<i>6</i>	<i>20</i>
	58	3	<i>13</i>	<i>4</i>	<i>6</i>	<i>23</i>
	59	1	<i>8</i>	<i>2</i>	<i>3</i>	<i>13</i>

Number of positive alleles for DR, DP, DQ, or all three (HLA class II) was predicted at a threshold of 20%. Values in italics represent  $\geq 33\%$  of alleles in each group (5/15 alleles for DR, 2/6 alleles for DP and DQ, and 9/27 alleles all class II alleles)

results shown in Fig. 2a show that 12/13 samples were completely restricted to DR and 1/13 was restricted by a combination of DR, DP, and DQ when DR had the strongest inhibition of the three. The difference in the restriction pattern that was observed in one sample can be explained by the longer peptide length used in the experiment. We extended the HLA typing of 12 of these samples to the DR3/4/5 in this study. We found that all 12 samples that responded to epitope 1 share a locus of DRB5\*101 or DRB5\*102. Furthermore, 11/12 samples also share DRB1\*15; this is not surprising because DRB5 and DRB1\*15 are genetically linked (52). We note that other factors in addition to the HLA binding can affect T-cell activation; these factors include a combination with variable alpha chains (for DP), T-cell availability, and regulatory functions (53).

To understand the mechanism of preferable DP presentation, we examined whether higher expression of DP molecules, which has been shown to be linked to a genetic polymorphism in the HLA-DPB1 gene regulatory region

(48), could affect the restriction of the epitopes in our *ex vivo* settings. We did not find a significant difference in DP, DR, or DQ membrane expression analyzing DP- and DR-restricted PBMC samples. We do not have sufficient DQ-restricted responses to make a similar determination regarding DQ.

The fact that epitope 3 was not predicted using the previous DR prediction analysis (26) is due to the fact that this epitope was 100% restricted to DP and because DR molecules have different binding cores and binding affinities from DP (54). However, it is surprising that predictions using reference DP alleles did not recognize this epitope as a binder. The threshold we used (of 20% percentile rank) is not a stringent one for epitope prediction and should support a low rate of false negative predictions.

Sidney *et al.* defined binding motifs for common DP alleles and also a super motif that is common for multiple DP binding cores (54). This motif includes a canonical P1-P6 main anchor spacing with aromatic/hydrophobic residues at

positions P1 and P6. This requirement for an aromatic/hydrophobic residue at position 6 is different from binding motifs for DRB molecules that are usually associated with a small or charged residue in this position (54). The common amino acid sequence between the overlapping, DP-restricted, peptides 8 and 9 is <sup>276</sup>RQPRGWEQLEQC<sup>287</sup>, which fits the canonical anchor of DPB1\*0101 with W281 at position P1 and Q286 at position P6 (54). Nevertheless, prediction of peptides 8 and 9 in DPB1\*0101 had a binding score of 50 and 49%, respectively. The fit of this peptide to the canonical anchor motif makes the inability of the DP algorithm to predict the binding of this epitope even more puzzling.

Because we found that 35% of the responses were non-DR, we expected that inclusion of DP and DQ alleles in the prediction query will improve the accuracy of the predicted epitopes, but that was not the case. We found that while seven out of the eight epitopes had at least one peptide predicted to bind in high affinity in the analysis of 15 DR alleles, only six out of the eight were predicted to bind in a high affinity in the analysis of 27 DR, DP, and DQ alleles. This discrepancy is due to the fact that the peptide needed to be predicted as strong binders in 33% of the alleles (9/27 alleles in DR, DP, and DQ analysis but only 5/15 in the DR analysis). It is possible that DP and DQ predictions need different and separate thresholds from DR for better accuracy.

Our observation that binding predictions of DP alleles missed an important epitope is supported by the findings of Paul *et al.* (55). They analyzed the accuracy of HLA binding predictions of 1151 peptides from 30 different proteins and found that inclusion of DP alleles in the *in silico* analysis resulted in a lower accuracy than when excluding them, although some of those experimental epitopes were indeed DP restricted. They suggested that the poor accuracy for DP predictions is a result of insufficient binding data available that drives the learning of the algorithm.

The second strongest epitope in PE38 (epitope 2) in amino acids PDARGRIRNGALLRVYVPRSSLPGFYR (peptides 74–78) is a complex epitope because T-cell stimulation was produced by more than three overlapping peptides. This indicates more than a single 9 mer core. Interestingly, most of the peptides at the N-terminus of the epitope (2B in peptides 74, 75, or 76) were DR restricted and most of the peptides at the C-terminus (2A, peptides 76, 77, or 78) were DP restricted. This is an example of both intralocus and interlocus cross reactivity, a feature that was documented in several antigenic epitopes including viruses (hepatitis B virus and hepatitis C virus), toxins (tetanus toxin), and allergen (Hay fever and German cockroach) (31,35,56–58). Additional examples of interloci cross reactivity were observed in this study in four donors that had co-presentation of the same epitope either by a combination of two loci (DR+DQ) or all three (DR, DP, DQ) (Table II). Intra- or interlocus cross-reactive epitopes may not always be due to the presence of a single promiscuous core region but rather may be caused by multiple overlapping core regions, each binding different HLA molecules (54). A similar phenomenon of epitope/motif clustering was reported by Berzofsky *et al.* who found that a few overlapping yet distanced peptides from the HIV envelope protein were recognized by 52–73% of 42 HLA diverse infected human donors and also by four MHC class II mice haplotypes (59).

From an immunogenicity-protein engineering perspective, the intra/interlocus cross reactivity by DR and DP of epitopes 2A and 2B poses a problem because it is difficult to identify point mutations that will prevent class II presentation of the epitope without affecting protein function. On the other hand, this same trait makes this epitope a valuable candidate to be included in a much needed vaccine design for *Pseudomonas aeruginosa* (60), because such epitopes will be useful to activate the immune system of a very diverse HLA class II population.

## CONCLUSION

In conjunction with recent discoveries of the important role that HLA-DP plays in multiple immune conditions, we provide an analysis of the relationship between T-cell epitopes in PE38, class II presentations of those epitopes, and a failure to predict an important T-cell epitope in an analysis that is based on class II HLA binding. We conclude that DP plays a critical role in the immunogenicity of PE38 and that widely used predictions that are based on HLA prediction algorithms are not sufficient to accurately predict some T-cell epitopes. We propose that inclusion of HLA-DP allele in prediction analysis will be useful and that DP alleles should be taken into consideration in population coverage cohorts. Furthermore, it is necessary to produce more DP binding datasets to try and increase the accuracy of DP binding algorithms. At the current time, an experimental approach such as that described by Sette and colleagues (31) is necessary to correctly identify T-cell epitopes.

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