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MHC allele-specific binding of a malaria peptide makes it become promiscuous on fitting a glycine residue into pocket 6

Luis Eduardo Vargas,[a,1] Carlos Alberto Parra,[a,b,*,1] Luz Mary Salazar,[a] Fanny Guzmán,[a] Martha Pinto,[a] and Manuel E. Patarroyo[a,b]

[a] *Fundación Instituto de Inmunología de Colombia (FIDIC), Carrera 50 No. 26-00. Bogotá, Colombia, USA*
[b] *Universidad Nacional de Colombia*

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Abstract

Peptide 1585 (EVLYLKPLAGVYRSLKKQLE) has a highly conserved amino-acid sequence located in the *Plasmodium falciparum* main merozoite surface protein (MSP-1) C-terminal region, required for merozoite entry into human erythrocytes and therefore represents a vaccine candidate for *P. falciparum* malaria. Original sequence-specific binding to five HLA DRB1* alleles (0101, 0102, 0401, 0701, and 1101) revealed this peptide's specific HLA DRB1*0102 allele binding. This peptide's allele-specific binding to HLA DRB1*0102 took on broader specificity for the DRB1*0101, -0401, and -1101 alleles when lysine was replaced by glycine in position 17 (peptide 5198: EVLYLKPLAGVYRSLKG$_{17}$QLE). Binding of the identified G$_{10}$VYRSLKGQLE$_{20}$ C-terminal register to these alleles suggests that peptide promiscuous binding relied on fitting Y$_{12}$, L$_{15}$, and G$_{17}$ into P-1, P-4, and P-6, respectively. The implications of the findings and the future of this synthetic vaccine candidate are discussed.

Keywords: *Plasmodium falciparum*; MSP-1 peptide; Pocket 6; Promiscuous peptide; Peptide binding assays; Binding motifs; Synthetic vaccines; HLA-DR molecules; Class II molecules; HLA-HLA DRB1*01 alleles

A T-cell response requires multiple engagement of the T-cell receptor on T-cells with major histocompatibility complex (MHC) molecules binding ligands on the surface of antigen presenting cells (APC). Whereas MHC class II molecules sample peptide antigens in professional APC endocyte pathway to present them to T CD4$^+$ helper cells [1], MHC class I molecules present peptides generated in the cytosol to T CD8$^+$ cytotoxic cells [1]. The key role of MHC molecules in restricting T-cell immune response relies on structural features of each allele's peptide binding region (PBR). Subtle differences in primary PBR amino-acid sequence (encoded in exon 2) dictate the fine specificity of MHC alleles for binding processed peptides [2].

Crystallographic analysis of MHC class II molecules showed that the peptide binding groove constituted by α1 and β1 chain domains is open at both ends [3]. This structural work led to the prediction that class II compared to class I molecules (whose binding groove is closed at both ends) [4] would bind longer peptides. Despite recent studies suggesting that some contact residues outside the peptide binding groove may contribute towards binding [5], a large body of evidence has indicated that a class II molecule binding motif is made up of nine residues all lying in the groove. Structural features of pockets P-1, P-4, P-6, and P-9, and to lesser extent P-7 [3], have been determined by both structural and functional studies of MHC class II molecules interacting with peptides in vitro [6]. The information gathered from all these studies has led to peptide binding motifs for different class II alleles being clarified [7].

Peptide-based vaccines represent an important alternative for microbe-based vaccines to be used in immune-prophylaxis of infectious diseases, due to simplified manufacturing low cost and their ability to elicit strong immune responses against whole microbes. The genetic

* Corresponding author. Fax: +57-1-481-52-69.
E-mail address: carlos_parra@fidic.org.co (C.A. Parra).
[1] These authors contributed equally to this work.

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restriction of the immune response has represented an important bottleneck for designing universal synthetic vaccines, due to the extreme polymorphism of MHC molecules. The ideal Th epitope would be "universal," meaning that they would be capable of interacting with a large number of class II molecules. The rules governing MHC–peptide binding have been identified through a combination of structure determination [3], library screening [7], and computational analysis [8]. The recent input of informatics to the field has enabled predicting allele $CD4^+$ T-cell epitopes (where direct structural or binding data are lacking) with encouraging results [8,9].

Both genetic restriction of the immune response to parasite antigens and antigenic variation have hampered identifying peptide epitopes to be used in a malaria vaccine, due to immune pressure biasing the immune response towards highly variable regions of parasite antigens [10]. Peptide 1585 (EVLYLKPLAGVYRSLK KQLE) has a highly conserved amino-acid sequence located in the *P. falciparum* merozoite surface protein (MSP-1) C-terminal region [11]. Other studies have proved that 1585 harbors a binding motif important for binding *P. falciparum* merozoites to human erythrocytes [12], to be a T-cell epitope in humans [11] and, when used as a vaccine this peptide's modified analogues have elicited high antibody titers that protected *Aotus* monkeys from challenge with a highly infective dose of *P. falciparum* [13]. Despite all these data suggesting that this peptide sequence deserves to be included as a subunit in a synthetic vaccine candidate specific for *P. falciparum* merozoite stage this peptide's specific binding to MHC molecules has yet to be determined.

In this work peptide 1585 specific binding to five HLA DRB1* alleles was explored by using purified molecules and peptides in in vitro competition assays. High specific HLA DRB1*0102 allele binding for this peptide was revealed when studying the original 1585 sequence specificity for five HLA DRB1* alleles. However, the original sequence's specific HLA DRB1*0102 binding became promiscuous binding to the other three alleles examined when a single lysine amino-acid was replaced by glycine in position 17 in the original sequence (peptide 5198: EVLYLKPLAGVYRSLKGQLE). The binding data from anchoring residues located towards the C-terminal in 5198 peptide suggest that G_{17} fitting into P-6 is required for P-1 and P-4 promiscuous binding to all four alleles.

Materials and methods

Peptides. Synthetic peptides included in this study were synthesized by the multiple-solid-phase technique, using *tert*-butoxycarbonyl (Boc) strategy as previously described [14]. They were then purified by reverse-phase HPLC on a C18 LiChrospher column (Merck, Germany). The products were assessed by analytical high performance liquid chromatography (HPLC) and mass spectrometry (MALDI-TOF). Biotinylated $HA_{306–318}$ (PKYVKQNTLKLAT), 1585 (EVLYLKPLA GVYRSLKKQLE), and Gly–Phe–Lys–$(Ala)_7$ [GFK$(A)_7$] indicator peptides, used for HLA binding and competition experiments, were synthesized by using biotin derivative sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford). This derivative incorporates a six carbon-spacer long chain to increase the distance between the biotin moiety and the peptide. Biotin was coupled by following the standard solid-phase peptide synthesis coupling procedure; its efficacy was evaluated by the Kaiser test and standard enzyme linked immunosorbent assay (ELISA).

HLA-DR molecule affinity purification. Human molecules were purified from DR1, WT100BIS (DRB1*0101); DR1, LWAGS (DRB1*0102); DR4, BSM (DRB1*0401); DR7, EKR (DRB1*0701); and DR11 BM21 (DRB1*1101) homozygous EBV-B cell lysates by affinity chromatography [15] using anti-HLA-DR mAb L-243 cross-linked to protein A–Sepharose CL-4B (Amersham–Pharmacia Biotech AB) as affinity support. Cells were lysed at 10^8 cells ml^{-1} density on ice for 60 min in 1% (v/v) Nonidet P-40, 25 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg ml^{-1} of each of the following: soybean trypsin inhibitor, antipain, pepstatin, leupeptin, and chymostatin in 0.05 M sodium phosphate buffer, 0.15 M NaCl, pH 7.5. Nucleic acids and debris were cleared from lysates by spinning at 27,000*g* for 30 min. After 0.2 volumes of 5% sodium deoxycholate (DOC) was added to the supernatant and mixed for 10 min, the lysate was centrifuged at 100,000*g* for 2 h and subsequently filtered through a 0.45 μm membrane. Lysates were first passed over a Sepharose CL-4B pre-column and subsequently over the A Sepharose-mAb L-243 protein column for class II molecule affinity purification. The affinity column was then washed with: (i) 20 column volumes of 50 mM Tris–HCl, pH 8, 0.15 M NaCl, 0.5% NP-40, and 0.5% DOC; (ii) 5 column volumes of 50 mM Tris–HCl, pH 9, 0.5 M NaCl, 0.5% NP-40, and 0.5% DOC; and (iii) 5 column volumes of 2 mM Tris–HCl, pH 8, 1% octyl-β-D glucopyranoside (Sigma, St. Louis). HLA-DR molecules were eluted with 0.05 M diethylamine in 0.15 M NaCl, pH 11.5, containing 1% octyl-β-D glucopyranoside, 1 mM EDTA. The eluate was immediately neutralized with 1/20 volume of 1 M Tris/HCl, pH 6.8. Preparations were kept in aliquots at −70 °C until use.

Direct binding assay. Peptide HLA-DR molecule binding was analyzed using methodology described by other authors [16,17] and modified by ourselves, as well as by ELISA-based assay using biotinylated-labeled peptide 1585 (EVLYLKPLAGVYRSLKKQLE) for all HLA-DR assays and biotinylated-labeled hemagglutinin $HA_{306–318}$ (PKYVKQNTLKLAT) peptide for DRB1*0101. Both peptides were +N-terminally labeled with sulfo-NHS-LC-Biotin (Pierce Chemical, Rockford, IL). Purified HLA-DR molecules were diluted in freshly prepared binding buffer containing 100 mM citrate/phosphate buffer (pH 7), 0.15 mM NaCl, 4 mM EDTA, 4% NP-40, 4 mM PMSF, and 40 μg ml^{-1} of each of the following: soybean trypsin inhibitor, antipain, leupeptin, and chymostatin. Ninety microliters of HLA-DR molecules (0.1 μM) was added to Eppendorf tubes together with 30 μl biotinylated-labeled peptide (5 μM) in DMSO:PBS (1:4). After 24 h of incubation at room temperature, the peptide/class II complexes were transferred to ELISA well plates (Nunc-Immuno Modules Maxisorp Loose Brand product, Denmark), which had been coated with a 10 μg ml^{-1} anti-HLA-DR mAb L-243 solution and subsequently blocked with PBS containing 5% bovine serum albumin. After 2 h incubation at room temperature, plates were washed with PBS, 0.05% Tween 20. After incubation with alkaline phosphatase-labeled streptavidin (Vector Laboratories, Burlingame, CA), labeled peptide/HLA-DR complexes were revealed with 4-nitrophenylphosphate substrate (Kirkegaard and Perry Laboratories. Maryland, USA). A Titertek MC Multiscan ELISA reader (Labsystems, Franklin, Mass) with 405 nm filter was used for determining peptide binding to HLA-DR molecules by measuring the optical densities (OD). The amount of peptide bound was normalized to the maximum observed binding.

The K_D values for the HLA DRB1*0102 and DRB1*0101 biotinylated peptide 1585 and $HA_{306–318}$ complex were determined by

binding assay, as described above, with varying concentrations of peptide (10^{-9}–100 μM). K_D was determined by fitting the values of the curve into a quadratic binding equation.

Relative binding affinities were determined for other peptides by competition assay. IC_{50} values were calculated and converted to K_D values by using the equation, $K_D = (IC_{50})/(1 + ([\text{biotinylated peptide}]/K_{D,\text{ biotinylated peptide}}))$.

Peptide-binding competition assays. Peptide binding competition assays were conducted to measure unlabeled peptide ability to compete with biotinylated indicator peptides for binding to purified HLA-DR molecules. The experiments were performed by using unlabeled mixed peptide excess, as described for the direct binding assay. A Titertek MC Multiscan ELISA reader (Labsystems, Franklin, Mass) with 405 nm filter was used for determining peptide binding to HLA-DR molecules by measuring the optical densities (OD) in the presence versus the absence of competitor peptide. Inhibition was calculated as a percentage by using the formula: $100 \times [1 - \Delta OD$ in the presence of competitor/ΔOD in the absence of competitor peptide]. According to this assay, a good competitor was a peptide which was capable of inhibiting more than 50% of the indicator peptide's binding to the HLA molecule being tested.

Dissociation assays. The biotinylated complex was prepared as described in the direct binding assay section. Dishes were left to stand for 2 h and then washed with fresh 0.05% Tween–PBS. Aliquot samples were taken at determined periods of time (each 6 h) and put into ELISA well plates (Nunc-Immuno Modules Maxisorp Loose Brand product, Denmark) for different periods of time (72, 48, 36, 24, 12, and 0 h). After incubation with alkaline phosphatase-labeled streptavidin (Vector Laboratories, Burlingame, CA), labeled peptide/HLA-DR complexes were revealed with 4-nitrophenylphosphate substrate (Kirkegaard and Perry Laboratories. Maryland, USA). Peptide binding to HLA-DR molecules was calculated as described above.

Molecular modeling. The crystal structure of the HLA DRB1*0101 molecule complexed to the $HA_{306–318}$ peptide (PDB: 1dlh [3]) was used as a template for further modeling. Amino-acids were replaced by using Insight II (2000) Biopolymer Group software (Accelrys Software, San Diego, CA) run on an Indigo 2 Station (Silicon Graphics). The Docking program (Accelrys) facilitates calculating the non-bond energy between two molecules. Determining intermolecular energy (IM) for DRB1*0101 with HA, 5198 and 1585 peptides, and putative residues in P-1, P-4, P-6, and P-9 gave the explicit van der Waals energy value for evaluating the probability of MHC–peptide complex formation.

Results and discussion

*Peptide 1585 binds with high specificity to the HLA DRB1*0102 allele*

A direct binding assay was used to study peptide 1585 binding capacity to HLA DRB1* alleles (HLA DRB1*0101, HLA DRB1*0102, HLA DRB1*0401, HLA DRB1*0701, and HLA DRB1*1101) using fixed concentrations of HLA DRB1* molecules with 17-fold excess of labeled peptide (0.1 μM HLA DRB1* allele and 5 μM 1585 biotinylated peptide). It was decided to characterize the stability of the complex formed between this peptide and the HLA DRB1*0102 molecule since, throughout different experiments, 1585 native sequence exhibited allele-specific binding to this allele molecule but not to HLA DRB1*0101, -0401, -0701 or -1101 alleles (Fig. 1A). The half-time stability of the complex formed was determined in association and dissociation profiles, monitored for up to 50 h, in which the quantity of bound peptide was normalized regarding maximum peptide binding obtained. Fig. 1A shows that maximum peptide binding to HLA DRB1*0102 was reached in around 15 h (half time formation and dissociation rate of about 4 and 18 h, respectively). The complex's binding affinity was determined by using a fixed concentration of HLA DRB1*0102 molecule mixed with varying concentrations of 1585 biotinylated peptide in a direct binding assay. The K_D value of 300 nM (Table 1) was determined by fitting the values of the curve (Fig. 1B) into a quadratic binding equation, as described in Materials and methods.

*Determining 1585 peptide anchoring residues for HLA DRB1*0102*

The experiments described above showed an intriguing preferential binding of peptide 1585 to HLA DRB1*0102 allele. These results prompted us to conduct experiments leading to identifying the anchor amino-acid residues responsible for 1585 specific binding to this allele. The binding competition assays between biotinylated 1585 peptide and a set of unlabeled single substitution glycine analogues derived from 1585 were used to determine the role of each residue within the sequence in this peptide's interaction with the HLA DRB1*0102 molecule (Table 1). The results obtained in these assays allowed key residues L_8, V_{11}, Y_{12}, L_{15}, and L_{19} to become identified (see 1585 residues signaled with black arrows in Table 1). High IC_{50} and K_D values seen for modified analogues 5190, 5193, and 5196 (>33 μM

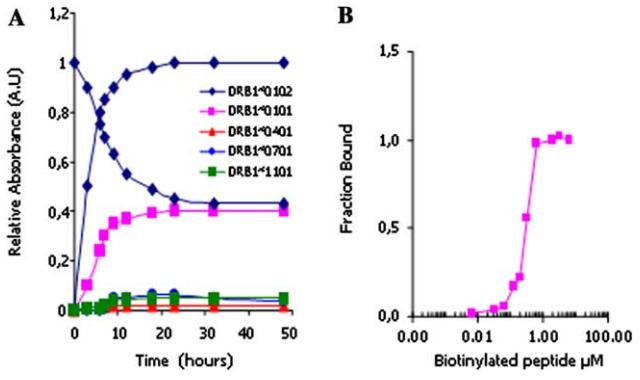

Fig. 1. (A) Allele-specific binding of 1585 peptide to HLA DRB1*0102. 1585 peptide DRB1* allele association and dissociation kinetics were studied by using 0.1 μM HLA DRB1* allele incubated with 5 μM of 1585 biotinylated peptide for 50 h as described in Materials and methods. The figure shows a representative experiment from three performed. (B) Biotinylated 1585 peptide binding affinity for the HLA DRB1*0101 molecule. A representative direct binding assay for biotinylated 1585 peptide, binding being detected by sandwich ELISA. K_D values were determined by substituting in the quadratic binding equation, as described in Materials and methods. A representative experiment is shown from three performed.

Table 1
Binding profile of 1585 glycine analogous to HLA DRB1* alleles

Peptide	Amino-acid sequence	HLA DRB1* 0102 %	0102 IC_{50} (μM)	0102 K_D (nM)	0101 %	0101 IC_{50} (μM)	0101 K_D (nM)	0401 %	1101 %	(c) %
	E V L Y L K P L A G V Y R S L K K Q L E		(a)			(b)				
5183	G -	94±1	-	-	7±4	-	-	7±3	62±4	72±1
5184	- G -	82±2	-	-	4±2	-	-	7±5	55±5	60±6
5185	- - G -	86±1	-	-	7±3	-	-	4±8	41±5	41±5
5186	- - - G -	70±3	-	-	4±4	-	-	13±2	42±3	42±4
5187	- - - - G - - - - - - - - - - - - - - - - - - -	78±2	-	-	2±3	-	-	−7±2	45±7	45±4
5188	- - - - - G - - - - - - - - - - - - - - - - - -	70±2	-	-	0±2	-	-	5±2	52±6	52±2
5189	- - - - - - G - - - - - - - - - - - - - - - - -	78±1	-	-	5±2	-	-	3±4	40±6	70±8
5190	- - - - - - - G - - - - - - - - - - - - - - - -	40±2	>33	>3x10^4	13±3	-	-	19±4	45±1	75±5
5191	- - - - - - - - G - - - - - - - - - - - - - - -	64±1	-	-	2±5	-	-	9±3	35±3	65±2
1585	- - - - - - - - - G - - - - - - - - - - - - - -	56±3	0.32	300	2±7	117.30	4233	11±5	23±1	27±3
5192	- - - - - - - - - - G - - - - - - - - - - - - -	43±2	-	-	0±4	-	-	9±2	40±3	62±4
5193	- - - - - - - - - - - G - - - - - - - - - - - -	24±2	>33	>3x10^4	0±4	-	-	11±4	28±9	58±7
5194	- - - - - - - - - - - - G - - - - - - - - - - -	53±1	-	-	39±5	-	-	29±3	64±2	84±3
5195	- - - - - - - - - - - - - G - - - - - - - - - -	70±3	-	-	2±5	-	-	11±3	50±6	56±3
5196	- - - - - - - - - - - - - - G - - - - - - - - -	20±2	>33	>3x10^4	2±1	-	-	2±8	38±4	48±9
5197	- - - - - - - - - - - - - - - G - - - - - - - -	48±2	-	-	0±2	-	-	1±3	51±3	81±5
5198	- - - - - - - - - - - - - - - - G - - - - - - -	**67±1**	-	-	**63±2**	4.20	152	**61±2**	**67±7**	**89±2**
5199	- - - - - - - - - - - - - - - - - G - - - - - -	67±1	-	-	5±5	-	-	−7±2	51±4	71±5
5200	- - - - - - - - - - - - - - - - - - G - - - - -	28±1	-	-	2±3	-	-	5±1	60±8	87±6
5201	- - - - - - - - - - - - - - - - - - - G - - - -	84±3	0.05	30	4±4	-	-	8±2	74±2	87±4
27714	E V L Y L K P L A G V Y	36±1	-	-	36±5	-	-	-	-	-
28097	E V L Y L K P L A G V Y R S	-	-	-	4±4	-	-	-	-	-
23483	Y L K P L A G V Y R S L K K	85±2	-	-	3±4	-	-	-	-	-
28095	P L A G V Y R S L K K Q L E	74±3	-	-	21±2	-	-	-	-	-
HA	P K Y V K Q N T L K L A T	18±1	-	-	86±2	0.2	15	-	-	-

Value of relative binding of peptide analyzed to a given allele. Underlined values correspond to critical residues for 1585 peptide binding to HLA DRB1*0102 (signaled by arrowheads). Values in bold correspond to those of 5198 analogue ($K_{17}G$); (-) not done. Biotinylated peptides used for binding assays were (a) 1585 (EVLYLKPLAGVYRSLKKQLE); (b) HA: $HA_{306–318}$ (PKYVKQNTLKLAT); and (c) GFKAA7 (GFKAAAAAAA).

IC_{50} and $>3 \times 10^4$ nM K_D), compared with those (0.32 μM IC_{50} and 300 nM K_D) from the native sequence, showed that these residues are important in mediating HLA DRB1*0102 molecule interaction (Table 1). Since a classical binding motif could not be established by using the full-length peptide, three short overlapped peptides were made: 27714 (EVLYLKPLAGVY), representing the amino terminal region; 23483 (YLKPLAG VYRSLKK), covering the central core; and 28095 (PLAGVYRSLKKQLE), located at the 1585 sequence C-terminal region. When these sequences were evaluated for their binding capacity to HLA DRB1*0102, the results showed that whereas the central and carboxy terminal cores play an important role in binding to HLA DRB1*0102, the amino terminal region did not bind to HLA DRB1*0102. There was 85% and 74% binding efficacy for peptides 23483 and 28095, respectively, compared to 36% for peptide 27714 (Table 1). The results of 1585 binding to HLA DRB1*0102 (suggesting the combined effect of five residues contributing towards the stability of the complex thus formed) together with results with truncated peptides 23483 and 28095 (with L_{15} and L_{19} contributing separately) and biphasic kinetics of dissociation led us to argue that native 1585 sequence becomes anchored to HLA DRB1*0102, probably through the use of more than one binding motif.

*Peptide 1585 binding to HLA DRB1*0101*

Fig. 1 shows that peptide 1585 bound well to HLA DRB1*0102 but not to HLA DRB1*0101. Peptide 1585 binding well to the HLA DRB1*0102 molecule but not to the HLA DRB1*0101 allele might have reflected the dimorphism effect between these two alleles at positions β85 and β86 [18]. Experiments with 1585 glycine analogue binding assays using HLA DRB1*0101 were conducted to evaluate the impact of dimorphism between HLA DRB1*0101 and DRB1*0102. The HLA DRB1*0101 allele's 1585 glycine analogue binding profile showed that all analogues excepting one (peptide 5198) bound poorly to this allele. At 150-fold excess, 5198 analogue ($K_{17}G$) displaced 63% of bound biotinylated HA peptide from the HLA DRB1*0101 molecule whereas HA itself displaced 86%. Peptide 5198 HLA DRB1*0101 allele relative binding values (Table 1) confirmed this peptide's high relative binding to the DR1*0101 molecule (4.2 μM IC_{50} and 152 nM K_D), one

log lower affinity than that exhibited by the HA peptide itself (0.4 μM IC_{50} and 15 nM K_D) (Table 1). Experiments with the 5198 peptide and truncated peptides thus confirmed that the 1585 peptide native sequence did not bind to the HLA DRB1*0101 molecule, suggesting esteric hindrance of K_{17} in its interaction with this molecule (Table 1).

Based on results with peptide 5198, it was decided to subject this peptide to glycine scanning and assess 5198 glycine analogue DRB1*0101 binding ability (Table 2). Peptides 28086 ($Y_{12}G$) and 28089 ($L_{15}G$) with relative binding affinity ($IC_{50} > 120\,\mu M$ and $K_D > 4500\,nM$) highlighted the importance of Y_{12} and L_{15} as HLA DRB1*0101 anchorage residues (Table 2) suggesting a putative binding motif (P-1, P-4, and P-6) in which Y_{12}, L_{15}, and G_{17} were, respectively, required for binding this peptide to the HLA DRB1*0101 molecule (Table 2). This motif implied that G_{17} and E_{20} in peptide 5198 would bind to P-6 and P-9, respectively. Previous studies have shown that HLA DRB1*0101 pocket 9 preferentially anchors relatively hydrophobic and aromatic residues (L, A, I, V, N, F, Y, and W) but not polar residues [19]. Since charged glutamic acid in pocket 9 did not destabilize the complex formed would suggest that either E_{20} does not play an important role in this peptide's interaction (see peptide 28093, 74% relative binding in Table 1) or alternatively this peptide's interaction with the HLA DRB1*0101 molecule is sufficiently stabilized for residues anchored to P-1, P-4, and P-6 annulling the negative effect introduced by residue E_{20} at P-9.

The 5198 peptide C-terminal motif binds to HLA DRB1*0101, in spite of having a suitable P-1, P-4, and P-9 motif, appropriate fitting of peptide could only be seen until optimum combination with P-6 was achieved, suggesting that K_{17} generates a destabilizing interaction when it interacts with HLA DRB1*0101 P-6 revealing the importance of pocket 6 in peptide 5198 interaction with this molecule. This result was unexpected in light of previous studies showing that for a restricted HLA DRB1*0101 peptide [20,21] it is sufficient to have an appropriate residue in P-1 (Y_{12} in the 5198 peptide). Other failures to this rule were peptide 27714 (E_1–Y_{12}), having the previously reported V_2, L_5, and P_7 motif fitting into P-1, P-4, and P-6, respectively, that did not bind to the HLA DRB1*0101 molecule (Table 1), suggesting that G_{10} did not provide a good fit into P-9, or 28097 (E_1–S_{14}), having Y_4, A_9, and Y_{12} fitting into P-1, P-6, and P-9, respectively, that neither bound (Table 1), suggesting P_7 in P-4 impaired binding. All these cases suggest that binding to DRB1*0101 was impaired despite having proper residues that would fit into three out of four pockets (P-1 being one of them).

This K_{17} destabilizing interaction in P-6 could be demonstrated by 1585 docking C-terminal sequence

Table 2
Binding efficacy of glycine analogues of 5198 peptide for DRB1* alleles

Peptide	Amino-acid sequence (P-1 ▼ P-4 ▼ P-6 ▼ P-9 ▼)	HLA DRB1* 0101 %	0101 IC_{50} (μM)	0101 K_D (nM)	0401 %	1101 % (a)	1101 % (b)
5198	**E V L Y L K P L A G V Y R S L K G Q L E**	64±3	4.20	152	64±7	85±4	95±12
28076	G - - - - - - - - - - - - - - - G - - -	63±3	-	-	64±2	84±5	96±7
28077	- G - - - - - - - - - - - - - - G - - -	64±2	-	-	59±4	70±5	81±7
28078	- - G - - - - - - - - - - - - - G - - -	60±5	-	-	66±5	63±7	73±2
28079	- - - G - - - - - - - - - - - - G - - -	60±2	-	-	65±6	60±7	73±3
28080	- - - - G - - - - - - - - - - - G - - -	57±3	-	-	73±2	50±3	61±2
28081	- - - - - G - - - - - - - - - - G - - -	57±4	-	-	67±1	75±5	82±3
28082	- - - - - - G - - - - - - - - - G - - -	63±5	-	-	70±5	81±6	94±2
28083	- - - - - - - G - - - - - - - - G - - -	52±1	-	-	74±4	53±4	66±4
28084	- - - - - - - - G - - - - - - - G - - -	68±3	-	-	72±3	62±3	78±3
28085	- - - - - - - - - G - - - - - - G - - -	55±4	-	-	35±2	49±1	55±5
28086	- - - - - - - - - - G - - - - - G - - -		>120	>4500	**28±5**	**24±5**	
28087	- - - - - - - - - - - G - - - - G - - -	74±5	-	-	58±3	80±6	93±2
28088	- - - - - - - - - - - - G - - - G - - -	45±8	-	-	47±5	51±8	60±4
28089	- - - - - - - - - - - - - G - G - G - - -	**–2±3**	>120	>4500	**38±5**	**40±6**	**45±6**
28090	- - - - - - - - - - - - - - G G - - - -	48±4	-	-	68±4	69±2	80±3
28091	- - - - - - - - - - - - - - - G G - - -	58±5	-	-	57±3	73±4	89±5
28092	- - - - - - - - - - - - - - - G - G - -	43±6	-	-	52±4	70±3	83±4
28093	- - - - - - - - - - - - - - - G - - G -	74±2	3.9	143	95±4	82±8	92±4
HA	P K Y V K Q N T L K L A T	86±2	0.2	15	-	-	-

Value of relative binding of peptide analyzed to a given allele. Values in bold correspond to those critical residues experimentally determined for P-1; P-4; P-6; and P-9 (signaled with arrowheads); (-) not done. Biotinylated peptides used for binding assays were (a) HA: $HA_{306–318}$ (PKYVKQNTLKLAT) and (b) GFKAA7 (GFKAAAAAAA).

(G_{10}VYRSLKKQLE$_{20}$) on HLA DRB1*0101 molecule. The predicted intermolecular energy (IM in Table 3) of this peptide–MHC interaction when compared to that of the $HA_{306–318}$ peptide (1.11 e^{+10} and 48.94 kcal mol^{-1}, respectively) suggests inappropriate interaction of HLA DRB1*0101 with this sequence. This is probably a consequence of K_{17} inappropriate interaction in P-6 (1.12 e^{+10} kcal mol^{-1}) (Table 3). In contrast, when K_{17}G substitution in peptide 5198 was analyzed, this peptide sequence's interaction with HLA DRB1*0101 substantially improved (IM value of 108,330 kcal mol^{-1}) as a consequence of Y_{12} and G_{17} proper fitting into P-1 and P-6 (−24.74 and −6.47 kcal mol^{-1}), respectively, very close to the values given by P-1 and P-6 residues within the original $HA_{306–318}$ sequence used in this analysis as reference (Table 3). Such analysis suggests that poor K_{17} contact in P-6 influences poor Y_{12} contact to fit into P-1. Hence, when evaluating whether a peptide binds to a class II molecule the global sequence and the possible destabilizing role of each one of the residues must be evaluated. These results thus suggest that, although binding motifs are useful, they do not provide sufficient conditions for tight binding. On the other hand, dominant negative interactions difficult to predict and usually underestimated by predictive algorithms seem to be a crucial parameter determining peptide–MHC interactions.

*Substitution of K_{17} for G prompted 1585 binding to HLA DRB1*0401 and HLA DRB1*1101 alleles*

Peptide 1585 glycine analogue binding capacity to HLA DRB1*0401 and HLA DRB1*1101 molecules was evaluated in competition assays using purified HLA DRB1*0401 and -1101 molecules, biotinylated allele-specific peptides (HA and $GFKA_7$ peptides), and a 150-fold excess of each unlabeled 1585 glycine analogue. Similar to what was found for HLA DRB1*0101, poor HLA DRB1*0401 binding capacity was observed in all glycine analogues except for the 5198 analogue that exhibited 61% relative binding (Table 1). In contrast, although K_{17}G remarkably improved 1585 binding capacity to HLA DRB1*1101 when biotinylated HA or $GFKA_7$ were used as reference peptides (Table 1), this analogue being the one with the highest relative binding for this allele in both cases (Table 1), several 1585 glycine analogues competed with biotinylated HA or $GFKA_7$ for binding to DRB1*1101 molecule (Table 1).

*Binding of 5198 peptide to DRB1*0101, -0401, and -1101 alleles is achieved by anchoring Y_{12} and L_{15} in P-1 and P-4, respectively*

Similar experiments using peptide 5198 glycine analogues with the HLA DRB1*0101 molecule were performed with DRB1*0401 and HLA DRB1*1101 molecules to assess which critical residues in 5198 were required for its binding to these two molecules (Table 2). Notably, data presented in Table 2 suggest that the uncovered anchoring residues used by the 5198 peptide to bind HLA DRB1*0101 (with Y_{12}, L_{15}, and G_{17} fitting into P-1, P-4, and P-6, respectively) are also required for binding this peptide to the HLA DRB1*0401 and -1101 alleles (Table 2). The mapping of 5198 peptide binding residues to HLA DRB1*0101, -0401, and -1101 alleles were assessed by using biotinylated HA and $GFKA_7$ reference peptides with similar results (Table 2).

Hence, the key role of pocket 6 in promoting 5198 sequence interaction with HLA DRB1*0101 leading to P-1 and P-4 exercising a suitable function was also shown by two additional alleles (HLA DRB1*0401 and 1101). Replacing K_{17} by Gly in 1585 made this peptide become suitable for binding to HLA DRB1*0101, -0401, and -1101 alleles; this prompted further binding assays with all three alleles using peptide analogues where K_{17} was replaced by different amino-acids. When 1585 K_{17} was replaced by different amino-acids, the result showed that voluminous residues, such as tyrosine and charged polar residues (be they acidic or basic), exercised a similar effect to that of lysine in this position, introducing a destabilizing interaction (Table 4). These experiments showed K_{17} substitutes' variable binding capacity to different alleles (S and P for HLA DRB1*0101; N and Q for -1101; and N for both -0401 and -1101). Only glycine in P-6 was capable of promoting this peptide's promiscuous binding to all three

Table 3
Docking of 5198 C-terminal register into DRB1*0101

Sequence	HLA DRB1*0101				
	IM[a]	P-1	P-4	P-6	P-9
HA (PK**Y**VK**Q**N**T**LK**L**AT)	48.94	−24.74[b]	−6.94	6.06	6.60
1585 (GV**YRSLKK**QL**E**)	1.11 e^{+10}	10.83	60950.90	1.12 e^{+10}	25.56
5198 (GV**YRSLKG**QL**E**)	108,330	−24.74	60961.10	−6.47	25.50

IM: intermolecular energy. DRB1*0101 pockets 1, 4, 6, and 9 are referred to as P-1, P-4, P-6, and P-9. Amino-acids shown in bold within peptide sequences correspond to residues modeled in DRB1*0101 allele P-1, P-4, P-6, and P-9.

[a] Values are given in kcal mol^{-1}.

[b] van der Waals forces.

Table 4
Binding efficacy for HLA DRBl* alleles of 1585 K_{17} peptide analogue

Peptide	P-6	% Binding to HLA DRBl*		
		0101	0401	1101
5198	**G**	**63 ± 6**	**62 ± 2**	**70 ± 8**
1585	K	2 ± 4	11 ± 5	23 ± 1
23919	D	−8 ± 7	19 ± 3	45 ± 8
23939	N	36 ± 5	**52 ± 4**	**68 ± 6**
23958	E	−6 ± 6	−13 ± 5	46 ± 3
23977	Q	41 ± 1	−3 ± 4	**55 ± 1**
23995	S	**57 ± 3**[a]	41 ± 9	35 ± 3
24031	P	**51 ± 3**	3 ± 4	36 ± 4
24049	Y	3 ± 5	−2 ± 6	16 ± 3
13709	A	41 ± 5	40 ± 5	10 ± 2

[a] Values shown in bold correspond to residues providing efficient binding to a given allele.

alleles (Table 4). Both the important role of P-6, as observed in these experiments, and the use of glycine as a proper anchor residue in P-6 for all three alleles are surprising. This is because a crucial role of P-6 in determining peptide binding has so far been underestimated and because anchor residues in P-6 have so far been described as being rather allele-specific [22].

This paper has explored the HLA DR binding specificity of highly conserved peptide 1585 by using in vitro binding assays. Evidence has been presented here for this synthetic malaria vaccine candidate's highly conserved MSP-1 sequence's tight binding capacity to several HLA alleles, which was achieved by changing a single amino-acid within the *P. falciparum* 1585 20-mer sequence.

Positive correlation between the affinity of peptide tightly bound to MHC molecules and in vivo immunogenicity for generating potent T-cell responses has been described in a number of experimental murine and human studies [23,24]. Identifying peptide epitope variants strengthen MHC–peptide complex stability (in vivo and in vitro) is thought to enhance the potency of intrinsically weak immunogenic peptides for activating and amplifying T cells. This concept, originally described in a murine $CD4^+$ T-cell model using HIV peptides [23], has been recently extended to a variety of viral [25,26] and tumor [27–29] immunological systems. Promiscuous 1585 peptide binding was prompted by engineering the peptide in the C-terminal region without distorting this peptide's putative erythrocyte binding motif located towards the N-terminal region (L_5, K_6, P_7, A_9, and S_{14}) ([13] and unpublished data). Eliciting *P. falciparum* antibody against peptide sequences involved in parasite invasion of erythrocytes might be a suitable pathway for identifying potent malaria vaccine candidates [30,31]. The DR binding site engineered in the 5198 peptide might provide the T-cell help required by B-cells to enhance antibody response blocking the infection of erythrocytes by merozoites. The fact that parasites do possess the native rather than the mutated sequence peptide might lead to it being thought that T-cell responses being elicited by altered ligands would be useless as vaccine. However, potent immune responses against native peptide sequences can be elicited by using peptides mutated for enhancing native MHC sequence binding capacity ([32] and references therein), suggesting that this may not be an important concern.

Furthermore, although the wild type 1585 peptide might bind weakly as shown by our results it nonetheless binds strongly enough to be recognized by immune T-cells in infected individuals as has been shown by others [11]. Although the immunogenicity of weak MHC binding peptides has been well documented in some autoimmune diseases [33], the relevance of T-cells that respond to peptides loosely bound by MHC molecules in infectious diseases is less clear. Boosting these T-cells primed during natural infection must become an important goal for any vaccine candidate. A tumor antigen mutated to enhance peptide binding to a MHC class I molecule enhanced the precursor frequency, the cytotoxic and proliferative activities of T-cells primed with the cognate peptide ligand [34]. Furthermore, the molecular and functional analysis of TCRs from CTL lines specific for both native and mutated peptide, respectively, revealed predominant use of the same Vα and Bβ chains and recognition of identical T-cell contact residues in native peptide [34]. These results together suggest that vaccination with a 1585 peptide mutated for improved MHC anchoring (i.e., peptide 5198) whilst boosting T-cell responses specific for the mutated peptide will perhaps maintain the integrity of TCR recognition for the naturally expressed epitope in the parasite. The same strategy used here with peptide 1585 could be followed to improve the immunogenicity of other peptides within highly conserved regions of *P. falciparum* proteins essential for erythrocyte binding that have been mapped in our laboratory and which are being currently studied as suitable malarial vaccine candidates [12,35–38].

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