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# Electrostatic potential as a tool to understand interactions between malaria vaccine candidate peptides and MHC II molecules

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ABSTRACT

One of the most important problems in vaccine development consists in understanding receptor–ligand interactions between Class II Major Histocompatibility Complex molecules (MHC II) and antigenic peptides involved in inducing an appropriate immune response. In this study, we used X-ray crystallography structural data provided by the HLA-DRβ1*0301–CLIP peptide interaction to compare native non-immunogenic and specifically-modified immunogenic peptides derived from the malarial SALSA protein, by analyzing molecular electrostatic potential surfaces on the most important regions of the peptide binding groove (Pockets 1, 4, 6 and 9). Important differences were found on the electrostatic potential induced by these peptides, particularly in MHC II conserved residues: Qα9, Sα53, Nα62, Nα69, Yβ30, Yβ60, Wβ61, Qβ70, Kβ71 and Vβ86, the same ones involved in establishing hydrogen bonds between Class II molecule-peptide and the recognition by T cell receptor, it correlating well with the change in their immunological properties.

The results clearly suggest that modifications done on the electrostatic potential of these amino acids could favor the induction of different immune responses and therefore, their identification could allow modifying peptides *a priori* and *in silico*, so as to render them into immunogenic and protection-inducers and hence suitable components of a chemically-synthesized, multi-antigenic, minimal subunit based vaccine.



## 1. Introduction

The development of computational chemistry, together with a deeper understanding on the physical and chemical laws governing the properties of molecules, is one of the most promising fields to comprehend the molecular interactions, in order to reach a logical and rational methodology for immunoprophylactic and chemotherapeutic methods to help in the control of infectious diseases that afflict 2/3 of population worldwide and kill 17 million people annually [1].

As a model for studying this type of interactions, we used the synthetic peptide 20608 (named according to our institute's sequential numbering system) derived from the sporozoite and liver stage antigen (SALSA), a 70 kDa protein expressed on the surface of *Plasmodium falciparum* malaria sporozoites and liver stage during the parasite's development inside the hepatic cells [2], representing a candidate to be included as a component of an effective vaccine against malaria [3].

By the use of a highly robust, specific and sensitive methodology suitable for the identification of receptor–ligand interactions between the parasite binding amino acid sequences of molecules involved in invasion of the host's cells, peptide 20608 was chosen due to its high binding capacity to Hep-G2 liver cells and its critical binding amino acids to hepatocytes were identified by a glycine scanning methodology [4].

Since these native conserved High Activity Binding Peptides (HABPs) are non-antigenic and non-immunogenic, as many other sporozoite [5] and merozoite protein derived HABPs [3], SALSA native HABP, 20608 (IWSA**E**KKDEK**EAS**EQG**EESH**), was modified in its critical liver cell binding residues (here shown in bold) replacing them for others having similar mass, surface and volume but opposite polarity in order to improve its immunogenicity and to induce long-lasting immune responses, according to principles previously established as and thoroughly shown by as [3].

As a result, after testing a large number of modified HABPs in *Aotus* monkeys [6], a non-human primate model capable of developing malaria in a analogs way to humans because its immune system is similar to humans [7,8], two modified peptide analogs (with modified residues written in bold and underlined hereafter for better clarity) named **24276** (IWS**SM**K**M**DEK**M**A**AM**QGEESH) and

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**24488** (IWS**SV**K**M**DEK**V**A**AV**QGEESH) were obtained highly immunogenic as was determined by the induction of high antibody titers against the sporozoite in immuno-fluorecent assays and against the recombinant SALSA protein in Western blot antibody assays.

The immune response elicited by SALSA-derived peptides is genetically restricted by their presentation in the context of Class I or Class II Major Histocompatibility Complex (MHC) molecules, as has been reported by other researches [9] and some of our previous studies, which show that native HABP 20608 and its modified-immune response inducers **24276** and **24488** have different binding affinities to HLA-DRβ1∗0301 allelic molecules [10]. The Peptide Binding Region (PBR) of these Class II molecules contains amino acids from both α and β chains which define special sites denoted as Pockets: 1, 4, 6 and 9 [11–14] (Fig. 1A). The fitting of residues from an antigenic peptide inside each PBR pocket is considerably mediated by receptor–ligand electrostatic interactions, to allow its presentation to the T-cell receptor (TCR). The stability and duration of such complex on the membrane of antigen presenting cell are largely defined by the type, strength and number of electrostatic interactions, which stabilize these interactions that will ultimately determine the immune response fate, *raizon d'être* of this work [15,16].

As previously shown by Cifuentes et al. [10], SALSA modified HABPs **24276** and **24488** bound with high ability (∼50%) to HLA-DRβ1∗0301 purified molecules (Table 1A) displaying the classical binding motifs and reading registers for this molecule (Table 1B), i.e., M for **24276** or V for **24488** in Pocket 1, D in Pocket 4, K in Pocket 6 and A in Pocket 9 which are in complete agreement for what has been experimentally found in isolated peptides bound to these purified HLA-DRβ1∗0301 molecules [17,18].

In our purpose to find clues for a methodology for vaccine design, we studied the modifications induced by native and modified SALSA peptides on the electrostatic landscape of HLA-DRβ1∗0301 molecules, which have been determined on the basis of the X-ray three-dimensional (3D) structure of this molecule, co-crystallized with the Class II-associated Invariant Chain Peptide (CLIP) [12].

Given that computational chemistry partly explains electrostatic phenomena inherent to receptor–ligand interactions, we have adopted this approach as a rational, logical, effective and high-speed methodology for explaining the absence of immunogenicity of some molecules and determine which modifications are needed to render such peptides into immunogenic- and protection-inducers, in an attempt to define a solid methodology for vaccine development.

## 2. Materials and methods

### 2.1. Electrostatic potential calculations

The geometries of each studied complex were obtained on the basis of the PDB structure corresponding to the HLA-DRβ1∗0301–CLIP complex (PDB ID: 1A6A) [12]. CLIP amino acids ($_{304}$PVSK**M**RM**AT**P**L**L**M**QA$_{318}$) were punctually replaced by each residue in the sequence of native SALSA 20608 HABP and its modified peptides, using the UCSF Chimera software package [19], which is programed with standard parameters for each amino acid. Each system was optimized by molecular mechanics using the CVFF force field [20] and a convergence parameter of 0.001 Root Mean Square Deviation (RMSD).

The "Pocket" concept [11–14] was used as a structural approximation for calculating electrostatic potentials, which is defined by those amino acids from HLA-DRβ1∗0301 molecules located in close proximity to the amino acid of the antigenic peptide (within 10 Å range), here denoted as "occupying amino acid". We centered our analysis on Pockets 1, 4, 6 and 9, taking into account the amino acids previously reported by Balbín et al. [21] to define each pocket and the peptide fragment which is occupying this region. Finally, an *ab initio* single-point HF/6-31G* calculation was performed based on each pocket and peptide fragment set of coordinates from the complex, followed by electrostatic potential calculations [22] using Gaussian98 software package [23]. The electrostatic potential was displayed over Michael Sanner's Molecular Surface (MSMS – [24]) by using VMD software package [25].

Biological properties such as binding capacity to purified HLA-DRβ1∗0301, immunological characteristics of these native and modified SALSA-derived peptides and the 3D structure of these molecules determined by $^1$H NMR, have been already described by Cifuentes et al. [10]. These results make the possibility to obtain a logical and rational methodology for vaccine development much more expedite.

## 3. Results

### 3.1. Molecular electrostatic potential (MEP)

Fig. 1C shows molecular electrostatic potentials on MSMS (volumetric) surfaces [24], in the most important 3D views analyzed with VMD program [25].

#### 3.1.1. Pocket 1

The electrostatic potential differences in Pocket 1 were mainly confined to the central portion of this pocket clearly observed in the top view (Fig. 1C). When the apolar residue M, by which CLIP is anchored to this pocket, was replaced to E in native non-immunogenic HABP 20608 (Table 1A), the positive electrostatic potential observable in CLIP (blue region, in fuchsia circle indicated by the arrow) changed to negative potentials (red color) in 20608 mainly on the surface of Vβ86. On the contrary, P1 residue electrostatic potential continued being positively charged (blue) in both modified immunogenic peptides **24276** (anchored by the apolar M) and **24488** (anchored by the apolar V). These data suggest that the presence of a polar negatively charged amino acid (E) in peptide 20608 hampers the proper electrostatic fit inside this pocket, the most important for peptide binding [17,18]. This fact could partially explain, its absence of immunogenicity.

Another important difference observed between CLIP versus 20608 and its immunogenic modified **24276** and **24488 analogs,** was the variation in Sα53 superficial potential (circled in yellow), which displayed a positive potential in CLIP, but showed a zero or negative potential in SALSA peptides (white or red region) and therefore could not be related with its different immunological activities. This difference is due to the presence in $P_{-1}$ of K in CLIP and A/S in the other peptides.

#### 3.1.2. Pocket 4

In the lateral view of these complexes a very broad negative potential (red) was observed in the pocket's central region when it interacts with amino acids from native or modified SALSA-derived HABPs, which is not observed when this pocket interacts with CLIP amino acids. While A is the occupying amino acid in CLIP (a less frequently found residue in this pocket as is shown in Table 1B), this pocket is occupied by D in SALSA-derived peptides, the most specific binding amino acid for this allele as seen in Table 1B. Thus, the CLIP peptide probably is not the best experimental molecule to crystallize the HLA-DRβ1∗0301.

On the contrary, the wide region of positive electrostatic potential (yellow circled), observed in CLIP, that is determined by the characteristics of residues Kβ71/Rβ74 for HLA-DRβ1∗0301 allele and define the preference in this pocket by glutamic acid (Table 1B), is strongly modified in the SALSA's derived peptides. This

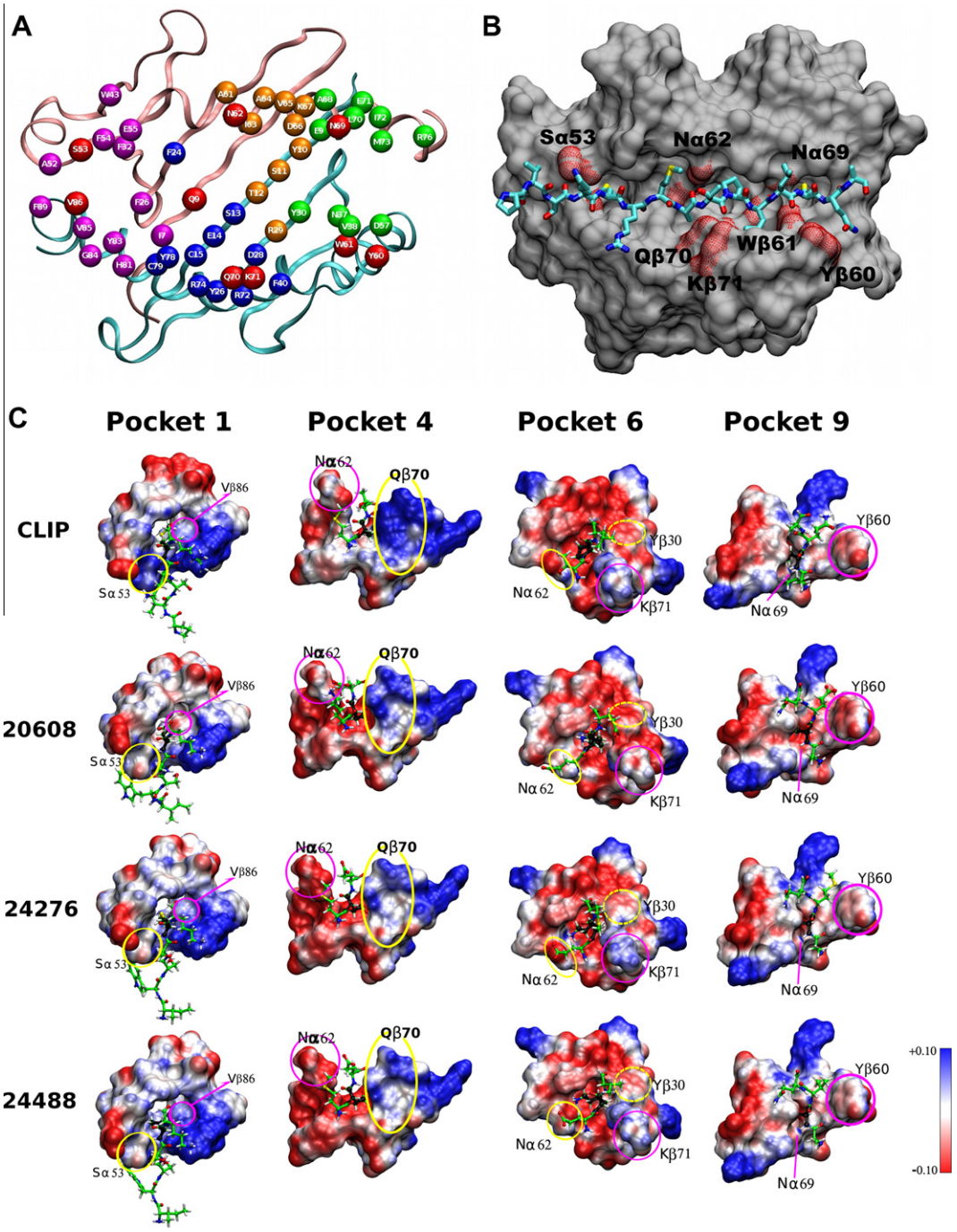



**Fig. 1.** (A) Residues forming the Pockets: Pocket 1 in fuchsia, Pocket 4 in blue, Pocket 6 in orange and Pocket 9 in green. In red are the residues that presented important electrostatic changes when the residues of the peptide are changed. (B) MSMS representation showing the residues (red surface) that are electrostatically modified by the changes in the binding peptide. As can be observed, those residues are accessible for the TCR recognition. (C) View of electrostatic potential on Pockets 1, 4, 6 and 9 respectively for each peptide. The most relevant differences are enclosed in circles. The occupying amino acid is shown in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

effect is extended to residue Q70β that is recognized as important in TCR footprint [26].

A prominent difference of the electrostatic potential was seen on Nα62, in modified antibody-inducing HABPs **24276** and **24488**, but not in 20608 or CLIP. This difference is not given by the occupying amino acid D, which is shared between the native and modified peptides. For 20608, the presence of K in the P3 peptide position (upwardly oriented in the TCR direction) affects the influence of E (P5, upwardly oriented too) on Nα62 residue and resembles the CLIP case in which the P3 and P5 are M and T, respectively. As is shown in Fig. 1C (in fuchsia circle), there is a strong electrostatic difference on the last two immunogenic (24276 and 24488 with M in P3) but not on the first two non-immunogenic complexes (CLIP and 20608).

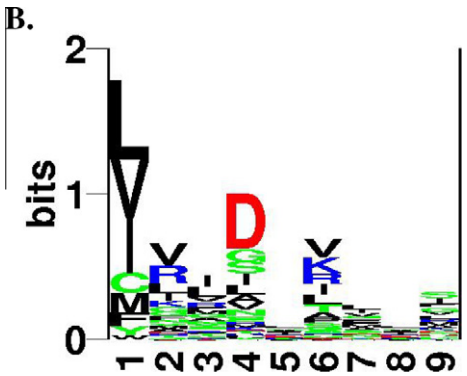
Another notable difference between the electrostatic potential induced by modified immunogenic and the native HABP in Pocket 4, was observed around amino acid Qα9 (not visible in the figure), resulting in a positive potential between the N atom in occupying peptide A (P4) amino acid and the side-chain carbonyl O from Qα9, which suggests the formation of a hydrogen-bond like interaction.

**Table 1**
(A) Peptides sequence and their HLA-DRβ1∗ binding percentage. Those are aligned according to Pockets 1, 4, 6 and 9 binding motifs and registers of HLA-DRβ1∗0301 molecule. Also the humoral immune response induced by the conserved SALSA-derived peptide (20608) and its modified analogs (24276 and 24488) in *Aotus* monkeys is presented. PI: Pre-immune monkeys. I: First dose, II: second doses. Subscripts are the bleeding days when antibody titers (in parenthesis) were determined. The prefix corresponds to the number of monkeys producing those antibody titers [10]. (B) Binding profile for HLA-DRβ∗0301 reported by Lund et al. [17].

**A.**

| Peptide no. | Peptide sequence | | | | | | | | | | | | | | | | | | | | % Binding HLA-DRβ1∗ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pf-SALSA | | | | | P1 | | | P4 | | P6 | | | P9 | | | | | | | | 0101 | 0301 | 0401 | 0701 | PI | $I_{20}$ | $II_{10}$ | $II_{15}$ | $II_{20}$ |
| 20608 | I | W | S | A | E | K | K | D | E | K | E | A | S | E | Q | G | E | E | S | H | 6 | 16 | 8 | 10 | 0/5 (0) | 0/5 (0) | 0/5 (0) | 0/5 (0) | 0/5 (0) |
| **24276** | – | – | – | S | M | – | M | – | – | – | M | – | A | M | – | – | – | – | – | – | 8 | **47** | 19 | 10 | 0/8 (0) | 3/8 (1:640) | 1/8 (1:320) | 1/8 (1:320) | 1/8 (1:320) |
| **24488** | – | – | – | S | V | – | M | – | – | – | V | – | A | V | – | – | – | – | – | – | 0 | **50** | 31 | 12 | 0/8 (0) | 1/8 (1:320) | 0/8 (0) | 0/8 (0) | 0/8 (0) |
| CLIP | $P_{304}$ | V | S | K | M | R | M | A | T | P | L | L | M | Q | $A_{318}$ | | | | | | | | | | | | | | |

**B.**



Given that Qα9 is conserved in all Class II molecules, crystallographic studies have shown that this amino acid stabilizes the MHC II–peptide complex by forming two hydrogen bonds with the peptide backbone corresponding to amino acid P4. It is also probable that the negative interaction between Qα9 and peptide 20608 backbone is one of the reasons this native HABP does not anchor appropriately to HLA-DRβ1∗0301 and hence together with the other electrostatic differences does not induce an antibody-mediated immune response.

*3.1.3. Pocket 6*

One of Pocket 6 main characteristics, defined mainly by α-chain residues, is the presence of two negatively charged amino acids: Eα11 and Dα66 which are in close proximity and facing each other, thus conferring an eminent electronegative character to this pocket, that defines the K/R preference in it (Table 1A and B).

Several clear differences are observed in the top view of the complexes but perhaps one of the most interesting electrostatic potential differences in Pocket 6 was found around amino acid Kβ71 (encircled in fuchsia Fig. 1), which is a polymorphic residue among the different HLA-DR alleles but is conserved in all HLA-DRβ1∗03 variants where it is well-known for establishing a hydrogen bond with CLIP backbone through residues P5/P6. In immunogenic peptides **24276** and **24488**, such interaction is mediated by K (P6) which is stronger in the long-lasting immunity inducer peptide **24276**, around which positive potentials highly resemble the ones observed in CLIP, while in native peptide 20608 such electrostatic potential are negative or close to zero due to E (P7), which was modified in derived peptide for M (**24276**) and V (**24488**). This provides further support to the lack of immunogenicity of peptide 20608.

Another important difference was observed again in the side-chain atoms of Class II canonically conserved amino acid Nα62 again. A large negative potential was found when this amino acid interacted with the antibody-production inducer peptides **24276** and **24488**. It has been found that this conserved amino acid in Class II molecules interacts directly with the peptide's backbone by establishing two hydrogen bonds with amino acids located in position 5 and 6 (E and K in 20068, **24276** and **24488**, but T and P, respectively in CLIP) on the occupying peptide. Similarly, a negative potential was found when amino acid Yβ30 interacted with non-immunogenic native peptide 20608 and with CLIP (dotted yellow circle), a behavior which was not observed with immunogenic modified HABPs **24276** and **24488**, these exhibited a more neutral than negative electrostatic potential tendency.

*3.1.4. Pocket 9*

The electrostatic potential observed on the top view of this complex, close to amino acids Yβ60 and Wβ61, which is defining the outermost boundaries of Pocket 9, was mainly negative both with CLIP (interacting with M) as well as with 20608 (which interacts via S), contrary to what occurs with peptides **24276** and **24488** (immunogenic peptides interacting via A); in which even though the potential is also negative, there are regions where the electrostatic potential is close to zero.

Moreover, a positive electrostatic interaction was observed between the Nα69 amino acid and NH backbone atoms from the occupying amino acid in all immunogenic peptides and in CLIP, but not in native peptide 20608. Nα69 has been characterized by crystallographic studies as being critical for the anchorage of immunogenic peptides since it establishes two hydrogen bonds with the backbone of occupying amino acids P8 and P9 (L and M, respectively for CLIP), hence further stabilizing the MHC II–peptide complex.

In brief, the results obtained from SALSA-derived HAPBs' interaction with HLA-DRβ1∗0301 molecules have shown that this allele's specificity resides mainly in amino acids Qα9, Sα53, Nα62, Nα69, Yβ30, Yβ60, Qβ70, Kβ71, Rβ74 and Vβ86, most of them corresponding to conserved positions involved in peptide binding via hydrogen bond interactions due their proximity with the backbone.

## 4. Discussion

This electrostatic-structure characterization could be a partial explanation for the contrasting immunological characteristics of these peptides, as shown by Cifuentes et al. work [10]. Native conserved HABP 20608 induced no detectable antibody titers in *Aotus* monkeys, when sera from these immunized monkeys were tested against the monomeric native peptide by ELISA, neither the parasite as determined by IFA, nor the recombinant SALSA protein in the Western blot assay. Meanwhile modified HABP **24276** induced high antibody titers (1:640), when determined by IFA, against sporozoites in 3/8 monkeys, which were detectable 20 days after the first immunization that persisted in 1/8 monkeys after the second immunization (Table 1A), suggesting that these sera were recognizing the parasite native protein on the sporozoites surface, as well as the induction of high long-lasting antibody titers by modified peptide **24276** which lasted in these monkeys for up to 120 days (not shown). Due to their long permanence these antibodies are highly relevant for inducing immune memory or persistent protective immunity against the parasite [5].

Modified HABP **24488** showed a different immunological behavior, inducing high IFA (1:320) antibody titers in 1/8 monkeys following the first immunization dose, these remained detectable 20 days after the immunization and disappeared after the administration of the second dose (Table 1A). This phenomenon, previously described by us and named "short lived antibodies", is associated to a particular modification and configuration in the modified peptide and has been found as an evasion mechanism displayed by microbes [27].

In consequence, conserved native HABPs have to be modified, as has been documented in other systems, to be rendered into immunogenic and protection inducing [3,6]. This work was intended to find a partial electrostatic explanation for non-antigenic and non-immunogenic phenomena and contributes to solve the conserved HABPs absence of immunogenicity and their consequent inability to induce protective immune response.

As the anchor of the peptide to the Pocket 1 is critical for binding to Class II molecules, we see that by replacing E (P1) on 20608 by M or V, a higher binding percentage of modified peptides (24276 and 24488) was achieved (Table 1A), which is correlated with the change of positive potentials in the Vβ86 amino acid. This was observed in the presence of immunogenic peptides **24276** and **24488**, as well as same as in CLIP (binding peptides), but not in native HABP 20608 which displayed a negative potential.

The **G**β86**V** genetic polymorphism occurring in this pocket determines the size of the occupying amino acid, since the **G**β86 genetic variant allows the fitting of large apolar aromatic residues such as W (228 $Å^3$), Y (194 $Å^3$) and F (190 $Å^3$). The genetic variant **V**β86 accepts apolar amino acids with intermediate size such as L (166 $Å^3$), I (163 $Å^3$), M (163 $Å^3$) present on **24276** and V (140 $Å^3$) present on **24488**. An analysis of these electrostatic potential data suggests that residue volume does not play a critical role in 20608 binding to HLA-DRβ1∗0301 since E volume (140 $Å^3$) is quite similar to that of V (140 $Å^3$). Therefore, critical role of E in P1 for this non-immunogenic peptide seems to be its influence on the electrostatic landscape of Pocket 1, more than its volume.

In Pocket 4, the critical relevance of conserved residue Nα62 resides in its ability to form two hydrogen bonds with CLIP's A (P4) and T (P5) amino acids backbone, as determined by crystallography studies, which showed that it is stabilizing peptides binding

to HLA-DRβ1∗0301 molecules. Due to such characteristic, this residue has been considered as a clue-anchoring residue in Class II molecules [11–14]. Additionally, this residue on the outside of the pocket plays an important role in the recognition by the TCR, in the central region (P3, P4 and P5) of MHC–peptide complex. The effect of the residues P3 (K on non-immunogenic peptide and M in the immunogenic peptide) and P5 is correlated with the immunological activities observed. A similar effect occurs with Qβ70, a residue that also involved in the interaction with the TCR.

The studies by Kersh et al. [28], demonstrated to have a high relevance showing that a minimal variation on the amino acid occupying Pocket 6 from the hemoglobin peptide (Hb 64–76), such as the E73D change (which implies the change of one amino acid for another one with a shorter side-chain), diminished this peptide's immunogenicity by 1000-fold. Such reduction is related to a modification induced in the modified peptide between lateral chains of residues P5 and P8 that results in a lateral residue orientation of 10 Å, twisting its direction and therefore modifying its contact with the TCR. These data presented here highlight the importance of the region surrounding Pocket 6 in MHC–peptide–TCR complex interaction in HLA-DRβ1∗0301 binding peptides, providing and additional explanation to the immunogenicity and antibody-production inducing properties of these two peptides.

Pocket 9 size is determined by the salt bridge established between Rα76 and Dβ56 generating a small pocket which allows the fit of small apolar amino acids in most Class II molecules as occurs in HLA DRβ1∗0301 and in these peptides, but when this salt bridge is broken by some allelic variants carrying in β56 position S, T and V an oxyanionic hole is formed, allowing the fit and interaction of negatively charged residues like D, E or neutral like Q and N. These weak electrostatic interactions observed with CLIP could also explain in part its instability. For this pocket the most interesting changes were observed in Yβ60 and Wβ61, amino acids that are located in an alpha-helical β chain exposed to recognition by the TCR (Fig. 1B). The effect on this residue is due to the change in P10:E for non-immunogenic and M/V immunogenic, again correlating with different immunological activities.

Thus, changes were made in the sequence of 20608 peptide to improve their binding to HLA-DRβ1∗0301 Class II molecules and to produce an important electrostatic variations in regions where the recognition by the TCR occurs. These peptides retain the minimum requirements to generate a subsequent recognition of the parasite by the antibodies generated.

A remarkable aspect of this computational analysis is that it has enabled finding the electrostatic potential changes occurring on the same amino acids (Qα9, Sα53, Nα62, Nα69, Yβ30, Yβ60, Wβ61, Qβ70, Kβ71, Rβ74 and Vβ86), recognized as important in peptide anchoring and recognition. Therefore, an analysis of these interactions from an electrostatic point of view could allow predicting and designing molecules in a rational, logic, fast and effective manner, so as to be used as immunogens or vaccine components for the control of infectious diseases which represent a tremendous burden to the health of humankind.

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